Role of neurosteroid modulation of $GABA_A$ receptors in the central actions of alcohol

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Declaration

I, Reka Penzinger, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in my thesis.

Abstract

Inhibitory GABA-mediated neurotransmission is considered to be vitally important for mediating the major effects of alcohol in the brain. Recent studies have suggested that certain behavioural and electrophysiological actions of ethanol are dependent on increased concentrations of brain-derived neuroactive steroids. These neurosteroids, such as allopregnanolone and tetrahydro-deoxycorticosterone, are potent endogenous positive modulators of GABA type-A receptors ($GABA_ARs$), and are synthesised *de novo* in the brain, or from the peripherally-derived hormones, progesterone and corticosterone.

To investigate the role of neurosteroid modulation of specific isoforms of $GABA_A$ receptors in alcohol-driven reward behaviour, we have assessed ethanol consumption in two novel knock-in mouse models, $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$, which removes neurosteroid potentiation from α2-$GABA_A$ and α4-containing $GABA_A$ receptors, respectively. This study shows that both α2- and α4- neurosteroid insensitive mice exhibit reduced ethanol intake when assessed by an intermittent access two-bottle choice protocol. By contrast, saccharin and quinine solution intakes remained unchanged.

The importance of neurosteroid levels was further demonstrated by using finasteride, a neurosteroid synthesis inhibitor. This reduced the preference for ethanol intake in C57BL/J6, $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ mice, as well as reducing blood ethanol concentrations.

To explore whether altered modulation of inhibitory synaptic transmission might underpin these behavioural phenotypes, whole-cell electrophysiological recordings were performed from dentate gyrus granule cells (DGGCs) during application of ethanol. Upon acute exposure to ethanol, increased miniature inhibitory post-synaptic current amplitude, frequency, decay kinetics and charge transfer were observed. However, no overall differences were detected between wild type and α2 and α4 mutant expressing neurons, indicating that these changes are not mediated via neurosteroid modulation of α2- and α4-$GABA_ARs$ at least in DGGCs.

In conclusion, this study demonstrates that neurosteroids are playing an important role in mediating the central actions of ethanol via α2- and α4-$GABA_ARs$ but such $GABA_A$ receptors in DGGCs are not majorly involved in these effects of the neurosteroids.

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Impact statement

Alcohol use disorders (AUD), encompassing both alcohol abuse and dependence, pose significant long-term challenges for individuals and society. Currently approved pharmacotherapies are over a decade old, limited, and often only moderately effective in clinical practice, underscoring the need for more efficacious novel treatments to address the multifaceted challenges presented by alcohol dependence. Understanding the mechanisms underpinning AUD is critical for developing effective and safe pharmacological therapies.

Ethanol's effects on the brain are closely linked to $GABA_A$ receptors, partly through the modulation by neurosteroids. These neurosteroids, which are potent endogenous positive modulators of $GABA_A$ receptors, amplify ethanol's impact by enhancing inhibitory GABAergic signalling.

The behavioural implications of genes have been explored using global gene knockout animals, a method applied to various $GABA_A$ receptor subunits such as α2, α4, and δ. However, eliminating any $GABA_A$ receptor subunit can trigger compensatory changes, potentially complicating behavioural interpretations. Our two novel knock-in mouse lines, $\alpha4^{Q246M}$ and $\alpha2^{Q241M}$, which lack just neurosteroid sensitivity, provide a unique opportunity to elucidate the roles of α4- and α2-containing receptor isoforms in the neurosteroid-mediated modulation of ethanol effects.

The findings of this research hold considerable promise for real-world applications in the field of neuroscience. By elucidating the intricate mechanisms through which ethanol exerts its effects on $GABA_A$ receptors and inhibition, particularly in relation to neurosteroid modulation, this study paves the way for the development of novel therapeutic strategies. Understanding these interactions can potentially lead to the creation of targeted pharmacological interventions aimed at mitigating alcohol use disorder and related neurological (often co-morbid) conditions. Such advancements are crucial for addressing the societal and health burdens associated with AUD, offering new avenues for improving treatment efficacy and patient outcomes after decades of relative therapeutic stagnation.

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5-HT	5-hydroxytryptamine
5α-DHP	5α-dihydroprogesterone
a.a.	amino acid
AC	adenylyl cyclase
ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
ALLO	allopregnanonole
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
ANOVA	analysis of variance
ASPA	Animal (Scientific Procedures) Act, 1986
AUD	alcohol use disorder
BEC	blood ethanol concentration
BK channel	large conductance potassium channel
bic	(-)-bicuculline-methiodide
bp	base pairs
CA1/3	*Cornu ammonis* (CA) region 1/3 of the hippocampus
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor 1
CIE treatment	chronic intermittent ethanol treatment
CNS	central nervous system
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
Ctrl	control
D1/2	dopamine 1/2 receptor
DG	dentate gyrus
DGGC	dentate gyrus granule cell
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DS2	4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide

EDTA	ethylene-diamine-tetra-acetic acid
EM	electron microscopy
ES cell	embryonic stem cell
EtOH	ethanol
FIN	finasteride
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
GIRK	G-protein coupled inwardly rectifying potassium channel
hab	habituation
HCN	hyperpolarisation-activated cyclic nucleotide–gated channel
HDL	high density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
het	heterozygous
hom	homozygous
HPA axis	hypothalamic-pituitary-adrenal axis
HSD	hydroxy-steroid dehydrogenase
HSL	hormone-sensitive lipase
i.p. injection	intra-peritoneal injection
IPSC	inhibitory postsynaptic current
kDa	kilo-daltons
KO	knock-out
LD	lipid droplet
LDL	low density lipoprotein
LHFPL4	Lipoma HMGIC Fusion Partner-Like 4
mGlu	metabotropic glutamate
mIPSC	miniature inhibitory postsynaptic current
NA	numerical aperture
NAc	nucleus accumbens
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
Neo	neomycin cassette
NGS	normal goat serum

NMDA	N-Methyl-D-aspartate
NO	nitric oxide
P450scc	cytochrome P450 cholesterol side-chain cleavage enzyme
P450c17	17α hydroxylase, c17,20 lyase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI	post-injection
PKA	protein kinase A
PKC	protein kinase C
Pref	preference
PS	pregnenolone sulphate
PTX	picrotoxin
Ra	access resistance
REML analysis	mixed effects model analysis
RM ANOVA	repeated measure analysis of variance
RMS	root mean square
ROI	region of interest
ROUT	robust outlier removal
Rs	series resistance
SEM	standard error of the mean
sIPSC	spontaneous inhibitory postsynaptic current
SK	small-conductance calcium-activated potassium channel
SR-BI	Scavenger Receptor Class B Type I
StAR	steroidogenic acute regulatory protein
TCA	trichloroacetic acid
THDOC	allotetrahydrodeoxycorticosterone
THIP	4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol
TK	thymidine kinase
TMD	transmembrane domain
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride
TSPO	translocator protein

TTX	tetrodotoxin
VEH	vehicle
VTA	ventral tegmental area
wt	wild type

Chapter 1 Introduction

1.1 $GABA_A$ receptors

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and it plays a central role in regulating neuronal excitability. GABA exerts its function via two main types of receptors: ionotropic type A receptors ($GABA_ARs$) and metabotropic type B receptors ($GABA_BRs$) (Olsen and Sieghart, 2009). This introduction gives insight into $GABA_ARs$ as the focus of the study is the $GABA_AR$ family.

1.1.1 $GABA_A$ subunit and receptor structure

$GABA_ARs$ belong to the family of Cys-Loop receptors, now known as pentameric ligand-gated ion channels, which also include glycine receptors, nicotinic acetylcholine receptors, 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors and zinc-activated ion channels (Miller and Smart, 2010). Upon binding of GABA, receptors undergo a conformational change and become permeable to negatively-charged chloride (Cl^-) and bicarbonate (HCO_3^-) ions (Bormann et al., 1987). In mature neurons, this influx of negatively charged ions hyperpolarises the membrane and increases the threshold for neuronal firing. This inhibitory effect helps regulate neuronal activity and is crucial for maintaining the balance between excitation and inhibition in the brain. Dysfunctional inhibition has been implicated in a multitude of disorders including alcohol dependence, depression, anxiety and epilepsy (Brickley and Mody, 2012, Nuss, 2015, Treiman, 2001, Dharavath et al., 2023).

$GABA_ARs$ are ligand-gated ion channels and co-assemble from 19 different subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) (Olsen and Sieghart, 2009). The individual subunits share a similar structure (*Figure 1.1*), consisting of a large extracellular amino (N)-terminal domain, four membrane spanning α-helices (M1-4), a large intracellular region between M3-4 and a short extracellular carboxy (C)-terminus (Schofield et al., 1987). Cryo-electron microscopy (EM) has resolved the quaternary structures of physiological αβγ

receptor proteins (Masiulis et al., 2019b, Laverty et al., 2019). Numerous studies have demonstrated that the N-terminal domain houses a binding pocket for GABA and that this pocket is located at the interface between β+ and α- subunits (Sigel and Buhr, 1997, Ernst et al., 2003, Smith and Olsen, 1995, Miller and Smart, 2010).

Given the large number of subunit isoforms, numerous potential receptor subtypes could occur theoretically. However, surprisingly only a small number of viable receptors exist *in vivo* as receptors follow certain rules of assembly (Olsen and Sieghart, 2009). The receptor subunit combinations that are likely to form *in vivo* have been delineated by studies using co-immunoprecipitation, immunohistochemistry, immunocytochemistry and *in situ* hybridisation (Fritschy et al., 1992, Wisden et al., 1992, McKernan and Whiting, 1996). Different expression patterns are noted for each subunit across various brain regions, and these patterns change during development (Pirker et al., 2000, Laurie et al., 1992b, Laurie et al., 1992a, Wisden et al., 1992). The most abundant $GABA_A$ receptor subtypes in the rat brain are α1β2γ2 (~40 %), α2β2/3γ2 (~20 %), α3βnγ2/3 (~20 %); other less common subtypes include α5β3γ2 (~5 %), α4βδ (<5 %) and α6βδ (< 5 %) (McKernan and Whiting, 1996).

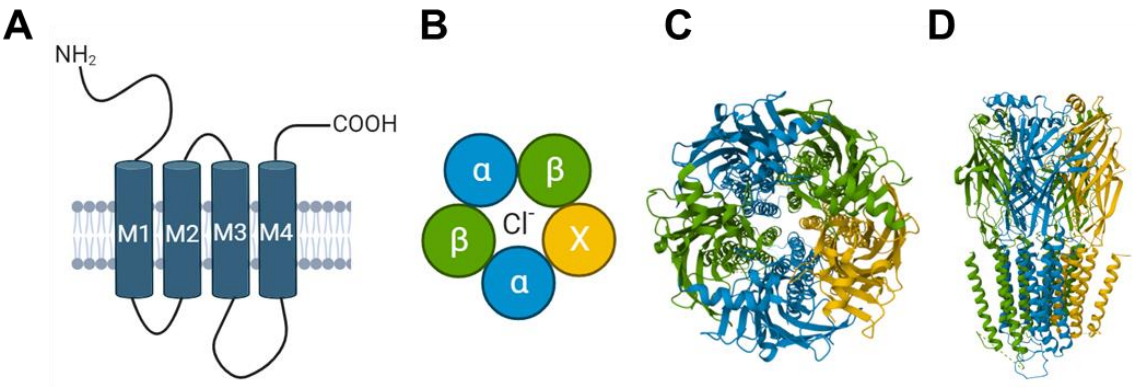


Figure 1.1 The structure of the $GABA_A$ receptor.

A, Schematic diagram of a subunit, displaying the large extracellular amino (N)-terminal domain, the four transmembrane helices (M1-4), the intracellular loop between M3 and M4, and a short extracellular carboxy (C)-terminus. ***B,*** Plan view schematic representation of the subunit assembly of the pentameric $GABA_A$ receptor. The most common pentameric receptor composition is two α (blue), two β (green) and a single γ or δ (X, yellow). ***C*** and ***D,*** Top (C) and side view (D) of a cryo-electron microscopy structure of the α1β3γ2L $GABA_A$ receptor (Laverty et al., 2019). The α1, β3 and γ2L are shown in blue, green and yellow, respectively. The PDB ID is 6I53.

1.1.2 $GABA_A$ receptor localisation and function

The most common pentameric receptor composition is two α, two β and a single γ or δ (*Figure 1.1 B*) (McKernan and Whiting, 1996). The subunit composition of the receptor determines its localisation, pharmacology, kinetics and function (Mortensen et al., 2024). Preferential assembly of subunits gives rise to two main categories of receptors: those located at synapses and those found extrasynaptically. Synaptic receptors typically comprise of α1-3, β and γ subunits, whilst extrasynaptic receptors consist of α4/6, β and δ subunits, α5βγ (see below), αβ heteromers, also α1-3βγ on transit to synapses (Stell et al., 2003, Mortensen et al., 2024, Thomas et al., 2005, Farrant and Nusser, 2005). Synaptic receptors mediate phasic inhibition through the generation of inhibitory postsynaptic currents (IPSCs), whereas extrasynaptic receptors are responsible for tonic currents. Phasic events, also known as IPSCs, occur from spontaneous GABA release and when action potentials trigger the orchestrated release of GABA into the synaptic cleft, leading to the opening of $GABA_A$ receptors located at the synapse. These events are short-lived due to the rapid clearance of GABA from the synaptic cleft via GABA transporters (GAT) (Scimemi, 2014). Conversely, tonic currents arise from sustained activation of $GABA_A$ receptors, which respond to the low ambient levels of GABA outside the synapse (Semyanov et al., 2004, Mody, 2001, Farrant and Nusser, 2005). Ambient GABA can originate from various sources, including spillover from synapses, reverse transport via GAT for example and non-synaptic release (Semyanov et al., 2004).

In general, the γ subunit targets $GABA_A$ receptors to synaptic sites where they are stabilised and anchored by $GABA_A$ receptor associated proteins, gephyrin (*Figure 1.2*) (Tretter et al., 2012), and Lipoma HMGIC Fusion Partner-Like 4 (LHFPL4/ GARLH4) (Davenport et al., 2017, Yamasaki et al., 2017), along with other transsynaptic proteins in the postsynaptic density like neuroligins (Chiu et al., 2019, Poulopoulos et al., 2009). However, α5βγ receptors have been found at extra- and perisynaptic locations in hippocampal neurons. It is because gephyrin directly interacts with the α1-3 subunits (Hausrat et al., 2015). Clustering of α5-containing receptors is regulated by primarily radixin (Loebrich et al., 2006), although they also possess a gephyrin binding site (Brady and Jacob, 2015). Radixin interacts with α5-$GABA_A$ receptors through a binding motif

located on the intracellular domain of the α5 subunit (Loebrich et al., 2006). When radixin is phosphorylated and activated, it effectively stabilises α5-containing receptors at extrasynaptic sites where they mediate tonic inhibition, whereas if radixin is dephosphorylated, α5-$GABA_A$Rs translocate to the postsynaptic density where they can contribute to synaptic inhibition (Hausrat et al., 2015).

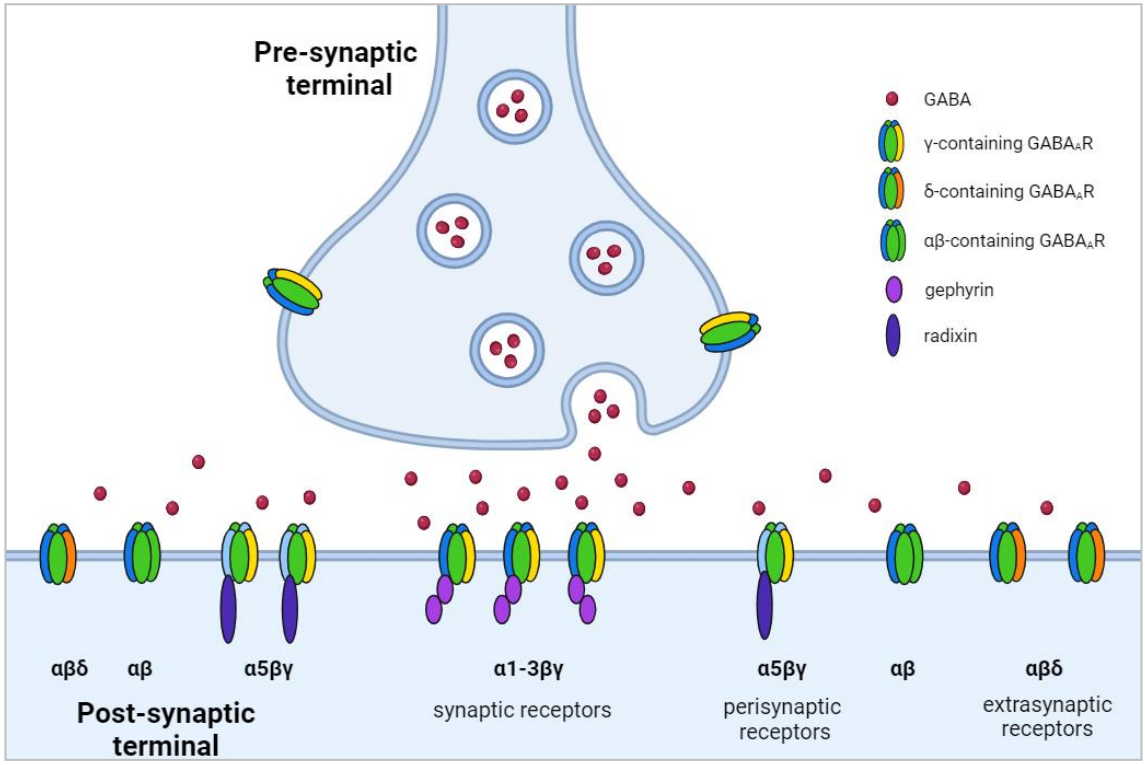


Figure 1.2 Synaptic and extrasynaptic $GABA_A$ receptors.

Upon the fusion of GABA-containing synaptic vesicles with the presynaptic membrane of a nerve terminal, GABA is released into the synaptic cleft. Synaptic receptors (α1-3βγ) encounter the highest concentration of GABA (~1-3 mM) and promptly become activated upon release, generating phasic currents. These receptors gather at the postsynaptic density due to interactions primarily with gephyrin (Luscher et al., 2011). Perisynaptic (α5βγ) and extrasynaptic (αβδ, αβ) receptors are exposed to a lower concentration of GABA (~100 nM) and produce a smaller yet more sustained tonic current. Receptors containing the α5 subunit aggregate outside the synapse through interaction with phosphorylated radixin (Hausrat et al., 2015). Upon dephosphorylation of radixin, α5-containing receptors can relocate to the postsynaptic density, contributing to synaptic currents.

The interaction between $GABA_A$ receptors and anchoring proteins (such as gephyrin and radixin) can be dynamically regulated by neuronal activity. Activity-dependent signalling pathways can modulate the phosphorylation state of these proteins and receptor subunits, influencing their binding affinity and clustering, allowing for dynamic

regulation of receptor localisation in response to changes in neuronal activity (Hausrat et al., 2015, Tretter et al., 2012). This modulation is crucial for synaptic plasticity and adaptive responses in the nervous system.

There is ample evidence to suggest that $GABA_A$ receptors also exist presynaptically. They have been found in the spinal cord and several subcortical structures, including the hippocampus, thalamus, retina and cerebellum (Bowery and Smart, 2006, Eccles et al., 1963, Kullmann et al., 2005). Presynaptic $GABA_A$ receptors are important modulators of synaptic activity, acting primarily to inhibit neurotransmitter release and thus influence neuronal communication and network dynamics (Kullmann et al., 2005). The specific effects of presynaptic $GABA_A$ receptors can vary depending on the brain region and the types of neurons involved. For example, in the hippocampus, these receptors play a role in regulating the release of glutamate from excitatory neurons, thereby impacting learning and memory processes (Kullmann et al., 2005).

1.1.3 $GABA_A$ receptor modulation by ligands

$GABA_A$ receptors have a rich pharmacology, and are modulated by various substances, including clinically relevant drugs such as barbiturates, benzodiazepines, general anaesthetics, as well as endogenous compounds, e.g. neurosteroids. These agents exert their effects by binding to the receptor and allosterically modulating the actions of GABA (Sieghart, 2015).

Barbiturates bind to $GABA_A$ receptors and enhance the receptor's response to GABA in several ways, including potentiation, direct activation and inhibition, in a concentration-dependent manner (Akaike et al., 1990, Evans, 1979, Akaike et al., 1985). The enhancement of GABAergic inhibition by barbiturates leads to prolongation and potentiation of IPSCs, increased neuronal hyperpolarisation producing sedative, hypnotic, anxiolytic, and anticonvulsant effects. These properties make barbiturates useful in clinical settings for anaesthesia, treatment of specific forms of epilepsy, and early management of anxiety (Macdonald and Olsen, 1994).

However, the use of barbiturates is limited now due to their toxicity and they have been superseded by benzodiazepines which show a more favourable safety profile (Sieghart, 2015). Benzodiazepines bind to a specific site on the $GABA_A$ receptor complex, located at the interface between the α+ and γ- subunits (Möhler et al., 2002). Specific residues in the α subunit, particularly in the α1, α2, α3, and α5 subtypes, are critical for benzodiazepine binding. These include a crucial histidine at position 101 (H101) in the α1 subunit, which is important for high-affinity binding (Benson et al., 1998, Wieland et al., 1992). Mutations in these residues can significantly alter the binding affinity and efficacy of benzodiazepines. The potentiation of GABAergic inhibition by benzodiazepines results in sedative, hypnotic, anxiolytic, muscle relaxant, and anticonvulsant effects. These properties make benzodiazepines widely used for treating anxiety disorders, insomnia, muscle spasms, and epilepsy.

Bicuculline is a well-known antagonist of $GABA_A$ receptors, exerting its effect by competitively blocking the binding of GABA and preventing chloride channel opening (Curtis et al., 1970, Jones et al., 2020, Krishek et al., 1996). It shows no receptor subtype selectivity but it shows negative allosteric properties (Ueno et al., 1997, Krishek et al., 1996). Another $GABA_A$ receptor competitive antagonist, SR-95531 (gabazine), does not exhibit selectivity for different β subunit-containing $GABA_A$ receptors (Ebert et al., 1997). However, at low concentrations, gabazine significantly reduces phasic currents without affecting tonic currents in CA1 neurons (Stell and Mody, 2002).

Picrotoxin (PTX), a non-competitive antagonist of $GABA_A$ receptors, does not bind between subunit interfaces. Instead, its binding site is within the channel pore, accessible only when the receptor is in its open state (Korshoej et al., 2010). Early electrophysiological studies suggested that PTX inhibits GABA-evoked currents by stabilising the closed/resting state of the receptor (Krishek et al., 1996, Newland and Cull-Candy, 1992). This idea was later confirmed by a cryo-EM structure of an α1β3γ2L $GABA_A$ receptor with PTX in the presence or absence of GABA (Masiulis et al., 2019a).

Neurosteroids represent a class of endogenous steroids that are synthesised *de novo* in the brain from cholesterol and from precursors synthesised in the periphery that then cross the blood-brain barrier due to their lipophilic properties. They have potent and

selective effects on $GABA_A$ receptors. The activities of neurosteroid are dependent on their structure, the brain regions and the types of neurons. Structural and functional properties of neurosteroids are further discussed in *Section 1.2*.

1.2 Neurosteroids as modulators of $GABA_A$ receptors

Sex hormones mediate their effects through genomic mechanisms by interacting with intracellular receptors located in the nucleus or cytoplasm. These hormones function as ligand-activated transcription factors, regulating gene expression (Paul and Purdy, 1992). However, certain metabolites of progesterone and various stress hormones elicit their effects via non-genomic mechanisms, acting on membrane-bound receptors (Rupprecht, 2003, Baulieu and Robel, 1995, Frye et al., 1992). While genomic actions typically unfold over minutes to hours and are constrained by the rate of protein biosynthesis, the effects mediated by membrane receptors are rapid (Colciago et al., 2020). Today it is widely accepted that metabolites of sex and stress hormones act non-genomically and play an important role in regulating neuronal excitability in the central nervous system (Lambert et al., 1995, Paul and Purdy, 1992, Majewska et al., 1986).

The term 'neurosteroid' was first coined by Étienne-Émile Baulieu, and is now used to refer to steroids that are synthesised *de novo* in the brain. Neurosteroids affect the function of several neurotransmitter systems, including GABA, NMDA (N-Methyl-D-aspartate), AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid), adrenaline, serotonin, nicotinic acetylcholine and sigma-type-1 (Wang, 2011). Therefore, endogenous neurosteroids play a vital role in regulating neuronal activity across both the central and peripheral nervous systems. These steroids exhibit dynamic fluctuations in response to various physiological states such as stress, pregnancy, the ovarian cycle, neural development, and aging (Porcu et al., 2016). Their modulation is crucial for maintaining proper neural function and behaviour.

Dysregulation of endogenous neurosteroid levels can contribute to the pathogenesis of neurological and psychiatric disorders, such as depression, anxiety and epilepsy (Wang, 2011). Neuroactive steroids also exhibit rewarding properties in rodents and can

influence ethanol and cocaine intake (Purdy et al., 2005). Consequently, neurosteroids have emerged as promising therapeutic targets in clinical practice (Pinna, 2020). They are utilised for their sedative, hypnotic, anticonvulsant, and antidepressant properties, offering effective management for a range of conditions.

1.2.1 Endogenous neurosteroids: synthesis and mechanism of action

Neurosteroids are synthesised *de novo* in glial and neuronal cells from cholesterol and from peripherally derived precursors (Baulieu et al., 2001). Based on structural features, neurosteroids can be classified as pregnane neurosteroids, such as allopregnanolone and allotetrahydro-deoxycorticosterone (THDOC), androstane neurosteroids, such as androstanediol and etiocholanone, and sulphated neurosteroids, such as pregnenolone sulphate (PS) and dehydroepiandrosterone sulphate (DHEAS) (Reddy, 2010). Pregnane neurosteroids, mainly allopregnanolone, are the primary focus here, therefore emphasis will be on their synthesis, mechanism of action and role throughout this thesis.

Cholesterol is the common precursor for all steroid hormones. The cleavage of the side chain of cholesterol to form pregnenolone was determined to be the first step in the biosynthesis of steroids, which occurs via the activity of the P450 side chain cleavage enzyme (P450scc) (Pikuleva, 2006). Via a series of enzymatic reactions mediated by cytochrome P450 or non-P450 enzymes, different classes of neurosteroids can be produced (*Figure 1.3*). Evidence from molecular and biochemical studies have shown that steroidogenic enzymes, including P450scc, 5α-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD) and 3β-hydroxysteroid dehydrogenase (3β-HSD), are present in the human brain (Stoffel-Wagner, 2001). Thus, the brain contains the enzymes required for the *in situ* synthesis of various pregnane steroids from cholesterol. Furthermore, it has also been shown that allopregnanolone levels persist in the brain following adrenalectomy and/or gonadectomy, suggesting that allopregnanolone can be synthesised *de novo* in the brain via the reduction of progesterone (Corpéchot et al., 1993). On the contrary, THDOC appears to be derived nearly exclusively from adrenal sources as adrenalectomy leads to the complete depletion of brain THDOC content (Purdy et al., 1991).

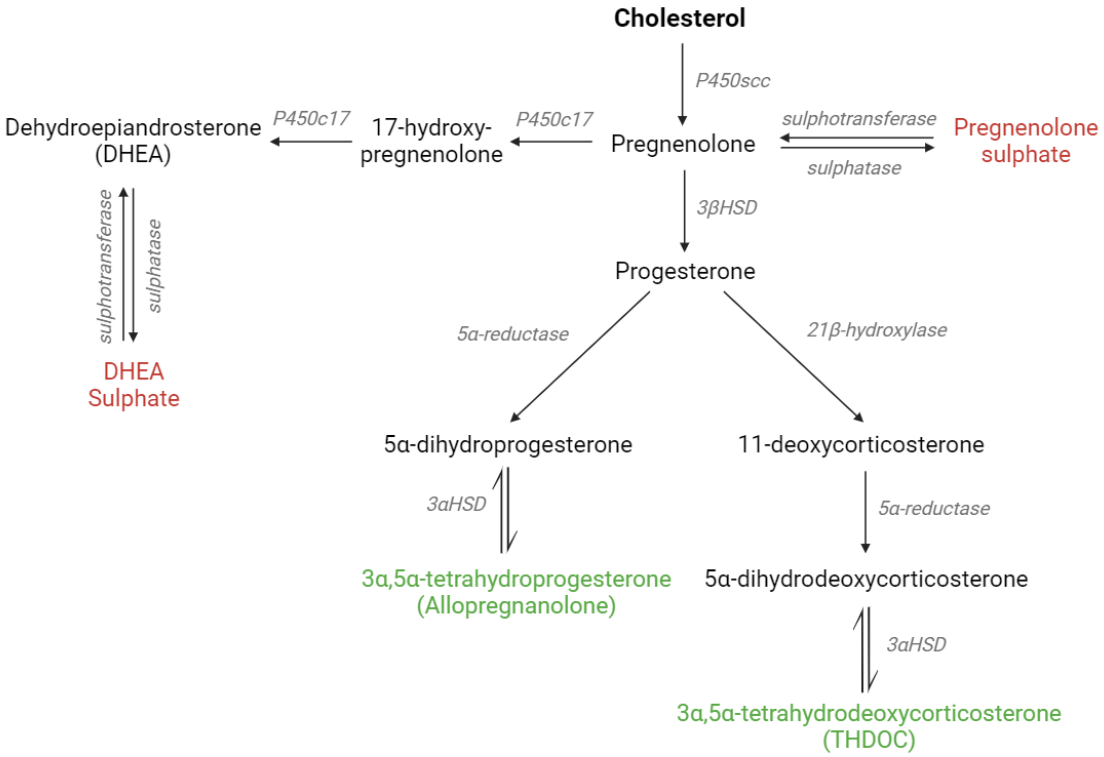


Figure 1.3 Neurosteroid synthesis pathways

The chart shows the conversion of cholesterol into various metabolites acting on $GABA_A$ receptors. The focus of this diagram is on the major positive (green) and negative (red) allosteric modulators of $GABA_A$ receptors, however, a number of additional intermediate compounds (black) are also produced (for more information, see Mellon and Griffin (2002)). The neurosteroid profile of a specific brain region is determined by the enzymes present locally. Neurosteroidogenesis occurs in the brain regions such as cortex, hippocampus, and amygdala (Reddy, 2010). Cholesterol is converted into pregnenolone in the mitochondria by cytochrome P450 cholesterol side-chain cleavage enzyme. This conversion requires the activity of two transporters: StAR (steroidogenic acute regulatory protein (Sierra, 2004)) and TSPO (18kDa translocator protein, formerly known as peripheral benzodiazepine receptor (El Chemali et al., 2022)). Soluble pregnenolone can enter into the endoplasmic reticulum unaided. Abbreviations: 3β-HSD, 3β-hydroxy-steroid dehydrogenase; 3α-HSD, 3α-hydroxy-steroid dehydrogenase; P450scc, cytochrome P450 cholesterol side-chain cleavage; P450c17, 17α hydroxylase, c17,20 lyase. Note that the HSD enzymes are also referred to as hydroxy-steroid oxido-reductase (HSOR) enzymes.

Cholesterol can be sourced from dietary intake, synthesised *de novo* within cells, or derived from circulating low-density lipoproteins (LDLs). Cholesterol can be trafficked to the mitochondria via several different pathways (outlined in *Figure 1.4*). StAR (steroidogenic acute regulatory protein) is a pivotal protein in steroidogenesis, facilitating the transport of cholesterol from the outer mitochondrial membrane to the inner membrane, where the first step of steroid synthesis occurs (Selvaraj et al., 2015).

This step is crucial because the inner mitochondrial membrane houses P450scc. The activity of StAR is tightly regulated and is often the rate-limiting step in steroid production (Manna et al., 2016).

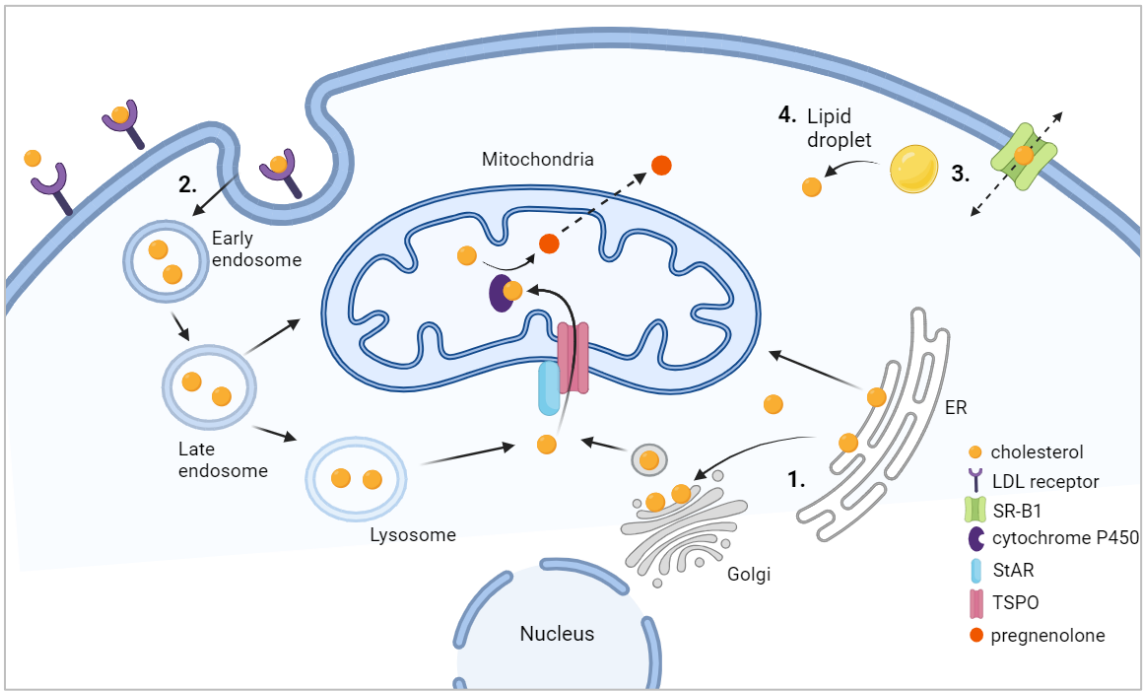


Figure 1.4 Trafficking of cholesterol to the mitochondria for steroidogenesis

Pathway 1: Cholesterol synthesised in the endoplasmic reticulum (ER) is trafficked to the Golgi apparatus where it can be targeted to the mitochondria. Another possible option is passive diffusion from the ER directly to the mitochondria. **Pathway 2**: Low density lipoprotein (LDL), containing cholesterol, binds to LDL receptors located on the cell surface. LDLs are then trafficked to the mitochondria through the endosomal pathway for steroidogenesis. **Pathway 3**: Cholesterol is transferred by the SR-BI (Scavenger Receptor Class B Type I) receptor. SR-BI facilitates the uptake of cholesterol esters from high density lipoprotein (HDL) into cells, a process that does not involve the entire HDL particle being internalised. Instead, cholesterol is selectively extracted and incorporated into the cellular lipid pool, where it can be utilised for various metabolic processes, including steroid hormone synthesis. **Pathway 4**: Hormone-sensitive lipase (HSL) interacts with esterified cholesterol present in the lipid droplets (LD), which converts esterified cholesterol to free cholesterol for use in steroidogenesis as well. Free cholesterol from the LD can interact with lipid-binding proteins present in the cytosol for delivery to the mitochondria. See review Rone et al. (2009).

TSPO (translocator protein), previously known as the peripheral-type benzodiazepine receptor, is another important player in steroidogenesis. Located on the outer mitochondrial membrane, TSPO is thought to be involved in cholesterol transport and regulation. Several biochemical and pharmacological studies have shown that TSPO

binds various ligands, including cholesterol (Papadopoulos et al., 2006). Structural studies have also revealed a five helical structure that forms a homodimer, and that ligand binding can promote cholesterol movement (Papadopoulos et al., 2015). It works in concert with StAR to ensure cholesterol is delivered efficiently to the inner mitochondrial membrane.

Despite numerous studies over the years providing strong evidence for TSPO's crucial role in steroidogenesis, recent research, primarily using genetic animal models, has challenged this conclusion. Global deletion of *Tspo* in mice does not impact upon steroidogenesis (Tu et al., 2014). However, cell-specific knockdown of TSPO in steroidogenic cells seems to affect cell lipid homeostasis (Fan et al., 2019). One explanation for the discrepancy between these findings could be that the global knock-out of TSPO may trigger the expression of a replacement protein or another already present cholesterol-binding protein may replace its function.

1.2.2 Mechanism of action of neurosteroids

Unlike steroid hormones produced by the endocrine glands, neurosteroids synthesised locally in the nervous system are likely to function in an autocrine or paracrine manner (Schumacher et al., 2009). It is commonly asserted that the majority of neurosteroid effects are facilitated through the modulation of neurotransmitter receptor systems. Neurosteroids produce various psychopharmacological effects, including anxiolytic, antidepressant, anticonvulsant, sedative, anaesthetic, analgesic, and amnesic effects, likely due to their interactions with $GABA_A$ receptors (Porcu et al., 2016).

The impact of neurosteroids on $GABA_A$ receptors is influenced by several factors, including whether the steroids are agonists or antagonists, the localisation of receptors (synaptic or extrasynaptic), the subunit compositions, and the intrinsic structure of the steroids. Recent research has identified at least two distinct actions of neurosteroids on $GABA_A$ receptors: an agonistic action and an antagonistic action. The antagonistic action is thought to be mediated specifically by sulphated and 3β-OH steroids (Wang, 2011). The agonistic action can be further categorised into two mechanisms: an allosteric

modulation (augmentation of GABA-evoked Cl^- conductance) and a direct activation of the $GABA_A$ receptor. At low nanomolar concentrations, which occurs during stress (Purdy et al., 1991), alcohol intoxication (Kumar et al., 2004) and pregnancy (Concas et al., 1999), they potentiate GABA currents (Stell et al., 2003, Belelli and Herd, 2003, Zhu and Vicini, 1997), whereas at submicromolar-to-micromolar concentrations, which may occur during parturition (Concas et al., 1999, Stoffel-Wagner, 2001), they directly activate the receptor (Majewska et al., 1986).

Most estimates of *in vivo* neurosteroid concentrations suggest that these levels are generally sufficient to potentiate $GABA_A$ receptors (Paul and Purdy, 1992, Belelli and Lambert, 2005). The estimates of rodent brain neurosteroid levels vary, with reports for allopregnanolone ranging from very low (< 3 nM;(Vallée et al., 2000, Purdy et al., 1991)), to levels sufficient for $GABA_A$ receptor potentiation (3-20 nM: (Sze et al., 2018, Uzunova et al., 2003, Meffre et al., 2007, Liu et al., 2003, Liere et al., 2000, Ebner et al., 2006, Caruso et al., 2010, Higashi et al., 2006, Bernardi et al., 1998, Uzunov et al., 1996)). However, under certain conditions, such as stress, menstrual cycle or in mothers and foetuses during late pregnancy, neurosteroid levels may be high enough to directly activate these receptors (Brunton and Russell, 2010, Nguyen et al., 2003, Hirst et al., 2008). Human brain allopregnanolone levels may be higher (10-40 nM) in women depending on progesterone concentrations in the serum (Bixo et al., 1997, Weill-Engerer et al., 2002).

On a molecular level, potentiating neurosteroids, such as allopregnanolone and THDOC, are believed to influence receptor kinetics, affecting the transition of receptors into and out of desensitised states (Zhu and Vicini, 1997), and potentially enhancing ion channel gating efficacy (Bianchi and Macdonald, 2003). On a cellular level, neurosteroid-induced enhancement of $GABA_A$ receptors amplifies both synaptic (Herd et al., 2007) and tonic currents (Stell et al., 2003). These effects are expected to induce membrane hyperpolarisation and/or reduce excitatory inputs, thereby diminishing the likelihood of neuronal firing.

Inhibitory neurosteroids, such as pregnenolone sulphate and DHEAS, are pro-convulsant in animals, operating as non-competitive antagonists at the $GABA_A$ receptor, and likely

bind to a different site than potentiating neurosteroids (Hosie et al., 2007, Laverty et al., 2017, Wang et al., 2002, Akk et al., 2001, Paul and Purdy, 1992). Their effects in animals may also result from the enhancement of excitatory glutamatergic transmission (Wolf and Kirschbaum, 1999, Park-Chung et al., 1997).

1.2.3 Neurosteroid binding to $GABA_A$ receptors

The effects of various neurosteroids and their analogues on $GABA_A$ receptor activity show stereospecificity and exhibit a biphasic action, with potentiation at low concentrations and activation at high concentrations. Consequently, it was suggested that the $GABA_A$ receptor contains two distinct neurosteroid binding sites: an "activation site" and a "potentiation site."

Hosie et al. (2006) compared the effect of THDOC on the mouse α1β2γ2 $GABA_A$ receptor and the Drosophila GABA receptor which is insensitive to neurosteroids. Chimeras were made that replaced the M1-M2 regions in the murine α1 and β2 subunits with the corresponding sequence from the Drosophila GABA receptor. Functional assessment of these chimeras revealed that the α subunit is likely to play a key role in neurosteroid binding, whereas the β subunit had no impact. They also pinpointed crucial residues, particularly Q241 and T236, in the transmembrane region essential for neurosteroid modulation of $GABA_A$ receptors. Mutagenesis of these two residues led to different outcomes: receptors with the mutation Q241W showed no potentiation by neurosteroids and direct activation was also markedly reduced, whereas the mutation T236I resulted in a decrease in direct activation, with little impact on GABA potentiation by neurosteroids. Therefore, it was concluded that there are likely two distinct binding sites, one involving Q241 for potentiation, and the other involving T236 for direct activation by neurosteroids. Homology modelling of α1 TMD revealed that these two residues are located at two discrete sites, placing T236 close to the outer surface of the receptor at the β(+)- α(-) interface, whereas Q241 was predicted to lie deep within the M1-M4 helices in the α1 subunit. By using mutant αβγ receptors, it was later shown that neurosteroid potentiation was universally dependent on the conserved glutamine residue in M1 of the respective α subunit (Hosie et al., 2009).

Photolabeling with etomidate (Li et al., 2009) and cysteine crosslinking (Bali et al., 2009) studies using a different homology model placed the Q241 residue at the β(+)- α(-) interface. The residue T236 no longer was assumed to be a part of the activation site. However, it is likely that this residue plays an important role in transduction mechanisms for allosteric modulation by neurosteroids. X-ray crystallography using different chimeras, namely GLIC (*Gloeobacter violaceus*; a bacterial cation channel that belongs to the same ion channel family as $GABA_A$ receptors) – GABA α1 (Laverty et al., 2017) and GABA α5 TMD – β3 ECD (Miller et al., 2017), showed five identical binding sites for potentiating neurosteroids located at the interfaces between the subunits (*Figure 1.5 A, B*). The hydrogen bond between the 3α-OH of the steroid and the α1(Q242) residue emerged as a crucial interaction, governing functional modulation (*Figure 1.5 C*). Taken together, these findings suggest that potentiating neurosteroids most likely bind to a single canonical site which is located at the β(+)- α(-) interface.

Recently, cryo-EM structures of $GABA_A$ receptors with allopregnanolone found that allopregnanolone was bound at the base of the TMD across each of the two β(+)- α(-) interfaces (*Figure 1.5 A, B and C*) (Sun et al., 2023, Legesse et al., 2023), which is in agreement with the chimera studies. Photolabeling studies have discovered two additional binding sites to the canonical binding site, and these are thought to be more important for mediating the effects of neurosteroids on desensitisation (Sugasawa et al., 2020, Chen et al., 2012, Chen et al., 2019), however, structural studies, including X-ray crystallography and cryo-EM, have not been able to identify these sites.

Overall, there is strong consensus that the canonical binding site located at the β(+)- α(-) interface is likely to be the one driving the activity of potentiating neurosteroids (see review for more detail Mortensen et al. (2024). Potentiating neurosteroid action is mediated by the canonical site, with modulation critically influenced by the Q241 residue. A mutation at this site could be employed to create knock-in mice lacking neurosteroid sensitivity in specific $GABA_A$ receptor subunits. These knock-in mouse lines enable the physiological roles played by endogenous positive allosteric neurosteroids when modulating specific $GABA_A$ receptor subtypes to be deduced and explored with precision.

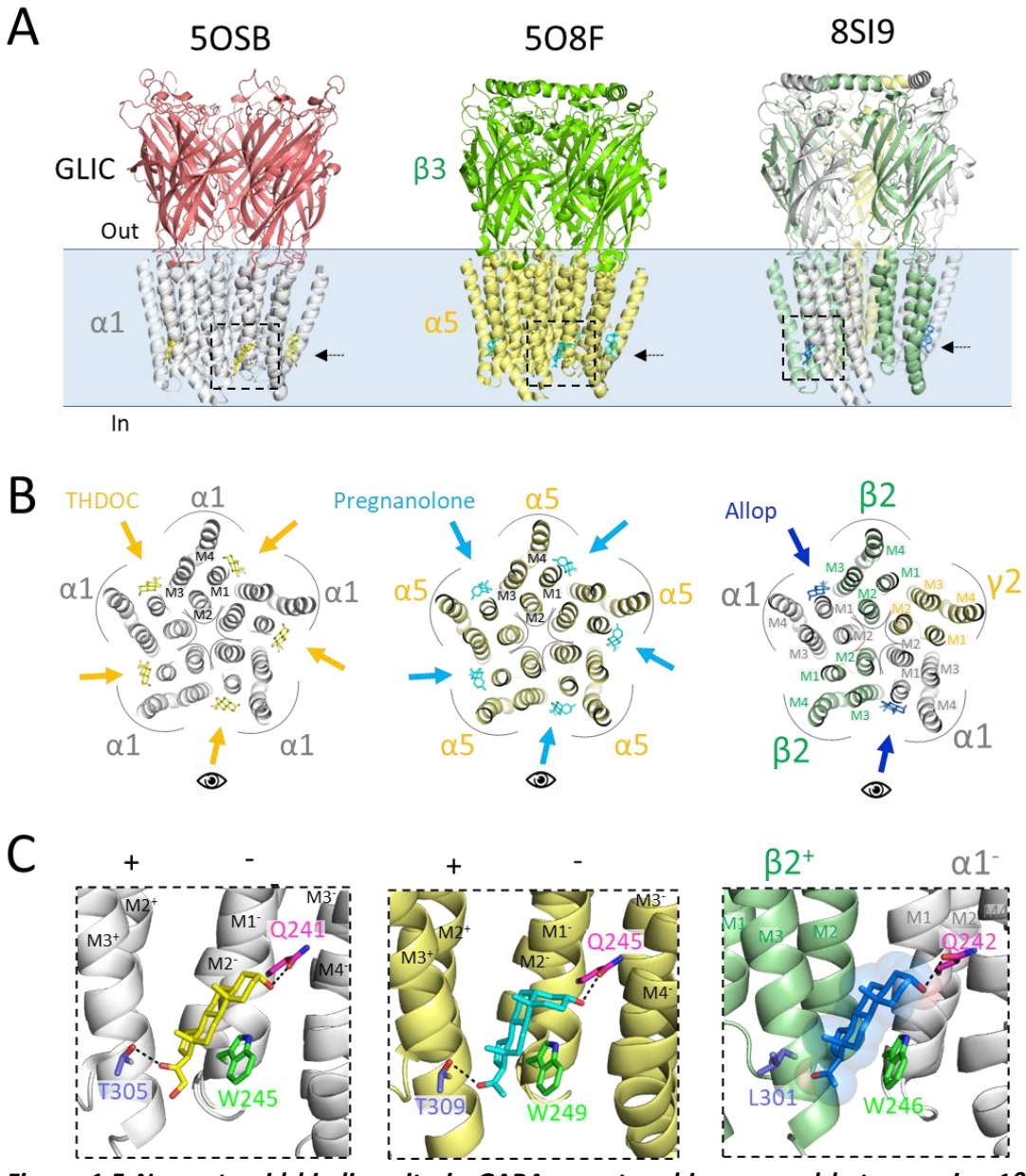


Figure 1.5 Neurosteroid binding site in GABA receptor chimeras and heteromeric α1β2γ2 GABA$_A$ receptors.

A, Side views of the two chimeric homo-pentameric structures: GLIC (red) – α1 GABA (grey; left side panel) with THDOC bound (yellow; PDB: 5OSB; (Laverty et al., 2017)) and GABA α5 TMD (yellow) – β3 ECD (green; middle panel) with pregnanolone bound (bright blue; PDB: 5O8F; (Miller et al., 2017)) and side view of the recombinant human α1(white) β2(green) γ2(yellow) GABA$_A$ receptor with allopregnanolone (Allop, dark blue) bound (PDB 8SI9; (Legesse et al., 2023)). ***B,*** Cross-section slices of the TMD taken at the canonical binding site showing the potentiating neurosteroids bound. Note that for the chimeras (left and middle panels) five neurosteroid molecules are bound as these are homomeric structures, whereas there are only two allopregnanolone molecules bound for the heteromeric receptor (right panel), which is consistent with the canonical sites being located at the β(+)- α(-) interface. ***C,*** High resolution side view of the binding site (see 'eye' in panel B for position) showing the key residues identified for neurosteroid binding: (α-Q241/5/2; α-W245/9/6; α-T305/9 (this threonine residue would be located in the β subunit in a heteromeric receptor) and β-L301)). Hydrogen bonds formed between the steroid and the glutamine residue are shown by a black dashed line. The figure was adapted from Mortensen et al. (2024).

1.2.4 Physiological modulation of $GABA_A$ receptors by neurosteroids

Potentiating neurosteroids, such as allopregnanolone and THDOC, enhance GABA currents by prolonging the decay of IPSCs (Harney et al., 2003, Belelli and Herd, 2003) and/or increasing the size of tonic currents (Stell et al., 2003). Low nanomolar concentrations of neurosteroids are sufficient for the slowing of decay times in various cell types, including CA1 hippocampal neurons (Harney et al., 2003, Lu et al., 2023), dentate gyrus granule cells (DGGCs) (Lu et al., 2020), hippocampal parvalbumin interneurons (Lu et al., 2023), cerebellar granule cells (Vicini et al., 2002) and Purkinje neurons (Cooper et al., 1999). However, hypothalamic neurons require micromolar concentrations to produce the same effect (Brussaard et al., 1997, Koksma et al., 2003). Furthermore, removing neurosteroid sensitivity from α2-$GABA_A$ receptors results in faster decay kinetics in both hippocampal CA1 neurons and dentate gyrus granule cells, which indicates basal prolongation by neurosteroids (Durkin et al., 2018). Additionally, decreasing endogenous neurosteroid levels in neocortical slices using SKF-10511 (a potent 5α-reductase inhibitor) significantly reduces IPSC decay times (Puia et al., 2003).

Ablating neurosteroid sensitivity from α4-containing $GABA_A$ receptors has no effect on IPSC kinetics in any of the following neurons: dentate gyrus granule cells, CA1 hippocampal neurons, medium spiny neurons in either nucleus accumbens or dorsal striatum (Minère, 2019). This is not surprising considering that α4-containing $GABA_A$ receptors most commonly assemble with the δ subunit, and these receptors are thought to be mainly involved in mediating tonic currents (Farrant and Nusser, 2005). Pharmacological and genetic deletions of δ or its preferred assembly partners (α4 and α6), has little to no impact on IPSC parameters (Brickley and Mody, 2012, Wei et al., 2003, Herd et al., 2013).

However, this classical viewpoint has recently been challenged by a new chemogenetic approach whereby populations of γ2- and δ-$GABA_A$Rs can be studied separately (Sun et al., 2018). Sun and colleagues introduced a point mutation in both the γ2 and δ subunits, which renders them picrotoxin resistant, allowing the isolation of their contribution to both phasic and tonic currents. They found that δ-containing $GABA_A$ receptors contribute to IPSCs (~10-15 %) in dentate gyrus granule cells (Sun et al., 2018). This

chemogenetic approach also showed that despite the δ subunit's contribution to phasic inhibition, the prolongation of GABA-mediated IPSCs by allopregnanolone in DGGCs is mainly driven by γ2-containing receptors (Lu et al., 2020), which also confirms the observations of others (Minère, 2019, Stell et al., 2003).

Neurosteroids, allopregnanolone and THDOC, also enhance GABA-mediated tonic currents in DGGCs and cerebellar granule cells (Farrant and Nusser, 2005, Stell et al., 2003). Removing neurosteroid sensitivity from α4-containing $GABA_A$ receptors showed that the α4 subunit is a key player in the modulation of tonic inhibition by neurosteroids, as the enhancement of tonic currents by THDOC was significantly reduced in neurosteroid insensitive mice compared to wild type across several different brain regions, including DGGCs, CA1, thalamus, dorsal striatum and nucleus accumbens (Minère, 2019). The contribution of α4-containing $GABA_A$ receptors to tonic currents ranges from 55 % to 90 % depending on the brain area (Minère, 2019). γ2- and δ-$GABA_A$Rs show an equal contribution to tonic currents potentiated by neurosteroids in DGGCs (Lu et al., 2020), which is in line with Minère (2019) who reported an approximately 60 % contribution by α4-containing $GABA_A$ receptors.

The effects of neurosteroids are dependent upon the subunit composition of the receptor – receptors containing the δ subunit show a much stronger GABA-modulatory effect by neurosteroids than γ2-containing ones (Belelli et al., 2002, Wohlfarth et al., 2002, Brown et al., 2002). Traditionally, this subunit-specific pharmacology would suggest that neurosteroids preferentially bind to receptors containing δ subunits. However, this selectivity is largely due to the functional characteristics, rather than the steroid-binding properties of δ-subunit-containing receptors, as the δ subunit makes no contribution to the canonical neurosteroid binding site. δ-$GABA_A$Rs exhibit a high affinity but low efficacy for GABA. Compared to agonists like tetrahydroisoxazolopyridinol (THIP), GABA acts only as a partial agonist, producing a lower maximal effect (Bianchi and Macdonald, 2003). Studies comparing the agonist-enhancing effects of THDOC with both full and partial agonists on receptors containing δ- and γ-subunits reveal that THDOC selectively enhances the low-efficacy gating induced by partial agonists, regardless of the receptor isoform (Bianchi and Macdonald, 2003).

There are many post-translation modifications that can affect $GABA_A$ receptor function, localisation and modulation; and phosphorylation appears to be particularly important. The effects of phosphorylation on $GABA_A$ receptor function are intricate, depending on the specific kinase or phosphatase isoforms, receptor subunit compositions and phosphorylated amino acid residues (Nakamura et al., 2015). In some instances, phosphorylation targets may include receptor-associated proteins rather than the receptor itself. The interaction between pregnane steroids and $GABA_A$Rs can be dynamically regulated by the activity of kinases and phosphatases, as observed in magnocellular oxytocin neurons of the hypothalamus (Brussaard et al., 1997, Koksma et al., 2003). Prior to parturition, these neurons' synaptic $GABA_A$ receptors are steroid-sensitive, a sensitivity that can be altered by manipulating kinase and phosphatase activities (Koksma et al., 2003). After parturition, steroid insensitivity can be reversed through phosphatase stimulation or protein kinase C (PKC) inhibition (Koksma et al., 2003).

In contrast, hippocampal neurons exhibit enhanced neurosteroid interactions with $GABA_A$ receptors through phosphorylation. Specifically, synaptic $GABA_A$ receptors in hippocampal CA1 neurons are more sensitive to pregnanolone than DGGCs (Harney et al., 2003). PKC stimulation in DGGCs enhances the prolongation of IPSCs by neurosteroids, whereas PKC inhibition reduces these effects in CA1 neurons (Harney et al., 2003), indicating phosphorylation's role in this differential sensitivity.

Studies reveal that the PKCε isoform is involved in neurosteroid and $GABA_A$ receptor plasticity (Hodge et al., 2002, Hodge et al., 1999, Song and Messing, 2005). Mice lacking PKC_ε ($Pkc_\varepsilon^{-/-}$) exhibit increased behavioural sensitivity to neurosteroids and other modulators, including benzodiazepines and alcohol, showing reduced anxiety compared to wild type mice (Hodge et al., 2002). This is linked to $GABA_A$ receptor function, as both benzodiazepines and neurosteroids more effectively enhance GABA-induced chloride uptake in cortical microsacs from $Pkc_\varepsilon^{-/-}$ mice than wild type mice (Hodge et al., 2002, Hodge et al., 1999).

Overall, phosphorylation contributes to the variability of neurosteroid interactions with $GABA_A$ receptors by potentially altering binding or modifying ion-channel kinetics, though the precise molecular targets and mechanisms remain to be fully elucidated.

1.3 Alcohol and GABAergic neurotransmission

1.3.1 Alcohol and related disorders

Alcohol is the most commonly abused drug in our society. Its misuse impacts not only public health but also economic output, costing billions of dollars annually in lost productivity (Rehm et al., 2009). Long-term alcohol abuse leads to premature death and a higher risk of serious illnesses (Shield et al., 2013). Moreover, foetal alcohol syndrome, a major global health issue, causes lifelong health problems (Popova et al., 2023). Additionally, alcohol can exhibit dangerous pharmacokinetic and pharmacodynamic interactions with other drugs, potentially resulting in fatal outcomes (Poikolainen, 1984, Sellers and Busto, 1982, Tanaka, 2003).

Ethanol (EtOH), the main alcohol present in alcoholic drinks, affects humans in various ways. It is a central nervous system depressant, sharing effects with other CNS depressants like sedatives, hypnotics, and anaesthetics. Despite its significant impact on the CNS, ethanol is not particularly potent; noticeable effects usually require relatively high blood concentrations (5–10 mM). However, consuming a single strong drink can introduce up to 12 grams of ethanol into the body, quickly reaching these concentrations.

The effects of ethanol are well documented (*Table 1.1*). Acute intoxication grows progressively stronger as blood ethanol concentrations rise. At low blood levels, it induces euphoria or disinhibition. As levels rise, motor function deteriorates and speech becomes slurred. Blood ethanol concentrations between 40 and 65 mM can cause vomiting and stupor. Higher concentrations may lead to coma, and at above 110 mM, there is a risk of respiratory failure and death.

Table 1.1 Blood ethanol concentrations (mM) and clinical effects in individuals
The table was adapted from Trevor (2018).

Blood ethanol concentration (mM)	Clinical effects
10-20	Disinhibition, sedation, slower reaction times
20-40	Impaired motor function, slurred speech, ataxia
40-65	Vomiting, stupor
65-110	Coma
> 110	Respiratory depression, death

Chronic ethanol exposure and alcohol use disorder (AUD) have even more profound negative impact on society, leading to loss of employment, psychiatric symptoms, liver failure and severe cognitive impairments (Abrahao et al., 2017). These chronic issues require substantial resources for psychiatric care, organ transplants, and long-term medical treatment. As tolerance to ethanol's acute effects develops, individuals can survive with blood ethanol concentrations up to eight times higher than those lethal to someone without such tolerance. There have even been reports of awake individuals with blood ethanol concentrations near 300 mM (Johnson et al., 1982).

Previously, it was believed that ethanol's effects were due to non-specific disruption of neuronal lipid bilayers (Ingólfsson and Andersen, 2011). However, it is now widely accepted that ethanol acts by binding to and altering the function of specific proteins, especially membrane-bound ligand-gated ion channels and voltage-dependent ion channels (*Figure 1.6*) (Abrahao et al., 2017). There is also growing evidence that ethanol affects the function of second-messenger proteins, including PKC (Pandey, 1998, Macdonald, 1995).

$GABA_A$ receptors have long been implicated in mediating the effects of ethanol in the mammalian brain. The molecular mechanisms underlying the actions of intoxicating doses of ethanol have always been of particular interest. Recently, ethanol research has shifted towards identifying specific receptor targets. Several ligand-gated ion channels, including glutamate receptors (Frye and Fincher, 2000, Lovinger et al., 1989), serotonin

5-HT_3 receptors (Lovinger and White, 1991), purinergic receptors (Davies et al., 2002), $GABA_A$Rs (Aguayo et al., 2002, Roberto et al., 2003), and G-protein coupled inwardly rectifying K^+ channels (Kobayashi et al., 1999), as well as voltage-gated ion channels like large conductance potassium (BK) channels (Davies et al., 2003), are implicated in mediating ethanol's actions in the brain. The mechanism of action of ethanol is not well established and whether ethanol acts directly or indirectly on these receptors is also controversial.

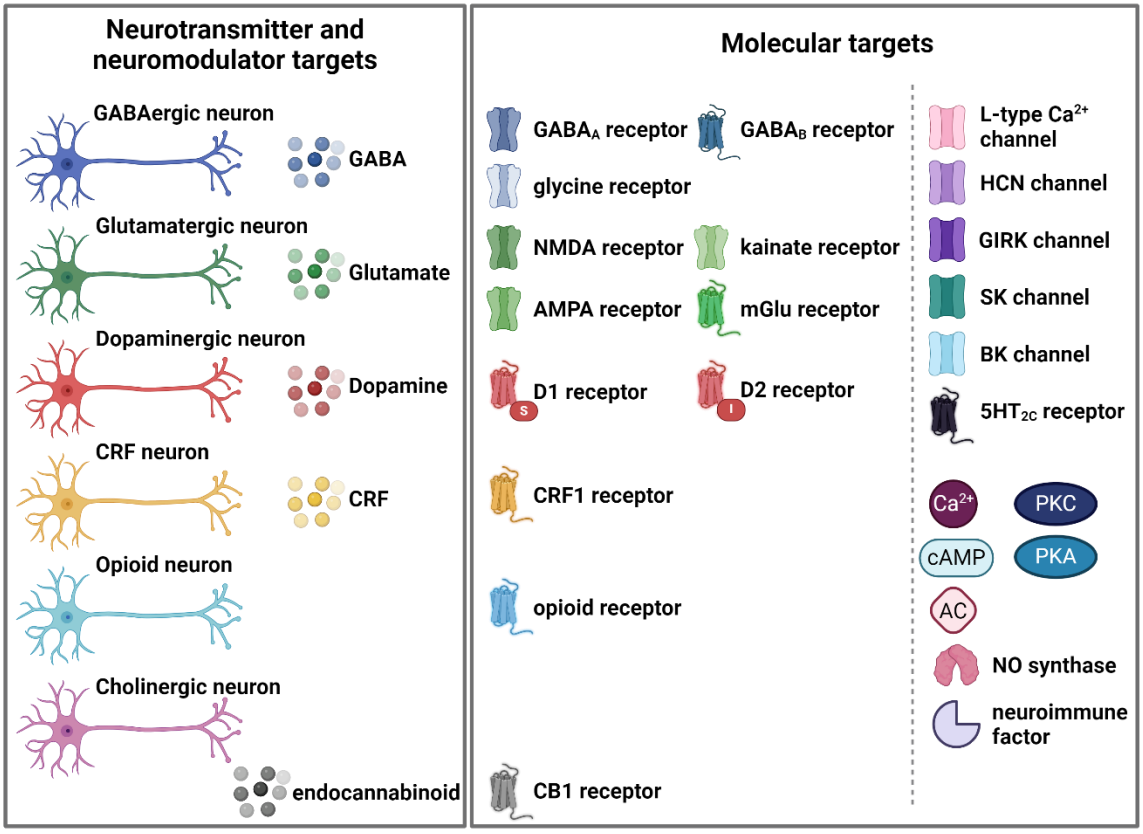


Figure 1.6 Molecular targets of ethanol

The diagram summarises all of ethanol's targets – including ionotropic and metabotropic receptors, ion channels and enzymes. The figure was adapted from Abrahao et al. (2017); see review for more detail. CRF: corticotropin releasing factor, NMDA: N-methyl-D-aspartate, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, mGlu: metabotropic glutamate, D1/2: dopamine 1/2, CB1: cannabinoid receptor 1,HCN: hyperpolarization-activated cyclic nucleotide–gated, GIRK: G-protein coupled inwardly rectifying K^+ channels, SK: small-conductance calcium-activated potassium, BK: large conductance potassium channel, 5HT: 5-hydroxytryptamine, PKC: protein kinase C, PKA: protein kinase A, cAMP: cyclic adenosine monophosphate, AC: adenylyl cyclase, NO: nitric oxide.

The mechanisms by which excessive ethanol consumption leads to alterations in the human brain that result in alcohol dependence remain unclear. The development of AUD is a chronic and complex process. Ethanol affects brain function by interacting with multiple neurotransmitter systems, notably disrupting the balance between excitation (driven mainly by glutamate) and inhibition (mediated mostly by GABA) (Valenzuela, 1997). Short-term alcohol exposure shifts this balance towards inhibition, while long-term exposure prompts the brain to compensate, attempting to restore equilibrium. These neurobiological changes manifest behaviourally – there is build-up of tolerance to the sedative effects of ethanol. When ethanol consumption is abruptly stopped, these compensatory changes are no longer counteracted by ethanol, leading to too much excitation and the development of alcohol withdrawal syndrome (Bayard et al., 2004).

Chronic alcohol consumption induces tolerance, defined as the reduced physiological and behavioural effects of alcohol due to repeated exposure. Metabolic tolerance occurs when alcohol is metabolised at an accelerated rate, requiring higher quantities to achieve comparable effects; this results from the body's increased efficiency in lowering blood alcohol concentrations through upregulated enzymatic pathways (Cederbaum, 2012). Functional tolerance, in contrast, involves adaptive changes in the nervous system's sensitivity to alcohol. Neurons may increase excitatory responses to counterbalance alcohol's inhibitory effects, allowing continued functionality under alcohol’s influence.

The focus of this study is the $GABA_A$ receptor family, long considered a major target for EtOH. Therefore, this section will explore the action of ethanol on $GABA_A$ receptors in greater detail. Ethanol is known to have a GABA-mimetic effect, though some significant effects on $GABA_A$R-mediated inhibition may be presynaptic (Breese et al., 2006, Weiner and Valenzuela, 2006, Siggins et al., 2005). Nonetheless, substantial evidence supports the modulatory/direct action of ethanol on $GABA_A$Rs.

1.3.2 Effects of ethanol on $GABA_A$ receptors

The understanding that ethanol mediates some of its effects through $GABA_A$ receptors has evolved over decades, with evidence from various scientific disciplines converging to support this conclusion. Early research in the 1970s and 1980s provided foundational insights into the role of GABAergic transmission in ethanol's effects. Initial studies observed that ethanol exhibited similar behavioural effects to those of GABAergic agonists, such as benzodiazepines and barbiturates, suggesting a similar mechanism of action (Ticku, 1989, Martz et al., 1983, Frye et al., 1979, Cooper et al., 1992).

Electrophysiological studies in the 1980s were pivotal in identifying ethanol's effects on $GABA_AR$ function. These studies demonstrated that ethanol enhances GABA-induced chloride currents in neurons in a dose-dependent manner (Nestoros, 1980, Bloom and Siggins, 1987). Subsequent *in vivo* electrophysiological studies revealed region-specific effects of ethanol on neuronal firing, with significant findings demonstrating ethanol's enhancement of GABA's inhibitory action in medial septal neurons, but not lateral septal neurons (Criswell et al., 1995, Givens and Breese, 1990b, Givens and Breese, 1990a, Bloom and Siggins, 1987). Wafford et al. (1991) showed that transfection of neuronal cultures with specific $GABA_A$ receptor subunits (α1β1γ2L) allowed ethanol to enhance GABA responses, whereas receptors containing the shorter version of γ2 (γ2S) were not affected, suggesting that subunit composition dictates ethanol sensitivity, in particular an 8 residue insert in γ2L that carries a PKC phosphorylation consensus sequence.

However, *in vitro* studies presented conflicting evidence. Siggins et al. (1987) and Palmer and Hoffer (1990) reported no direct ethanol effect on $GABA_A$ receptors in neurons from regions responsive to ethanol *in vivo*. Frye et al. (1994) and others found ethanol enhancement of GABA function only at lethal concentrations or not at all (Criswell et al., 2003, Mori et al., 2000, Peoples and Weight, 1999). Thus, it was concluded that the mechanism by which ethanol enhances GABA function is not present in isolated neurons. This conclusion aligns with the idea that ethanol's effects are mediated through more complex, region-specific neural pathways rather than solely through direct interaction with $GABA_A$ receptors (Criswell and Breese, 2005).

The following section aims to delineate the multiple mechanisms through which ethanol exhibits its GABA-mimetic effects. These mechanisms encompass: (1) the direct interaction of ethanol with specific δ-containing $GABA_A$ receptor subtypes, (2) ethanol's modulation of neural circuits that regulate GABAergic transmission, (3) ethanol-induced presynaptic release of GABA, (4) the elevation of neurosteroid levels in the brain by ethanol, which can modulate $GABA_A$ receptor function. These actions collectively contribute to ethanol's complex modulation of GABAergic signalling.

1. *Direct activation*

The debate over whether ethanol directly activates $GABA_A$ receptors, particularly those containing δ subunit, has been a significant area of research and controversy. The initial interest in this topic emerged from electrophysiological studies in the 1980s that showed that ethanol enhanced chloride influx through the channel in brain synaptosomes (Allan and Harris, 1986, Morrow et al., 1988, Suzdak et al., 1988) and cultured neurons (Ticku and Burch, 1980).

Several studies have demonstrated that recombinant $GABA_A$ receptors comprising specific subunit combinations exhibit sensitivity to ethanol at concentrations comparable to those achieved during moderate social drinking (3-30 mM) (Wallner et al., 2003, Sundstrom-Poromaa et al., 2002, Hanchar et al., 2005). These findings indicate that certain subunit compositions, particularly those involving the δ subunit, are responsive to ethanol within the physiological range of blood ethanol concentrations.

γ-containing $GABA_A$ receptors typically exhibit a response to ethanol only at concentrations exceeding 100 mM, if they respond at all (Wallner et al., 2006b). In contrast, $GABA_A$ receptors composed of α1, α4, or α6 subunits, in combination with β3 subunits and a δ subunit, exhibit unique sensitivity to ethanol.

Cerebellar granule cells expressing α6βδ (Hanchar et al., 2005), dentate gyrus granule cells expressing α4βδ (Wei et al., 2004, Liang et al., 2006, Fleming et al., 2007), medium spiny neurons in nucleus accumbens also expressing α4βδ (Liang et al., 2014) and hippocampal interneurons expressing the novel α1βδ assembly (Glykys et al., 2007)

display substantial increases in tonic currents under the influence of 'low dose' ethanol. This enhancement of tonic GABA currents is noticeable even at low concentrations of ethanol (< 10 mM) (Hanchar et al., 2005). However, there is no effect on $GABA_AR$-mediated synaptic responses at ethanol concentrations below 100 mM.

In specific brain regions, ethanol can trigger increased release of GABA (Carta et al., 2004, Hanchar et al., 2005), which may elevate tonic GABA current indirectly by elevating ambient GABA concentration rather than directly modulating postsynaptic $GABA_ARs$. However, the increase in tonic current persists even in the presence of tetrodotoxin (TTX), which blocks presynaptic activity, and when GABA levels are stabilised with added GABA and uptake blockers, suggesting that ethanol acts directly on native extrasynaptic $GABA_ARs$ containing α6 subunits (Hanchar et al., 2005). Furthermore, mice carrying ethanol hypersensitive polymorphism in the α6 subunit, show significantly increased tonic currents (Hanchar et al., 2005). Since this subunit is exclusively expressed postsynaptically in granule cells, these effects are inferred to be postsynaptic. Overall, these findings strongly support the notion that ethanol directly influences extrasynaptic $GABA_ARs$ on cerebellar granule cells.

Furthermore, it was shown that a benzodiazepine derivative, Ro15-4513, binds with high affinity to both recombinant and native δ-$GABA_ARs$ (Hanchar et al., 2006), potentially uncovering a previously uncharacterised benzodiazepine binding site on extrasynaptic receptors. Competition studies demonstrated that ethanol is able to displace Ro15-4513 in recombinant α4βδ receptors and in immunopurified δ-$GABA_ARs$, but not in γ-$GABA_ARs$ (Wallner et al., 2006a, Hanchar et al., 2006). Moreover, Ro15-4513 (300 nM) reverses the effects induced by ethanol (30 mM) in α4βδ receptors expressed in *Xenopus* oocytes. Ro15-4513 was also reported to reduce ethanol's anxiolytic and intoxicating effects (Suzdak et al., 1986, Paul, 2006). These results reinforced the idea that ethanol directly targets δ subunit-containing $GABA_A$ receptors, rather than acting through an unknown intermediate protein.

However, other groups have failed to replicate these findings (Yamashita et al., 2006, Valenzuela et al., 2005, Casagrande et al., 2007, Borghese et al., 2006). Borghese et al. (2006), which was a collaborative effort from four independent labs, found that rat and

human α4βδ receptors expressed in *Xenopus* oocytes and human α4βδ receptors in mammalian cell lines are only responsive to high doses of ethanol (> 100 mM). Furthermore, low dose ethanol had no impact on GABA-mediated chloride currents in α4/6βδ receptors expressed in Chinese hamster ovary cells (Yamashita et al., 2006). Additionally, these studies have also claimed that native $GABA_A$Rs containing the δ subunit in DGGCs (Borghese et al., 2006), cerebellar granule cells (Carta et al., 2004) or in cultured neurons are not directly sensitive to ethanol (Yamashita et al., 2006, Casagrande et al., 2007).

The discrepancies between the recombinant findings could potentially be explained by receptor subunit compositions; either the lack of δ subunit expression (in the Yamashita et al. paper) or the presence of a different, higher efficacy form of δ-$GABA_A$Rs (Borghese et al. (2006) observe much higher currents and different EC_{50} values compared to the original papers). The findings about native receptors are harder to reconcile – potential sources for differences include different preparations (Borghese et al. reported much larger mIPSCs than other studies under similar conditions) or the age of animals/ different stages of maturation (in cultured neurons P7-14 tonic current may have only reached one-third of its adult level, as tonic currents have been shown to increase five-fold between post-natal days 7 and 35 (Brickley et al., 2001, Brickley et al., 1996)). Another factor that might contribute to the differing observations on ethanol's effects on $GABA_A$ receptor currents is the phosphorylation status of the $GABA_A$ receptor. Choi et al. (2008) found that the enhancement of tonic GABA currents mediated by α4δ-$GABA_A$Rs requires phosphorylation by PKCδ. Knocking out PKCδ prevented ethanol from enhancing tonic GABA currents in the hippocampus and thalamus and reduced behavioural sensitivity to ethanol. While the reasons for varying phosphorylation states of $GABA_A$ receptors across different laboratories remain unclear, this mechanism could offer insights into the inconsistent findings concerning ethanol's direct effects on $GABA_A$ receptor currents. Interestingly, while PKCδ expression aligns with $GABA_A$ δ subunit expression in the hippocampus and thalamus, it is absent in the cerebellar granule cell layer. This suggests that if phosphorylation status accounts for the discrepancies in ethanol-induced potentiation of tonic GABA currents, alternative kinases beyond PKCδ might play a role in cerebellar granule cells.

2. Modulation of GABAergic interneurons

Recent research has utilised brain slices and electrophysiology to study the acute effects of ethanol on GABAergic interneurons in the hippocampus and cerebellum. In the hippocampus, kainate receptors, which are part of the glutamate-gated ion channel family, modulate interneuronal excitability in the CA1 region. Kainate receptor activation increases interneuronal firing, which leads to a significant increase in frequency of spontaneous IPSCs in pyramidal neurons. It was demonstrated that moderate concentrations of ethanol (20-80 mM) inhibit kainate receptor-mediated IPSCs in CA1 pyramidal neurons, indicating that these receptors are sensitive to ethanol (Crowder et al., 2002). Ethanol did not affect AMPA receptor activation or action potential firing triggered by AMPA receptor-mediated depolarising responses. Without kainate, ethanol did not alter spontaneous IPSC parameters in CA1 pyramidal neurons (Carta et al., 2003). This suggests that ethanol increases CA1 pyramidal neuron excitability indirectly by inhibiting kainate receptor-driven GABAergic interneurons, potentially explaining ethanol's paradoxical excitatory effects as a CNS depressant.

In the cerebellum, ethanol modulates GABAergic transmission to granule cells (Carta et al., 2004). These cells receive GABAergic input from Golgi cells in the form of phasic and tonic currents mediated by synaptic receptors (α1βγ) and extrasynaptic receptors (α6βδ), respectively. Ethanol (20 mM) increases the frequency of spontaneous IPSCs but not their amplitude, a finding corroborated by Hanchar et al. (2005). Ethanol also increases tonic currents at this concentration. However, in the presence of TTX, which blocks presynaptic activity, ethanol did not significantly alter tonic current amplitude. This suggests that under the given recording conditions, ethanol does not directly impact extrasynaptic $GABA_A$ receptors. This finding is in contrast with Hanchar et al. (2005) but the two studies employed different experimental conditions (difference in supplementation of GABA and GABA uptake blockers), which might in part explain the discrepancy.

Additionally, studies examined ethanol's modulation of molecular layer interneuronal function. Recordings from Purkinje neurons show that ethanol increases quantal GABA release from molecular layer interneurons without affecting postsynaptic $GABA_A$ receptor function (Carta et al., 2006, Criswell and Breese, 2005, Criswell et al., 2003).

Ethanol has minimal impact on the spontaneous firing of Purkinje neurons; however, it reduces the amplitude of excitatory postsynaptic potentials evoked by stimulating granule cell axons, which is dependent on $GABA_A$ receptor function as it is blocked by bicuculline (Mameli et al., 2008).

In summary, ethanol has varied effects on interneurons across different brain regions. In the cerebellum, it increases GABAergic input to granule cells by enhancing Golgi cell firing (Carta et al., 2004, Hanchar et al., 2005). In the Purkinje cell layer, it increases GABA release in an action potential-independent manner (Ming et al., 2006, Mameli et al., 2008, Carta et al., 2006, Criswell and Breese, 2005, Criswell et al., 2003). In the hippocampus, ethanol reduces excitability to CA1 interneurons by reducing kainate receptor activation, leading to disinhibition of pyramidal neurons. Thus, ethanol's impact on GABAergic interneurons varies by mechanism and effect.

3. Presynaptic effects

Recent studies have demonstrated that ethanol enhances the release of GABA from presynaptic terminals, contributing to increased synaptic inhibition. Enhanced fast GABAergic synaptic transmission during ethanol exposure has been observed in several brain regions, including the ventral tegmental area (Theile et al., 2008), cerebellum (Kelm et al., 2007, Ming et al., 2006), hippocampus (Ariwodola and Weiner, 2004) and amygdala (Zhu and Lovinger, 2006, Roberto et al., 2003). These studies reported increased miniature and/or spontaneous IPSC frequencies (indicative of presynaptic changes) in the presence of ethanol; these effects reverse after drug wash-out.

The precise mechanism by which ethanol potentiates GABA release is not known, however, several potential mechanisms have been suggested. Some studies proposed that ethanol affects intracellular calcium release, leading to increased Ca^{2+} concentrations in the presynaptic terminal (Theile et al., 2009, Kelm et al., 2007).

The role of intracellular signalling pathways in the potentiating effects of ethanol has been extensively studied. Activation of adenylate cyclase (AC) or protein kinase C (PKC) is known to enhance synaptic transmission throughout the nervous system (Leenders

and Sheng, 2005). Consequently, it is reasonable to propose that these key signalling enzymes could be involved in the acute effects of alcohol. Studies have shown that the increase in GABA release onto cerebellar Purkinje neurons is prevented by inhibitors of AC and protein kinase A (PKA) (Kelm et al., 2008), and is also influenced by substances targeting phospholipase C and PKC (Kelm et al., 2010). In the basolateral amygdala, ethanol potentiation was blocked by AC, but not PKA, inhibitors (Talani and Lovinger, 2015). In the central amygdala, the enhancing effect of ethanol is diminished in mice that lack PKCε (Bajo et al., 2008). Interestingly, in PKCε knockout mice, GABA release is already elevated even before ethanol exposure (Bajo et al., 2008). In cerebellar granule cells, ethanol-induced GABA release is blocked via inhibition of nitric oxide synthase (Kaplan et al., 2013). It is still unclear whether ethanol affects these key signalling enzymes directly or indirectly.

Presynaptic effects of ethanol at certain synapses occur indirectly through the release of neuromodulators that directly mediate increased vesicle fusion. For example, the central amygdala contains various neuropeptides, such as corticotropin-releasing factor (CRF), that are influenced by ethanol. These peptides play a pivotal role in processing aversive stimuli, thus rendering the central amygdala crucial in the negative emotional states associated with substance (including alcohol) abuse and addiction (Koob, 2015). CRF enhances GABAergic transmission in the central amygdala via presynaptic CRF1 receptors (Roberto et al., 2010, Cruz et al., 2012, Bajo et al., 2008). Acute ethanol exposure potentiates this transmission, indicating that ethanol indirectly increases GABA release by promoting local CRF release (Bajo et al., 2008, Nie et al., 2004). Conversely, in the dorsolateral striatum, ethanol reduces GABA release by increasing enkephalin release and activating presynaptic δ-opioid receptors (which negatively couple to adenylyl cyclase to suppress GABA release) (Patton et al., 2016). Consequently, ethanol-induced neuropeptide release modulates GABA release in a synapse-specific manner.

4. Neurosteroid modulation of ethanol actions

Another mechanism, and of most relevance to this project, and likely to be of major importance, by which ethanol exerts its functional effect on $GABA_A$Rs is proposed to be mediated by neuroactive steroids. The effects of acute ethanol administration, such as

anxiolysis, sedation, hypnosis, are similar to the effects of $GABA_A$ receptor modulators, including neurosteroids. Therefore, it was hypothesised that ethanol may exert some of its actions indirectly through the modulation of neurosteroid levels and their subsequent modulation of $GABA_A$ receptors.

There is extensive evidence indicating the involvement of neurosteroids in mediating and modulating the effects of ethanol, supported by findings from diverse experimental approaches and methodologies.

Firstly, acute ethanol administration increases the concentration of THDOC and allopregnanolone in the plasma, cerebral cortex and hippocampus, in a time- and dose-dependent manner (Barbaccia et al., 1999, Khisti et al., 2005, O'Dell et al., 2004, VanDoren et al., 2000). Furthermore, oral consumption of ethanol leads to an increase of allopregnanolone levels in the brain of C57BL/J6 male mice (Finn et al., 2004b). In humans, alcohol intoxication increases allopregnanolone levels in adolescent male and female subjects (Torres and Ortega, 2003, Torres and Ortega, 2004).

Secondly, a number of studies demonstrate that exogenous administration of allopregnanolone or its synthetic analogue, ganaxolone, increases ethanol consumption in mice (Ramaker et al., 2014, Morrow et al., 2001a, Ford et al., 2005) and in rats (Nie and Janak, 2003, Janak and Michael Gill, 2003). Conversely, other studies provide evidence for decreased ethanol drinking following allopregnanolone or ganaxolone treatment (Besheer et al., 2010, Cook et al., 2014c, Ramaker et al., 2015).

Thirdly, manipulations of endogenous neurosteroid biosynthesis alter ethanol-induced effects. Pre-treatment of animals with finasteride (5α-reductase inhibitor), which inhibits the biosynthesis of allopregnanolone, reduces the extent of the ethanol-induced increase in neurosteroid levels (VanDoren et al., 2000, Khisti et al., 2002). Additionally, pre-administration of finasteride diminishes alcohol-induced inhibition in the hippocampus (Tokunaga et al., 2003).

Taken together, these findings suggest that increased levels of neurosteroids largely account for many of ethanol's GABAergic effects *in vivo*, and that the presence of neurosteroids may influence sensitivity to ethanol's behavioural effects.

One mechanism by which ethanol seems to exert its effects on neurosteroid levels involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis. In response to acute stress, the HPA axis is activated, leading to an increase in corticotropin-releasing hormone (CRH) release from the hypothalamus (Morrow et al., 2006) (*Figure 1.7*). This triggers the pituitary gland to release adrenocorticotropic hormone (ACTH), which then stimulates the adrenal cortex to produce glucocorticoids, neurosteroid precursors and neurosteroids. In humans and non-human primates, cortisol is the primary glucocorticoid, whereas corticosterone is the main congener in rodents (Joëls et al., 2018). These glucocorticoids provide negative feedback to the hypothalamus and pituitary. Additionally, GABAergic neuroactive steroids inhibit the production and release of CRH, the release of ACTH, and subsequently reduce levels of corticosterone in rodents (Patchev et al., 1996, Owens et al., 1992). The ability of neurosteroids to reduce HPA axis activation helps restore homeostasis after stress, a crucial process for mental health. Disruptions in this response are linked to various mood disorders, such as depression, post-traumatic stress disorder, and premenstrual dysphoric disorder (Morrow et al., 2006).

Adrenalectomy blocks the effects of ethanol on cerebral cortical allopregnanolone concentrations of rats (Korneyev et al., 1993, O'Dell et al., 2004), and these effects can be restored by the administration of 5α-DHP, a precursor of allopregnanolone (Khisti et al., 2003). Ethanol also leads to elevated plasma and brain neurosteroid levels by stimulating precursor synthesis. These precursors can readily cross the blood-brain barrier. Pregnenolone and progesterone levels are more rapidly increased than allopregnanolone following acute ethanol administration, supporting the idea that increased allopregnanolone levels may be a result of increased pregnenolone and progesterone (Korneyev and Costa, 1996, O'Dell et al., 2004). Ethanol also stimulates *de novo* steroidogenesis within the brain in neurons and glial cells (Chisari et al., 2010). Incubation of rat hippocampal brain minces and slices in ethanol increases neurosteroid biosynthesis, and can be inhibited by finasteride administration (Sanna et al., 2004, Tokuda et al., 2011).

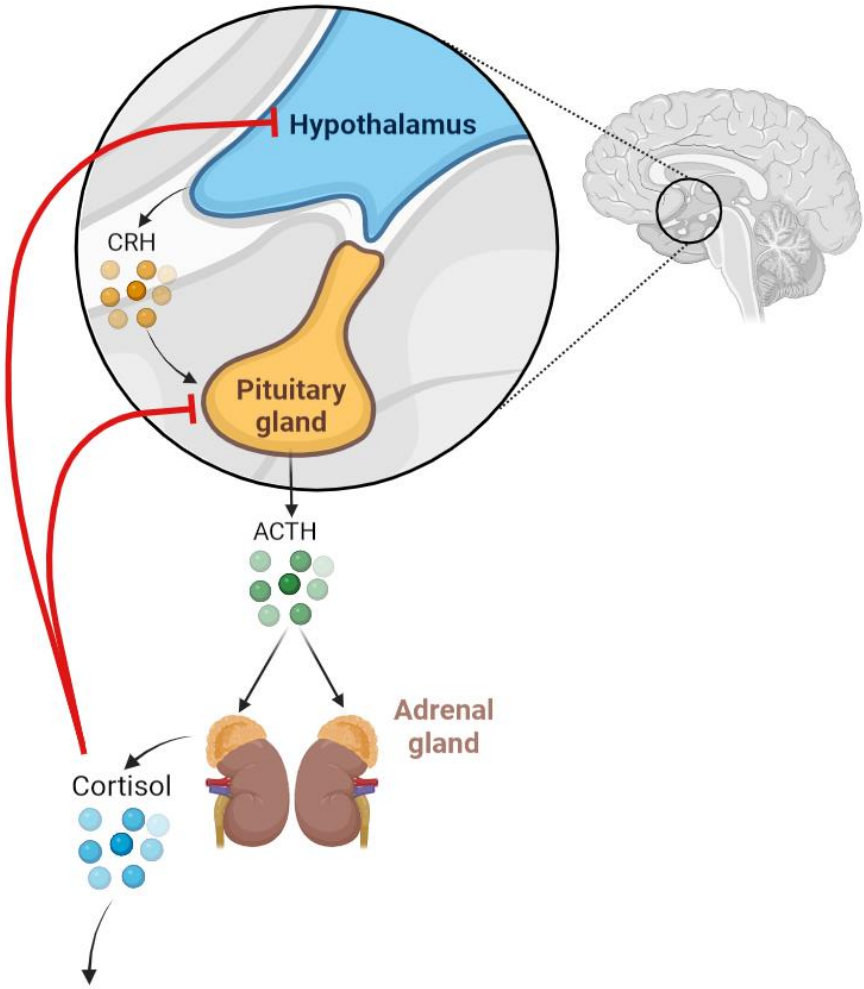


Figure 1.7 Hypothalamic-pituitary-adrenal (HPA) axis

In response to acute stress, the HPA axis is activated, resulting in increased corticotropin-releasing hormone (CRH) release from the hypothalamus. This prompts the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to produce glucocorticoids. In humans and non-human primates, cortisol is the primary glucocorticoid, while corticosterone predominates in rodents. These glucocorticoids provide negative feedback to the hypothalamus and pituitary. The red lines illustrate the negative feedback loops.

Sanna et al. (2004) provided direct evidence that neurosteroids contribute to ethanol's potentiation of GABAergic synapses. In their study using rat hippocampal slices, they found that ethanol at 50 and 100 mM concentrations significantly increased allopregnanolone levels within 20 minutes. Whole-cell patch-clamp recordings showed that 100 mM ethanol increased the amplitude of mIPSCs within 3 minutes, with a slight decrease at 10 minutes but remaining significant for 30 minutes of bath application of ethanol. Ethanol also significantly increased mIPSC frequency, but this presynaptic effect appeared only after 10 minutes. Pretreatment with finasteride abolished ethanol's

sustained effect on mIPSC amplitude after 3 minutes but did not affect the increase in mIPSC frequency. Similar transient effects were observed on evoked IPSCs in hippocampal slices, with initial potentiation diminishing within 10 minutes and then reappearing. Paired-pulse facilitation of evoked IPSCs indicated a sustained increase in release probability, consistent with the rise in mIPSC frequency. Finasteride pretreatment blocked the sustained effects of ethanol on evoked IPSCs but not the initial potentiation.

These findings suggest that ethanol interacts with neurosteroid biosynthesis and GABAergic transmission. Ethanol has a direct, transient postsynaptic effect on $GABA_A$ receptors, with acute tolerance developing within 10 minutes. A neurosteroid-mediated postsynaptic enhancement reemerges later, along with a sustained presynaptic facilitation of GABA release independent of neurosteroids. These results highlight the role of neurosteroid biosynthesis in ethanol's long-lasting effects on GABAergic synapses. Further research is needed to understand neurosteroid contributions to ethanol actions on GABAergic synapses in the hippocampus and other brain regions.

1.3.3 Roles of different α subunits in alcohol-related behaviours

Studies in the 1990s started to use genetically modified animals to elucidate the role of different $GABA_A$ receptor isoforms in mediating the effects of ethanol (Homanics et al., 1999, Homanics et al., 1998, Mihalek et al., 2001, Dixon et al., 2012, Homanics et al., 1997, Stephens et al., 2005). δ subunit knockout (δ-KO) mice showed a drastic reduction in neurosteroid sensitivity; thus, it was hypothesised that those mice may have altered behavioural responses to ethanol (Mihalek et al., 1999). Mihalek et al. (2001) showed that δ-KO mice significantly decreased their ethanol consumption and preference compared to their wild type counterparts. A similar impact was achieved by viral mediated RNAi knockdown of either α4 and δ subunit expression in the nucleus accumbens (NAc) shell of rats (Nie et al., 2011, Rewal et al., 2009). Moreover, regional knockdown of the δ subunit in the VTA lead to reduced ethanol drinking in female mice (Darnieder et al., 2019, Melón et al., 2017). Similarly, intraperitoneal injections of THIP and finasteride dose-dependently reduce ethanol consumption in male mice (Moore et

al., 2007, Ramaker et al., 2011, Ramaker et al., 2014). Furthermore, spontaneous mutations in the β1 subunit strongly associated with alcohol preference lead to increased tonic current in the NAc shell and increased ethanol self-administration (Anstee et al., 2013). However, global genetic deletion of α4 leads to increased ethanol consumption in mice (Olsen and Liang, 2017). The mechanism behind this is unclear, however, caution should be taken when interpreting global gene deletion studies. The knockout of α4 results in compensatory increases in the expression of other $GABA_AR$ subunits, and this change alters the pharmacological sensitivity of both synaptic and extrasynaptic $GABA_AR$ currents (Liang et al., 2008). Taken together, these studies suggest that α4βδ receptors, perhaps expressed in brain reward centres, play an important role in alcohol drinking behaviour.

Chronic ethanol exposure leads to a lasting reduction in α1 and δ subunits, while increasing α4 and γ2 subunit expression in the hippocampus (Liang et al., 2006). This observed shift in subunits (from α1 to α4 and from δ to γ2) suggests a decrease in primarily synaptic α1βγ2 $GABA_ARs$ and extrasynaptic α4βδ $GABA_ARs$, with an increase in α4βγ2 receptors. Additionally, the α4 subunit expression was increased at synapses shown by electron microscopy (Liang et al., 2006). While tonic current and its enhancement by ethanol are diminished, mIPSCs become responsive to ethanol enhancement (increase in charge transfer due to increase in decay times). These changes are accompanied by behavioural shifts consistent with alcohol dependence, such as heightened anxiety and susceptibility to seizures, and tolerance to the sedative-hypnotic effects of ethanol (Liang et al., 2009). Co-immunoprecipitation studies demonstrated that after acute ethanol intoxication and/or chronic exposure to ethanol, α2 and γ1 subunit expression is highly upregulated in the hippocampus, indicating that α2βγ1-$GABA_ARs$ could mediate the maintained anxiolytic response to ethanol in dependent individuals, contributing to elevated ethanol consumption (Lindemeyer et al., 2017).

Numerous studies have identified a link between alcohol dependence and variations in *GABRA2*, the gene encoding the $GABA_A$ α2 subunit (Bierut Laura et al., 2010, Covault et al., 2004, Edenberg et al., 2004, Li et al., 2014). Alcohol dependence in African Americans is also linked to variations in the $GABA_AR$ γ1 subunit gene *GABRG1* and *GABRA2* (Ittiwut

et al., 2012). Pierucci-Lagha et al. (2005) found that finasteride reduces the subjective effects of ethanol in both healthy subjects and individuals possessing single-nucleotide polymorphism in *GABRA2* associated with alcohol dependence. Mice harbouring the Q241M point substitution in the α2 subunit, which renders α2-$GABA_A$Rs insensitive to neurosteroids, show reduced ethanol intake and preference compared to wild type mice (Newman et al., 2016). Global deletion of α2 leads to decreased ethanol consumption in mice, which is apparent after three weeks (Olsen and Liang, 2017). This finding, however, is in conflict with other studies, where there were no differences in ethanol self-administration between wild type and $\alpha2^{-/-}$ mice (Dixon et al., 2012). The discrepancy between the two studies could be explained by the different methodologies employed, Olsen and Liang looked at chronic effects (weeks), whereas Dixon et al. only looked at the impact of ethanol acutely (< 1 hour). Collectively, these studies support the role of neurosteroids in mediating some of ethanol's effects and suggest that some of these effects may occur via α2-$GABA_A$Rs.

Considerable evidence indicates the involvement of neurosteroids in mediating and modulating the effects of ethanol, with substantial evidence implicating $GABA_A$ receptors, particularly the α2 and α4 subunits. However, comprehensive evidence linking these components together remains limited.

1.4 Thesis aims

1.4.1 Alcohol consumption of α2^{Q241M} and α4^{Q246M} mice

The present study aims to assess the behavioural impact on ethanol consumption following the removal of neurosteroid sensitivity from distinct $GABA_AR$ populations. To achieve this, we have used two novel knock-in mouse lines, α2^{Q241M} and α4^{Q246M}, which express a targeted point substitution of glutamine to methionine within these $GABA_A$ subunits, thus rendering them insensitive to modulation by endogenous neurosteroids. The impact of these mutations on alcohol consumption was examined using the two bottle choice intermittent access paradigm. To test whether changes in consumption are ethanol-specific, and to confirm that the difference is not due to the taste of the ethanol solution, two bottle choice consumption of ascending concentrations of two different tastants, saccharin and quinine, was also assessed.

1.4.2 Functional effect of α2^{Q241M} and α4^{Q246M} mutations on ethanol modulation of $GABA_A$ receptors

To investigate whether specific neuronal populations of α2-$GABA_ARs$ and/or α4-$GABA_ARs$ underpin the behavioural consequences of removing neurosteroid sensitivity, we used electrophysiological recordings in dentate gyrus granule cells from these animals to examine inhibitory transmission, both phasic and tonic currents. Slices were treated with 100 mM ethanol to evaluate the effect of the knock-in mutation on sensitivity to acute exposure to ethanol. Chronic effects of ethanol on inhibitory neurotransmission were also investigated by recording from animals that took part in the intermittent two bottle choice paradigm.

1.4.3 Effect of finasteride on alcohol consumption in three different mouse lines

Finasteride, a neurosteroid synthesis blocker (5α-reductase inhibitor), has been shown to affect ethanol's action. We assessed whether a single peritoneal injection of finasteride alters alcohol consumption of three different mouse lines, C57BL/J6, $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ knock-in mice, in a limited access two bottle choice paradigm. We also examined the impact of finasteride on blood ethanol concentrations of these animals using a colorimetric assay.

1.4.4 Summary of thesis aims

1. To determine whether rendering α2- or α4-$GABA_A$Rs insensitive to modulation by endogenous neurosteroids alters voluntary ethanol consumption in mice (Chapter 3)

2. To examine the consequences of $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ $GABA_A$R knock-ins for inhibitory neurotransmission and their response to ethanol (Chapter 4)

3. To investigate the effect of finasteride, a neurosteroid synthesis blocker, on ethanol consumption, and subsequently on blood ethanol concentrations in wild type (C57BL/J6) and $GABA_A$R α2-, and α4-knock-ins. (Chapter 5)

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Polymerase Chain Reaction (PCR) was performed using the Phusion Hot Start High-Fidelity DNA Polymerase kit (Thermo Fischer Scientific Inc.) and primers from Eurofins MWG Operon (Ebersberg, Germany). All other chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) or VWR International (Leuven, Belgium) unless indicated otherwise.

2.1.2 Antibodies

Table 2.1 List of antibodies used throughout this project.
Primary antibody was used for immunohistochemistry. The secondary antibody was conjugated to Alexa Fluor® 488.

	Antibody	Species	Dilution	Catalogue Number	Source
Primary antibody	Polyclonal anti-allopregnanolone antibody	Rabbit	1:500	Ab45164	Abcam
Secondary antibody	Alexa Fluor® 488 anti-rabbit IgG	Goat	1:750	A32731	Invitrogen

2.2 Animals

All procedures were performed in accordance with the *Animal (Scientific Procedures) Act, 1986* (ASPA) working under our laboratory project licence (PP6960143) and had obtained local ethical approval. Animals were decapitated under isoflurane (5 %) anaesthesia when obtaining tissue for electrophysiology. After the two bottle choice (intermittent access paradigm), animals were either used for electrophysiological

recordings or culled by cervical dislocation according to Schedule 1 of ASPA. Also following after two bottle choice limited access paradigm, animals were decapitated under anaesthesia by isoflurane (5 %) followed by blood and tissue sample collection. Due to monthly fluctuations in steroid hormone levels during the oestrus cycle (Corpéchot et al., 1997), only male mice were used in these experiments.

2.2.1 Generation of the mutant $GABA_A$ receptor mouse lines

Both the $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ mutant mouse line generation was performed by GenOway (Lyon, France) as previously described in Durkin (2012) and Minère (2019), respectively. In short, targeting vectors containing a base pair change for the point mutations Q241M and Q246M into exon 8 of $GABA_A$ receptor α2 (*Gabra2*) and α4 (*Gabra4*) subunit genes, respectively, were created by Michael Lumb in our laboratory (*Figure 2.1 A and B*). The mutation Q241M was inserted into exon 8 of *Gabra2*, while the mutation Q246M was inserted into exon 7 of *Gabra4*. The position for the residue exchange was based on the numbering of the mature protein. The vector was then introduced into embryonic stem (ES) cells by electroporation using a commercial facility (GenOway) for homologous recombination. Positive and negative selection procedures were employed to enrich cells that had successfully undergone homologous recombination. A neomycin cassette (Neo) flanked by loxP sites was used for positive selection. A thymidine Kinase (TK) negative selection cassette was inserted at the 5' end in order to reduce the isolation of non-homologously recombined ES cell clones. ES cell lines containing the mutations, $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$, were identified by PCR and Southern blotting techniques. These ES cell lines were used to generate transgenic mice with the $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ mutations. The introduction of the mutations was verified by full-length DNA sequencing (*Figure 2.2*). Germline-transmitted pups from a chimera x C57BL/6J cross (defined as the F0 generation) were bred with C57BL/6J Cre-recombinase expressing mice to remove the neomycin resistance cassette (*Figure 2.1 C*). Further backcrosses were performed between heterozygous (het) $\alpha2^{Q241M\ +/-}$ and $\alpha4^{Q246M\ +/-}$ mice and C57BL/6J mice to eventually establish the homozygous and heterozygous mouse lines, including wild type littermates.

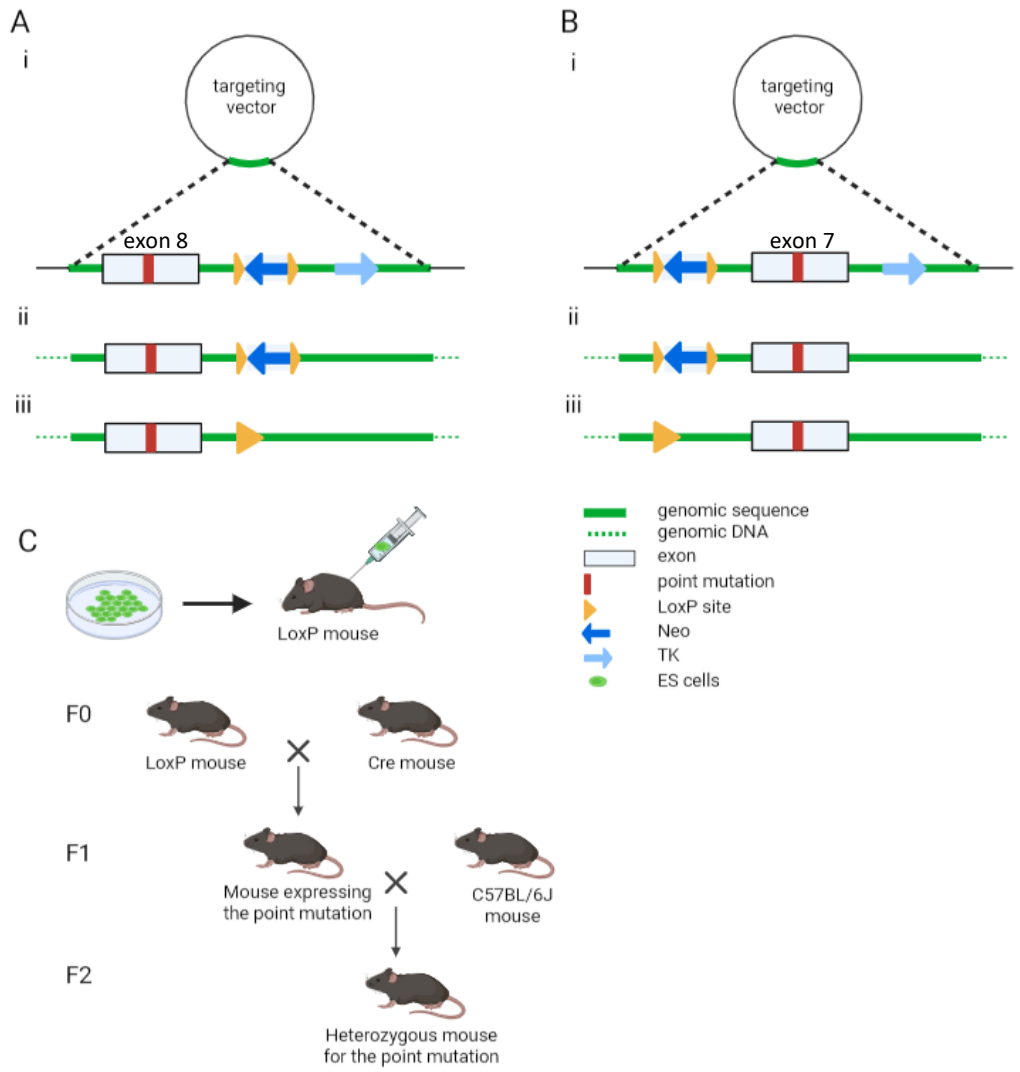


Figure 2.1 Generation of the mutant mice

A and **B** – i A targeting vector housed in a plasmid backbone including the mouse genomic sequence (green) with a point mutation (red) in either the Gabra2 (**A**, Q241M) or Gabra4 (**B,** Q246M) gene, together with positive and negative selection markers. A neomycin cassette (Neo, dark blue) flanked by loxP sites (yellow) was used as a positive marker, whereas thymidine kinase (TK) was used as a negative marker.

ii The result of successful homologous recombination; the mutation and Neo have been incorporated into the genomic DNA (dashed green line). TK and the plasmid are removed. This corresponds to the genomic DNA of F0 generation mice.

iii The result of breeding recombinant mice with Cre-recombinase mice (outlined in C). This corresponds to the genomic DNA of F1 generation mice and beyond. **C** – Embryonic stem (ES) cells that had undergone successful homologous recombination (as shown in B) were injected into blastocysts of C57BL/6J mice (LoxP mouse – as the genomic DNA still contains Neo flanked by loxP sites). The loxP flanked neomycin cassette was removed in vivo as the result of breeding with Cre-recombinase expressing mice. The deletion of Neo left behind a single loxP site (by the fusion of the two original loxP sites – as shown in C). These mice (F1) expressing the point mutation without Neo were bred again with C57BL/6J mice to produce heterozygous animals which were then used for further breeding for at least 5 generations.

2.2.2 Breeding

Homozygous (hom) and wild type (wt) mice were bred from heterozygous pairs. Experimental male mice were housed up to five per cage under an artificial 12-hour light/dark cycle (starting at 07:30 h). The cages were environmentally enriched and were kept at a constant temperature of 22°C and a relative humidity of 65 %. Animals had *ad libitum* access to water and standard laboratory rodent chow. Animals were used between postnatal days 42 (P42) and 120 for electrophysiology experiments. For behavioural analysis, animals aged between P56 and P84 were used.

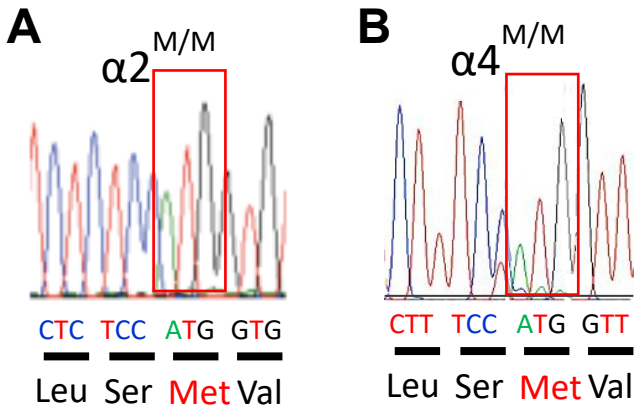


Figure 2.2 Sequencing of the mutant mouse lines

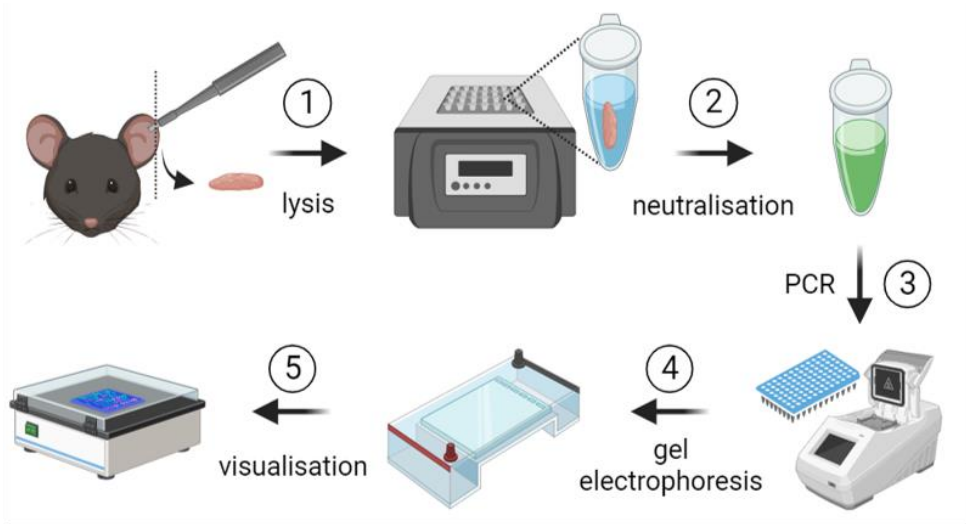
A and **B** – Sequencing data verifying the successful introduction of the point mutations Q241M and Q246M into *Gabra2* (α2) and *Gabra4* (α4), respectively. Exons containing the mutations were PCR-amplified from mouse genomic DNA. Red boxes highlight the sequencing signal corresponding to the introduced point mutations within the recombined alleles.

2.2.3 Genotyping

The protocol is diagrammatically outlined in *Figure 2.3 A*. Genomic DNA was isolated from ear notches by incubating in an alkaline lysis buffer (25 mM NaOH, 0.2 mM disodium ethylenediamine-tetraacetic acid (EDTA), pH 12.0) at 95 ^{o}C for at least 1 hour. The mixture was then inactivated to stop digestion by adding a neutralisation buffer (40 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-HCl), pH 5).

Genomic DNA was subjected to PCR using 1 µl of DNA according to the protocol shown in *Figure 2.3 G*. The mixture and primers used for the PCR reaction are shown in *Figure 2.3 B, D* and *F*. The PCR products were separated using a 2 % w/v agarose gel (100 V for 45 min). Ethidium bromide (0.2 µg/mL) was used as a fluorescent probe to label DNA. All PCR products were mixed with a 6X loading dye (New England Biolabs, B7024) for visualisation of the gel front alongside a 100 bp DNA ladder (New England Biolabs, B7025). The PCR products were visualised under UV light. The mutant alleles can be distinguished from the wt allele by the presence of the loxP site remaining after removal of the neomycin cassette on the transgenic allele. The presence of the lox P site results in a band 100 bp larger in the mutant allele compared to the wt allele, while heterozygous animals express both fragments (*Figure 2.3 C, D*).

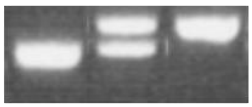
A



B

α2 - Forward primer:
5'-GCATAGACTACACAAAGTCTCTAGAAC-3'
α2 - Reverse primer:
5'-GGAGGTGGTGGTGATATCAAGTATAC-3'

C



D

α4 - Forward primer:
5'-TGTCTAAGGACTGAACTCTGACTCCCACAT T-3'
α4 - Reverse primer:
5'-TGCATATTTCACTGAGAATTGCATAGCTCC-3'

E

F

PCR mix	
Reagent	**µL**
DNA	1
H_2O	37
GC buffer	10
10 mM dNTPs	1
Forward primer	0.25
Reverse primer	0.25
Polymerase	0.5

G

PCR protocol			
Step	**Temperature**	**Time**	
Melt	98 °C	10 min	
Denature	98 °C	10 s	repeat 35X
Anneal	72 °C	10 s	
Extend	72 °C	60 s	
Extend	72 °C	5 min	

Figure 2.3 Genotyping protocol

A – Outline of the genotyping protocol: collection of tissue sample, isolation of DNA by lysis and neutralisation, amplification of DNA by PCR, separation of DNA fragments by gel electrophoresis and visualisation by UV light.

B and **D** – Forward and reverse primers used for PCR genotyping α2 (B) and α4 (D) mouse genomic DNA.

C and **E** – Representative images after running the PCR products on a 2 % w/v agarose gel (C: α2; E: α4). The images show the different DNA length obtained for wt (wild type) and homozygous mutant mice (hom). Heterozygous (het) animals for the mutations produce both bands.

F – Summary of the content of a single PCR reaction.

G – Summary of the PCR protocol used for genotyping.

2.3 Behavioural Analyses

2.3.1 Animal handling and drug administration

All animals were used in a single behavioural test, and were experimentally naïve. Before behavioural testing, mice were subjected to routine handling for husbandry and ear notching.

Where drugs were administered, mice received substances by intraperitoneal injection (27 gauge ½" needle, 1 ml syringe) at a volume of 10 ml per kg body weight. The timing of injection was 2-20 hours before behavioural testing. Gauging the dose-ranges and timings required for injection were determined by examining several protocols used and published in the literature (e.g. for finasteride (Gorin et al., 2005, Hirani et al., 2005)).

2.3.2 Two bottle choice – intermittent access paradigm

Eight-to-twelve week old wild type and homozygous mutant mice were assessed for voluntary alcohol intake and preference under a reversed light-dark cycle (12hr/12hr; lights off at 0600 hrs) according to the intermittent access protocol adapted from Hwa et al. (2011), as outlined in *Figure 2.5 A*. During assessments of fluid intake, solutions were presented in drinking bottles fabricated from 50 mL falcon tubes fitted with Hydropac (Plexx B.V, Elst, Netherlands) sterile disposable valves to prevent unintentional fluid loss (*Figure 2.4 A*). Mice were habituated from group to single housing, and to using the novel drinking bottles for at least 4 days prior to the start of the experiment. Three hours into the dark cycle, every other day, mice received 24-hour access to two bottles, one containing drinking water and the other a 20 % w/v EtOH solution for two weeks (*Figure 2.4 B*). On all other days, mice were presented with two bottles filled only with drinking water. The two bottles were positioned next to each other in the home cage. To control for whether mice had a bias for drinking from the left or right-side bottle (side-preference; *Figure 2.4 B*), the location (left or right) for the first EtOH containing bottle was initially randomised and subsequent presentations of the EtOH bottle were then alternated between the right and the left positions

(counterbalancing). The study was also blinded (to the genotype) to reduce operator bias. Mice were weighed prior to every EtOH access day before receiving the bottles. Daily fluid intake was measured in terms of mass (grams) in order to calculate EtOH consumption in grams of EtOH per kilogram of body weight. The measurement of EtOH preference was defined as the volume of 20 % (w/v) EtOH intake divided by the volume of total fluid intake expressed as a percentage.

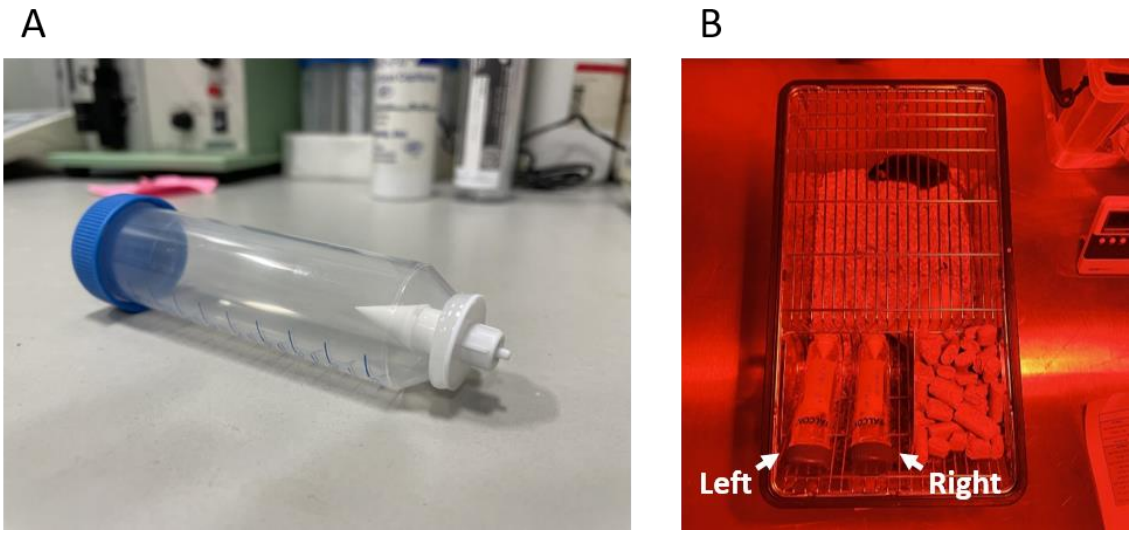


Figure 2.4 Equipment used in the two bottle choice experiments
A – Drinking bottles used for the presentation of solutions to mice. The bottles were manufactured by attaching a sipper valve to a modified 50 mL Falcon tube. **B** – Layout of the cage used for two bottle choice experiments. Mice had *ad libitum* access to rodent chow. The presentation of the bottles was alternated between the left and right side (referring to the experimenter's point of view as indicated).

2.3.3 Two bottle choice – tastants used in control experiments

To determine if there was a different preference for palatable and aversive tastants between wild type and mutant mice, animals were tested for their intake of saccharin (0.25 mM, 0.5 mM and 1 mM) and quinine hemisulphate (0.025 mM, 0.05 mM and 0.1 mM). Prior to the experiment, mice were acclimatised to the reverse light/dark schedule, the use of drinking bottles and single housing. The study was blinded and the initial presentation of the tastant solution bottles was randomised exactly as for the EtOH experiments. Ascending concentrations of the tastants were then presented three hours into the dark period as a two-bottle choice to eight-to-twelve week old male mice

with drinking water in the other bottle, as shown in *Figure 2.5 B, C*. Each concentration was made available for four consecutive days, with bottle presentation alternating from left to right daily. Fluid intake was monitored daily by weighing the bottles.

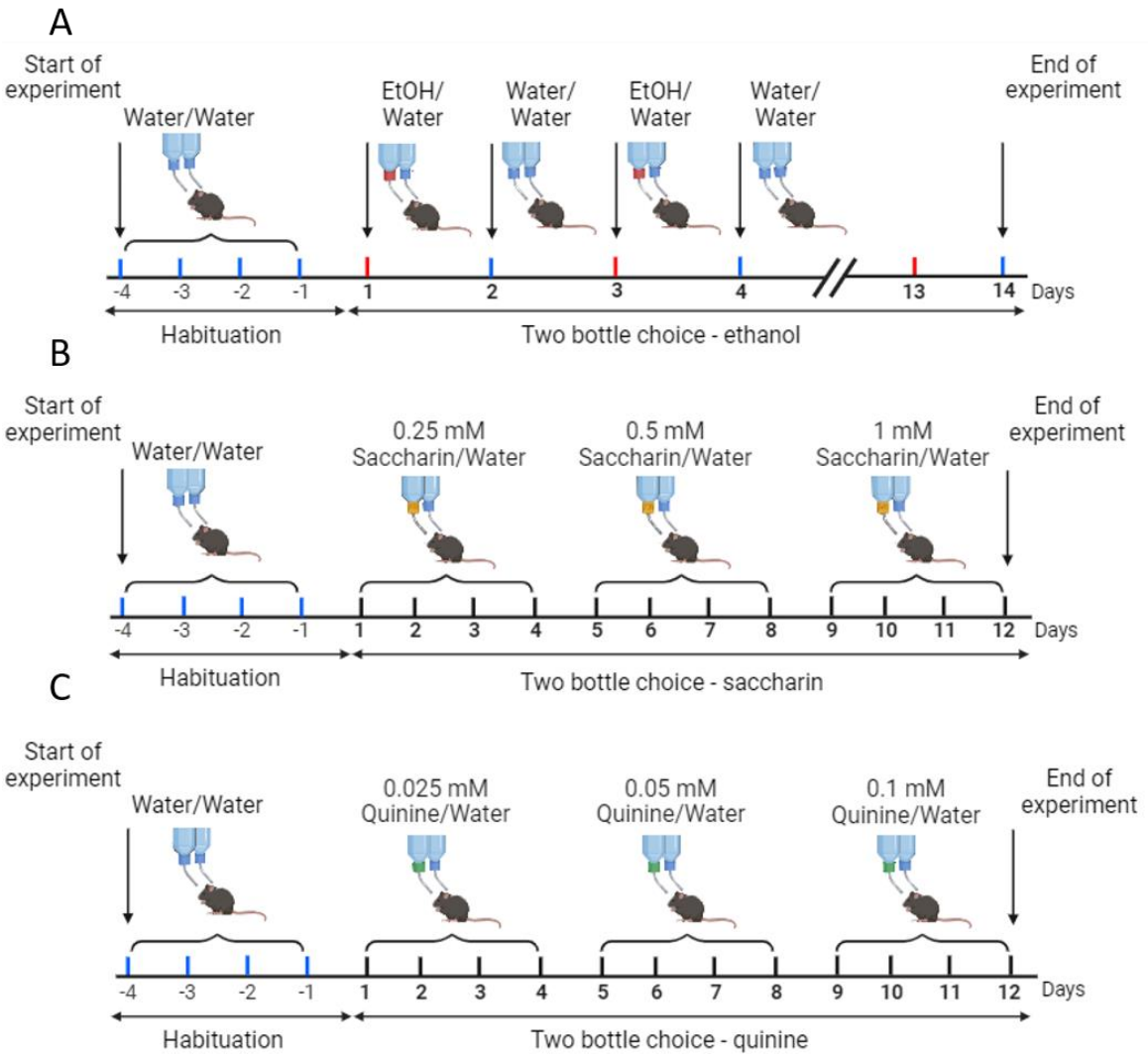


Figure 2.5 Two bottle choice protocols – ethanol, saccharin and quinine

A – Intermittent access protocol adapted from Hwa et al. (2011) that was used in the ethanol experiments. In short, after habituation, mice received access to ethanol every other day for two weeks. In-between the access to ethanol days, mice were presented with two drinking water bottles.

B and **C** – Ascending concentration protocol of tastants – saccharin (B) and quinine (C). After an acclimatisation period, mice were presented with two bottles – one containing the tastant and the other drinking water. Each concentration of the tastant was available for a period of four days.

2.3.4 Two bottle choice – limited access paradigm

Each mouse (8-12 week old C57BL/6J, $\alpha2^{Q241}$ and $\alpha4^{Q246}$ wt and hom) was individually housed and acclimatised to a reverse light/dark schedule (12hr/12hr; lights off at 0600 hrs) for a minimum of 5 days. All mice were provided *ad libitum* access to rodent chow and tap water. Mice were weighed and handled daily throughout the habituation and experimental phases of the study. The protocol is outlined in *Figure 2.6*.

Mice were designated to one of two treatment groups. One group received an intraperitoneal injection (i.p.) of finasteride (FIN; 50 mg/kg) whereas the other group was administered a vehicle control injection (VEH; i.p.; 20 % w/v 2-hydroxypropyl-β-cyclodextrin; 0.01 ml/g). The FIN dose was selected based on studies which demonstrated that a 50 mg/kg dose decreased plasma and brain allopregnanolone levels by 66 % and 80 %, respectively, determined at a 24-hr post-injection time point (Finn et al., 2004a). Furthermore, this FIN dose was found to be effective in altering ethanol-related behaviours in other studies (Gorin et al., 2005, Hirani et al., 2005).

Results from the intermittent access paradigm suggested that the largest difference in ethanol consumption between wild type and homozygous mutant mice occurs during the first 24 hours. Therefore, the experiment aimed to assess the difference in EtOH consumption and preference only during those initial few hours. We conducted three different versions of this paradigm (Protocol A, B and C; *Figure 2.6*).

A study documented that greater ethanol intake is apparent for C57BL/J6 mice when access begins either 2 or 3 hrs after lights out (compared to 1 hr; (Rhodes et al., 2005). A 6-hour limited access time (0800-1400, starting 2 hours after lights out) was therefore selected based on this finding (Protocol A and C). On the day of the experiment, mice received 6-hour access to two bottles, one containing drinking water and the other a 20 % w/v EtOH solution. We also conducted an exploratory 24-hour version of this experiment (Protocol B), where mice received 24-hour access to one water bottle and one ethanol bottle (20 % w/v) starting 2 hours after lights out.

To control for side-preference, the ethanol containing bottles were counterbalanced between the left and right sides across the cages. The study was blinded as to the treatment applied and for the genotype (i.e. VEH vs FIN; wt vs hom) to reduce bias. Mice

were weighed prior to the drinking session. Consumption of ethanol was assessed every 2 hours by weighing the bottles.

Blood and brain tissue samples were collected after the conclusion of the 6-hour drinking session (see *Section 2.6*).

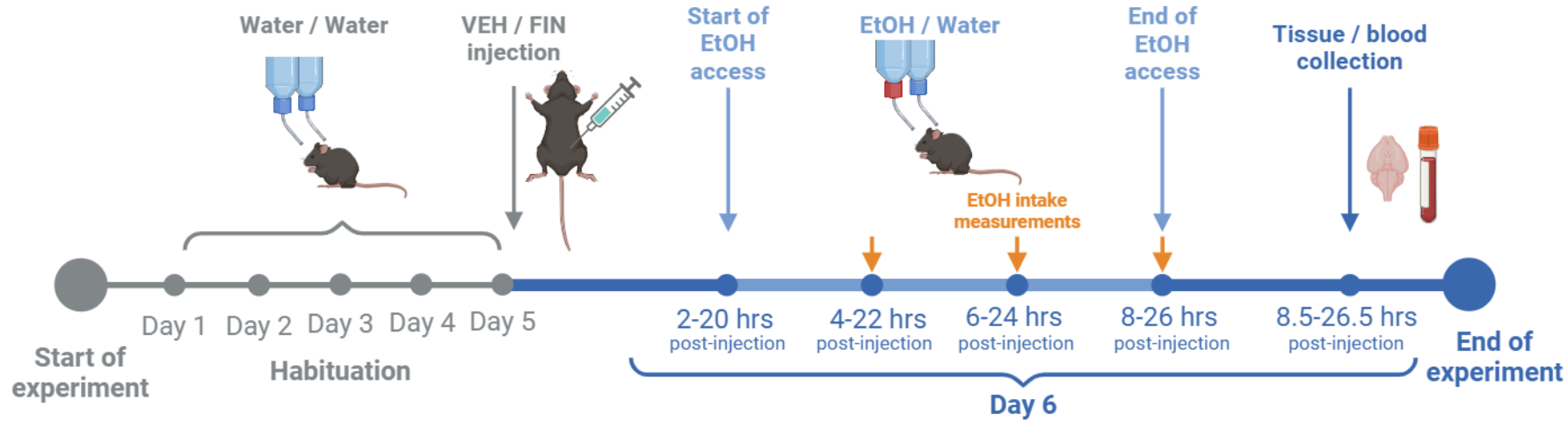


Figure 2.6 Two bottle choice - limited access protocol

Schematic diagram depicting the limited access two bottle choice paradigm. Briefly, mice were habituated to the experimental conditions. On the last day of habituation, mice either received an intraperitoneal injection of vehicle (VEH) or finasteride (FIN). 2-20 hours post-injection mice were given access to ethanol. Their intake was measured every two hours for a 6-hour period. The experiment was concluded by tissue and blood sample collection to determine EtOH levels.

Protocol A: Ethanol access started at 20 hours post-injection; ethanol intake measurements were taken at 22, 24 and 26 hours post-injection, tissue and blood samples were collected at 26.5 hours post-injection

Protocol B: Ethanol access started at 2 hours post-injection; ethanol intake measurements were taken starting from 4 hours to 26 hours post-injection every 2 hours. Tissue and blood samples were collected at 26.5 hours post-injection.

Protocol C: Ethanol access started at 2 hours post-injection; ethanol intake measurements were taken at 4, 6 and 8 hours post-injection; tissue and blood samples were collected at 8.5 hours post-injection.

2.4 Brain slice electrophysiology

2.4.1 Brain slice preparation

Adult male mice (> 6 week of age) were anaesthetised by 5 % isoflurane, and the brain was removed rapidly into ice-cold slicing solution containing (in mM): 85 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 75 sucrose, 1 $CaCl_2$, 4 $MgCl_2$, 25 glucose and 2 kynurenic acid (pH 7.4 equilibrated with 95 % O_2 and 5 % CO_2). Coronal slices of 250 µm of the dorsal hippocampus were cut with a Leica VT 1200s vibroslicer (Leica Microsystems GmBH, Wetzlar, Germany) and then incubated in a holding chamber first at 37°C for 60 min and then for 30 min at room temperature in artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose and 2 kynurenic acid (pH 7.4 equilibrated with 95 % O_2 and 5 % CO_2). Slices were then maintained in the holding chamber at room temperature.

2.4.2 Whole-cell patch clamp recording

Whole-cell recordings were performed from dentate gyrus granule cells (*Figure 2.7 A*) at room temperature with glass pipettes of 3-6 MΩ tip resistance when filled with an internal solution containing (in mM) 140 CsCl, 2 NaCl, 10 HEPES, 5 EGTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 2 Na-ATP, 0.5 Na-GTP, 2 QX-314 (pH 7.4 with CsOH). The neurons were visualised using infra-red differential interference optics (Nikon Eclipse E600FN, Nikon Instruments Europe B.V. Surrey, UK) fitted with a Basler SLA750-60fm Camera (Basler Vision Technologies, Ahrensburg, Germany). Membrane currents were recorded using a Multiclamp 700A amplifier (Axon Instruments), filtered at 2 kHz (Bessel filter, 6^{th} order) and digitised using a Digidata 1322A (Axon Instruments). Slices were continuously perfused with ACSF supplemented with kynurenic acid (2 mM) to isolate GABA-mediated events. Miniature IPSCs (mIPSCs) were recorded in the presence of 500 nM tetrodotoxin (TTX) at a holding potential of -60 mV. In paired ethanol experiments, mIPSCs were recorded for 10-20 minutes before, for 30 min during and for 10-15

minutes after 100 mM ethanol application (*Figure 2.7 B*). In unpaired ethanol experiments, hippocampal slices were pre-incubated for 30 min in 100 mM ethanol prior to recording. Experiments were completed by the bath application of 50 µM (-)-bicuculline-methiodide (bic), to confirm that all events were GABAergic, and to allow any GABA-mediated tonic currents to be measured. Recordings were made in 5 min epochs, and access resistance (typically 10-20 MΩ) was monitored every 5 minutes; if it changed by more than 25 % during an experiment, the recording was discarded.

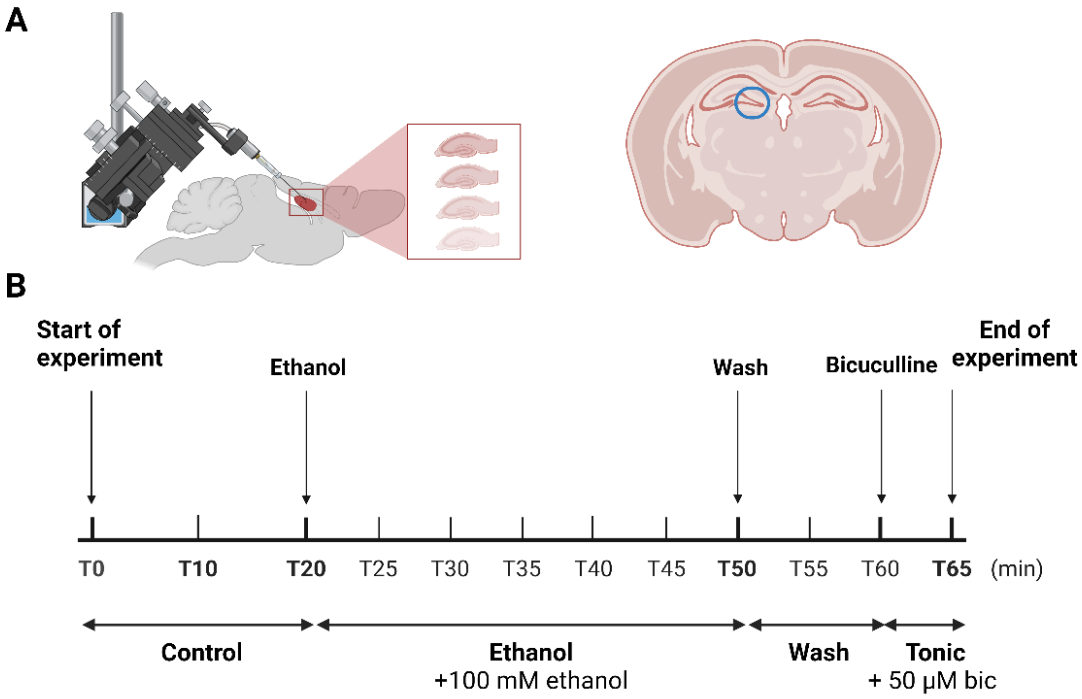


Figure 2.7 Whole cell patch clamp recordings

A – Schematic diagram showing the set up and location, dentate gyrus granule cells (blue circle depicts DG brain area), of electrophysiological recordings performed in the study.

B – Timeline of the paired ethanol recordings. Recordings of membrane current under voltage clamp held at -60 mV were made initially for 20 min under control conditions, followed by 30 min of bath application of 100 mM ethanol. Cells were then washed for 10 minutes. 50 µM bicuculline (bic) was applied at the end of the experiment.

2.4.3 Analysis of miniature inhibitory post-synaptic currents (mIPSCs)

An amplitude-threshold method was utilised to detect mIPSCs using WinEDR (version 3.9.4, John Dempster, University of Strathclyde) for event detection and WinWCP (version 5.5.5, John Dempster) for further mIPSC analysis. Firstly, recordings were imported into WinEDR where parameters were set for optimal synaptic event detection. Depending on baseline stability of the recording and noise, the following threshold parameters were used: amplitude of 4-8 pA negative deviation from the baseline, duration of 0.8-1 ms and rise time of 1-2 ms. Synaptic events were then confirmed by visual inspection. For paired ethanol experiments, each recording was broken down into five epochs – control, EtOH 0-10 min, EtOH 10-20 min, EtOH 20-30 min and wash. For unpaired ethanol experiments, 10-15 min of control and 10-15 min in the presence of EtOH segments were analysed. Mean mIPSC amplitudes were calculated for each epoch by averaging the amplitudes of all events in that epoch. Mean frequencies were calculated from all events in epochs of ~10 min.

For kinetic analysis, individual uncontaminated mIPSCs were isolated and averaged (>50 events) to measure rise and decay times. These events were aligned at the start of their rise phase before averaging to create a mean mIPSC waveform. Decay times were reported as weighted decay times (τ_w) to factor in both mono- and bi-exponentially decaying events according to the equation:

$$\tau_w = \frac{(A_1 \times \tau_1 + A_2 \times \tau_2)}{(A_1 + A_2)}$$

where τ1 and τ2 are exponential decay time constants, A1 and A2 are the relative amplitude contributions of τ1 and τ2, respectively (mono-exponential events: A2 = 0).

Mean charge transfer (pC/s) was calculated by multiplying the area under the mean mIPSC waveform (pA·s) by the mean IPSC frequency.

2.4.4 Analysis of tonic GABA currents

Tonic currents were measured by determining the holding current shift upon application of a competitive $GABA_A$ receptor antagonist, 50 µM bicuculline. The change in tonic

current size was determined by measuring the average holding current for a 60 s epoch before and after drug application. mIPSC currents were removed digitally in WinEDR to ensure that the measurement only relates to the baseline current.

Changes in holding current were often small (less than 10 pA) and changes in root mean square (RMS) noise (a measure of membrane current variance) proved to be a more robust and reliable measure of changes in tonic current in this study. WinEDR software was employed to measure RMS noise over 100 or 200 ms epochs of a recording. The presence of any synaptic events in a single epoch will increase the RMS value, and so such epochs were excluded from the analysis. Microsoft Excel was employed to compare the RMS value of each epoch to a user-defined threshold, and automatically exclude those with values above the threshold as 'contaminated'. The threshold is defined as a proportion of the median RMS current over a local 5 s window of recording (50 x 100 ms epochs).

2.5 Immunohistochemistry

2.5.1 Brain slice preparation and antibody labelling

Hippocampal slices of adult male mice (> 6 weeks of age) were prepared as previously described in *Section 2.4.1*. The staining protocol is outlined in *Figure 2.8*. After the incubation in ACSF, slices were fixed in fresh 4 % paraformaldehyde (PFA) / 4 % sucrose (w/v) in phosphate buffered saline (PBS) for 30 min. Samples were then thoroughly washed with PBS and incubated in 1 % normal goat serum (NGS) in PBS for 2 hours at room temperature to block any non-specific binding of primary antibodies. Slices were incubated with a primary antibody raised in rabbit against allopregnanolone (*Table 2.1*) diluted 1:500 for 48 hours at 4 °C. After incubation with primary antibody, slices were extensively washed with PBS and incubated with a secondary antibody, Alexa Fluor® 488 goat anti-rabbit IgG (diluted 1:750; *Table 2.1*). After staining, slices were washed with PBS and mounted onto glass microscope slides using the Prolong Gold mounting reagent (Thermo Fisher Scientific, P36930) and left at room temperature to cure overnight. For storage, slides were kept in the dark at 4 °C.

2.5.2 Image acquisition and image analysis

Fluorescence images were acquired using a Zeiss Axiscope LSM510 confocal microscope (Carl Zeiss Ltd.) and Zeiss acquisition software. The wavelength of the laser used for image collection was 488 nm (Alexa Fluor 488). A Plan-NEOFLUAR 40X/1.4NA (numerical aperture) oil differential interference contrast (DIC) objective (Zeiss) was used to acquire the images. 8-bit images were taken at 1024x1024 pixel resolution. Normally, scan speeds 6 or 7 were used translating to pixel dwell times of 7.68 µs and 3.84 µs respectively. For dentate gyrus, two images showing different regions were taken, whereas for CA1 and CA3 regions of hippocampus, three images were taken per hemisphere.

All confocal images were analysed using Image J software (version 1.52p). For dentate gyrus, the fluorescence was determined by selecting a region of interest (ROI) around the granule cell layer. A measure of background fluorescence was quantified using an area in the slice with no cells. This was subtracted from the average value of fluorescence to get a background-adjusted fluorescence intensity. The fluorescence of the two images taken of DG was averaged to give the mean fluorescence per hemisphere.

For the CA1 and CA3 regions of hippocampus (*Figure 2.9*), images were first 'thresholded' manually to create binary images. A threshold range was set to distinguish fluorescent particles of relevance from the background. All pixels in the image whose values fell below threshold were converted to black and all pixels with values above the threshold were converted to white. Dilation and erosion functions were then used to enhance the cells in the image and to remove noise. Then the analyse particle function was applied to the segmented image to count and outline the objects (cells) in the image. The parameter, pixel size, was adjusted manually for each image to ensure optimal detection. The application of the analyse particle function created a mask which was then overlaid onto the original image to measure fluorescent intensities. The average of the fluorescence of the three images of CA1/CA3 was then used to calculate the mean fluorescence intensity per hemisphere.

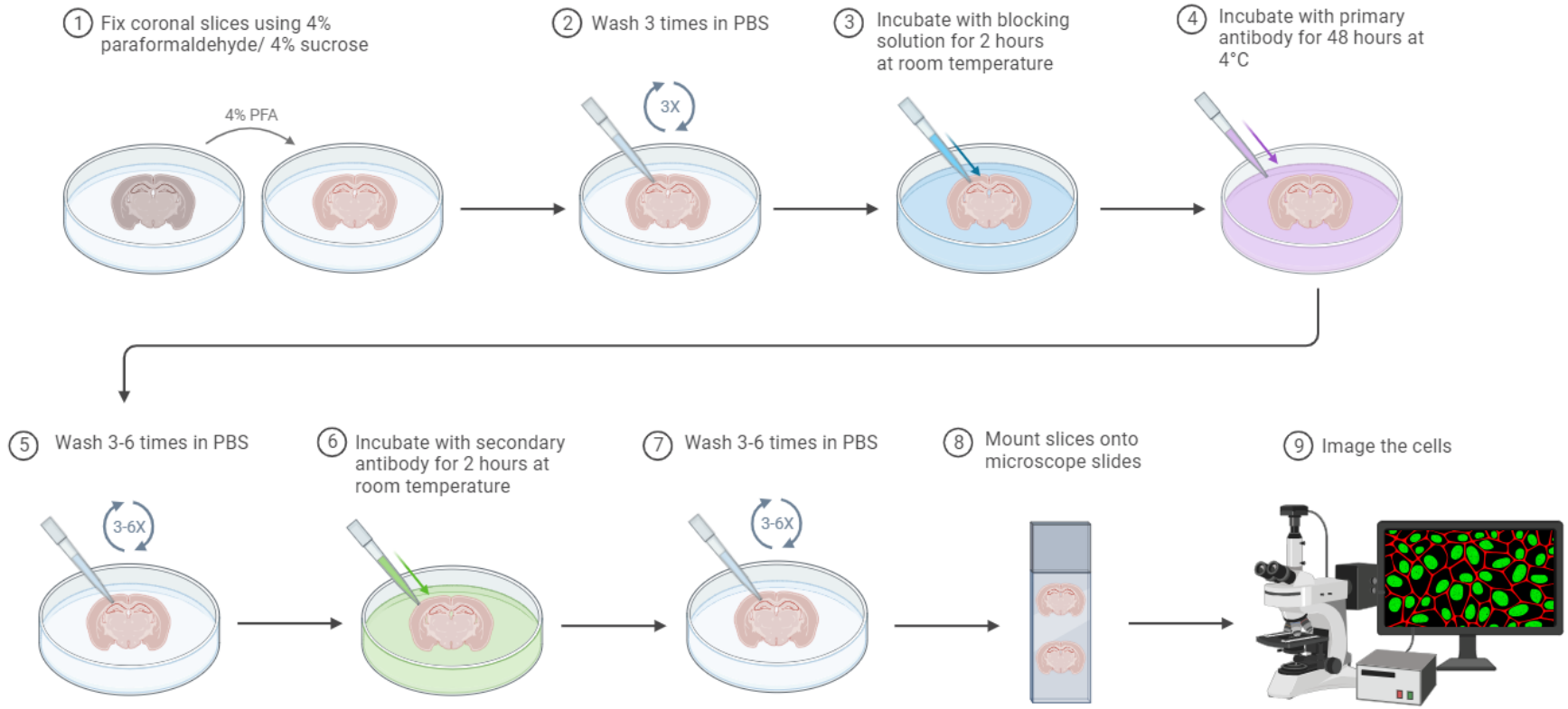


Figure 2.8 Staining protocol of hippocampal brain slices

Schematic diagram showing the antibody labelling protocol used for immunofluorescence measurements. PBS: phosphate buffered saline.

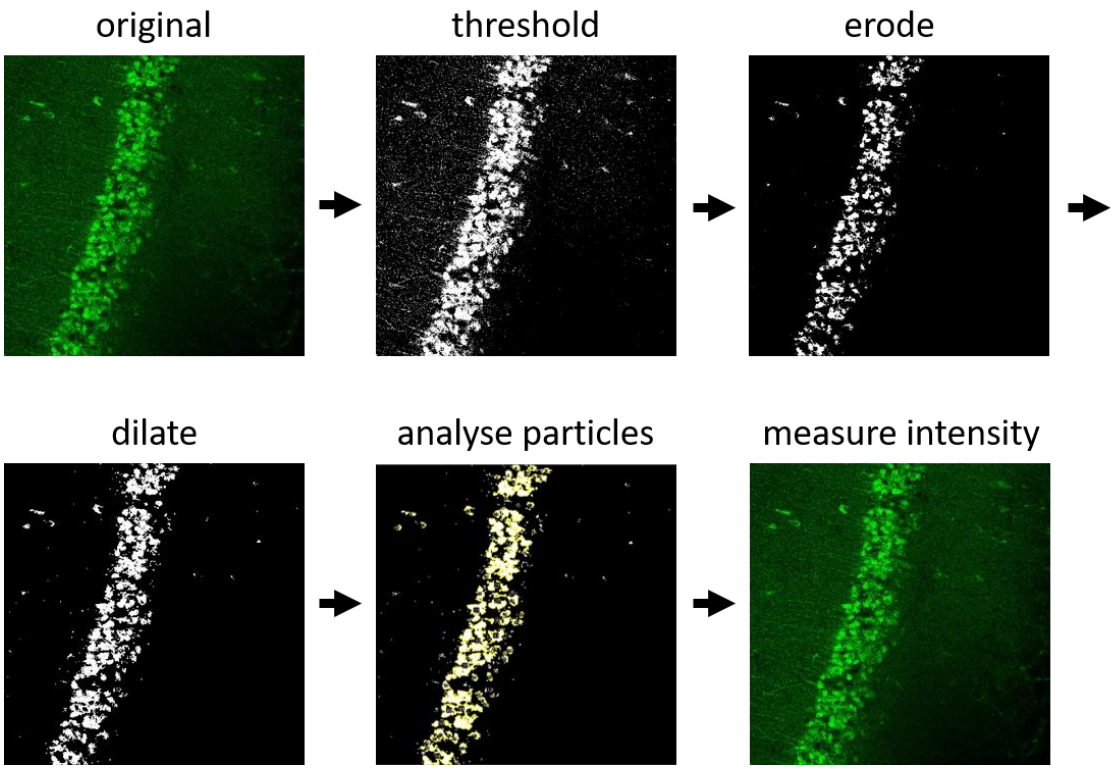


Figure 2.9 Image analysis of CA1 and CA3 hippocampal regions

A workflow chart showing the process of fluorescent intensity measurements of CA1 and CA3 neurons. Briefly, images were thresholded to create binary images. Erode and dilate functions were used to remove noise and enhance cells. Analyse particle function was applied to create a mask and count the objects. The mask created was overlaid on the original image and mean fluorescent intensity was measured.

2.6 Blood ethanol concentration measurement

Blood samples were collected at the end of the 6-hour session of the limited access protocol for blood ethanol concentration (BEC) measurements. Trunk blood samples were collected in 1 mL lithium/heparin coated tubes (VetWay Ltd, York, United Kingdom). Samples were centrifuged immediately for 15 min at 3000 rpm and the supernatant was transferred to a clean 1.5 mL Eppendorf and was stored at -80°C until testing. Plasma ethanol concentrations were determined using a colorimetric ethanol assay kit (Abcam, ab65343). The kit is based on a dichromate method, in which dichromate is reduced by ethanol to a bluish chromic product that can be quantified against prior calibration values.

Stored samples were first thawed on ice, then 10 % trichloroacetic acid (TCA) was added in equal ratio by volume to each sample (1 sample : 1 TCA ratio) to denature and precipitate soluble proteins. The mixture was centrifuged at 14000 rpm for 5 minutes and the supernatant was subsequently used for the assay.

A fresh set of calibration standards was prepared for every experiment by serially diluting a 10 % ethanol solution as described in the table below.

Table 2.2 Standard curve preparation for blood ethanol concentration measurements

Standard #	*EtOH concentration (%)*
1	1.2
2	1
3	0.8
4	0.6
5	0.4
6	0.2
7	0.1
8	0

Duplicates of standards and samples were added to separate wells in a 96-well plate. The assay is based on a kinetic reaction which is started by the addition of Reagent A. The mixture was incubated for 30 min at room temperature before adding Reagent B to stop the reaction. Absorbance was read at 570 nm (peak absorbance is at 580 nm) with a standard microplate reader (Multiskan, Thermo Fisher Scientific, United Kingdom).

Duplicate readings were averaged for each standard and sample. The mean absorbance value of the blank (Standard #8) was subtracted as a background reading from all EtOH containing standard and sample readings. This is the corrected absorbance. Corrected absorbance values for each standard were plotted as a function of the concentration of ethanol (*Figure 2.10*). Linear regression analysis was used to fit the data to find the best-fit values for the slope and y-intercept. Sample readings were then interpolated from the standard curve plotted using GraphPad Prism (version 9.2.0, GraphPad Software, La Jolla, California, USA). Note that 1 % (v/v) ethanol equals 170 mM or 785 mg/dL.

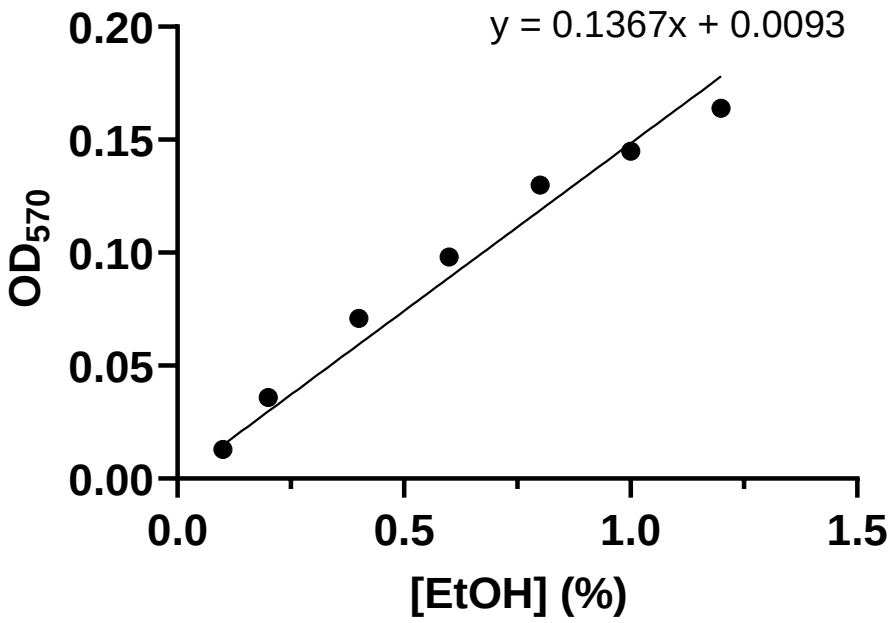


Figure 2.10 Example ethanol standard calibration curve using colorimetric readings

2.7 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Graphical representations of data were plotted using GraphPad Prism. For most of the data analyses, statistical analysis relied on paired and unpaired t-tests or analysis of variance (ANOVA) if more than two groups were compared.

The D'Agostino-Pearson test was used to determine if data sets were normally distributed. Unpaired t tests were performed for normally distributed, equal variance data. Where data sets had unequal variances, Welch's correction was applied. Where data was not normally distributed, Mann-Whitney U test was used.

Where applicable, two-way ANOVA with uncorrected Fisher's Least Significant Difference (LSD) post hoc multiple comparisons test, two-way repeated measures (RM) ANOVA (two-way RM ANOVA) with Šidák's test (to compare genotypic differences) and/or Dunnett's test (to determine the effect of ethanol treatment), or in cases where values were missing, mixed effects model (REML) analysis, were used. All data sets for group analyses were normally distributed.

The threshold for statistical significance for all tests was set to p-value ≤ 0.05 (denoted by one asterisk (*)). P values lower than 0.05 were denoted as ** (p-value ≤ 0.01), *** (p-value ≤ 0.001) or **** (p-value ≤ 0.0001).

2.7.1 Two bottle choice – analytical considerations

Data was collected every day for the duration of the experiment. If there was leakage of solution from either bottle, the data point was removed for that animal for that particular day. Outliers from all data sets were excluded using the Robust Outlier removal (ROUT) method (GraphPad Prism software). The ROUT coefficient, Q, was set to the default value of 1 % to avoid the false detection of outliers.

In ethanol experiments, if data from an animal was an outlier in one of the parameters, it was then subsequently removed from the analysis of all other parameters, since they are all inter-dependent. Two outliers were removed from experiments involving $\alpha2^{Q241M}$ mice, and an additional two outliers were excluded from experiments with $\alpha4^{Q246M}$ animals.

In tastant experiments, outliers were detected in the same way. However, data with different concentrations of saccharin and quinine were handled separately, meaning that outliers were only removed from the parameters for the same concentration. One outlier was removed from experiments with 1 mM saccharin involving $\alpha4^{Q246M}$ mice, and another outlier was excluded from experiments with 0.1 mM quinine using the same mouse line.

Chapter 3 Alcohol consumption of $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ mice

3.1 Introduction

As discussed in *Section 1.3*, some of ethanol's action are mediated by neurosteroid modulation of $GABA_A$ receptors. Systemic administration of ethanol in rodents elevates brain and plasma levels of neuroactive steroids such as allopregnanolone and THDOC, as well as their precursors (Barbaccia et al., 1999, Khisti et al., 2005, Korneyev et al., 1993, O'Dell et al., 2004, VanDoren et al., 2000). This ethanol-induced increase in neuroactive steroids is in part mediated by the HPA axis, evidenced by the absence of this effect following adrenalectomy/gonadectomy in rats (Khisti et al., 2003, O'Dell et al., 2004). However, ethanol has also been shown to increase neuroactive steroids in hippocampal slices from both intact and adrenalectomised/gonadectomised rats, correlating with enhanced GABAergic inhibition, which is blocked by the neuroactive steroid biosynthesis inhibitor finasteride (Follesa et al., 2006, Sanna et al., 2004).

Ethanol-induced neuroactive steroids reach physiologically significant concentrations that enhance GABAergic transmission (Kumar et al., 2009). Research from multiple laboratories indicates that these steroids contribute to ethanol's behavioural effects in rodents. Neurosteroids modulate ethanol's anticonvulsant (VanDoren et al., 2000), sedative (Khisti et al., 2003), spatial memory impairing (Morrow et al., 2001b), anxiolytic (Hirani et al., 2005), and antidepressant effects (Hirani et al., 2002). These behaviours are typically mitigated by finasteride pretreatment or prior adrenalectomy (Hirani et al., 2002, Hirani et al., 2005, VanDoren et al., 2000). Administration of 5α-dihydroprogesterone, a precursor of allopregnanolone, restores ethanol's effects in adrenalectomised rats, indicating that brain synthesis of neuroactive steroids modulates ethanol's effects (Khisti et al., 2003).

Collectively, these studies indicate that increased levels of neurosteroids are responsible for numerous GABAergic effects of ethanol *in vivo*, and that these steroids may influence sensitivity to ethanol's behavioural effects.

The free-choice water/alcohol drinking procedure, initially established in the 1940s, became the first widely adopted method for studying voluntary ethanol intake (Richter and Campbell, 1940). In this procedure, animals are provided with unrestricted access to bottles containing either water or ethanol solution (offered at one or multiple concentrations). Ethanol consumption and preference (ethanol intake relative to total fluid intake) are subsequently measured during a 24-hour period of unlimited access. To enhance voluntary ethanol intake by animals, several procedures have been developed, including intermittent access protocols. This method leverages the observation that, after brief periods of enforced abstinence—during which ethanol and water are alternated daily — voluntary ethanol consumption progressively rises (Sinclair and Senter, 1967, Wayner and Greenberg, 1972), potentially resembling the alcohol deprivation effect. Indeed, Hwa et al. (2011) demonstrated that the intermittent access paradigm in C57BL/J6 mice leads to increased voluntary and preferential ethanol consumption. This methodology has been widely used in studies investigating alcohol consumption.

There is substantial evidence that GABAergic systems play a role in mediating ethanol self-administration in rodents, likely by activating reward pathways in the mesolimbic system (Davies, 2003). Generally, drugs that activate or enhance $GABA_A$Rs are correlated with increased ethanol intake, while those that block or inhibit GABAergic systems are associated with reduced ethanol consumption (Davies, 2003).

Traditionally, the method for investigating the behavioural significance of a gene has involved studying global knockout mice. Indeed, this has been done for multiple $GABA_A$ receptor subunits, including α2, α4 and δ (reviewed in Boehm et al. (2004)). However, eliminating any $GABA_A$ receptor subunit might prompt compensatory alterations in other subunits (e.g. upregulation of expression, differential localisation) or other ion channels (Brickley et al., 2001), potentially complicating the analysis of behavioural findings (Ponomarev et al., 2006). To circumvent such compensatory adjustments and other drawbacks associated with global knockouts, constructing knock-in mice offers a solution. In knock-in mice, the wild type gene is substituted with a mutant sequence harbouring a drug-insensitive, otherwise functionally normal protein.

Various line of evidence suggest that α4-containing $GABA_A$ receptors play a role in alcohol-related behaviours. Mihalek et al. (2001) found that δ-KO mice consumed less ethanol compared to wild type mice. Similar results were observed with viral-mediated RNAi targeting α4 and δ subunits to lower their expression in the nucleus accumbens (Nie et al., 2011, Rewal et al., 2009). Furthermore, injecting the δ-preferring $GABA_AR$ super- or full agonist THIP into the VTA or knocking down δ subunits in the VTA reduced ethanol intake in mice (Darnieder et al., 2019, Melón et al., 2017). Additionally, intraperitoneal THIP and finasteride injections reduced ethanol consumption in male mice (Moore et al., 2007, Ramaker et al., 2011, Ramaker et al., 2014). In contrast, α4-knockout mice show increased alcohol consumption compared to wild type littermates (Olsen and Liang, 2017). However, deleting α4 leads to compensatory upregulation of other $GABA_AR$ subunits, altering the pharmacological responsiveness of synaptic and extrasynaptic $GABA_AR$ currents, which could explain the discrepancy between these studies. Nevertheless, all these results combined point towards α4βδ receptors playing an important role in behaviours related to alcohol consumption.

Multiple studies suggest that α2-containing $GABA_A$ receptors also contribute to alcohol-related behaviours. Global deletion of α2 results in reduced ethanol consumption in mice, evident after three weeks (Olsen and Liang, 2017). However, this contrasts with findings from Dixon et al. (2012), where no differences in ethanol self-administration were observed between wild type and $\alpha2^{-/-}$ mice. The disparity between the studies may stem from methodological differences; Olsen and Liang examined chronic effects over weeks, while Dixon et al. only assessed acute ethanol impact (< 1 hour). Mutant animals homozygous for the Q241M steroid site point substitution in α2 subunits exhibit reduced ethanol intake and preference compared to wild type animals (Newman et al., 2016). Together, these studies corroborate the role of neurosteroids in mediating some of ethanol's effects and indicate that some of these effects may occur via α2-$GABA_AR$s.

The aim of this chapter is to explore the effect of neurosteroid modulation of different populations of $GABA_AR$s on ethanol consumption. We have used two novel knock-in mouse lines, $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$, which express a targeted point substitution of glutamine to methionine within these $GABA_A$ subunits, thus rendering them insensitive to modulation by endogenous neurosteroids. The impact of the point mutation in the α4 subunit on behaviour has not been characterised; whereas the α2 knock-in animals have

been subjected to multiple behavioural assays. α2-$GABA_A$ receptors are key targets for neurosteroid modulation, and they are crucial for the anxiolytic function of endogenous neurosteroids, yet they are not necessary for their antidepressant or analgesic effects (Durkin et al., 2018). Our objective was to characterise ethanol consumption in the $\alpha2^{Q241M}$ mouse line to verify previous findings (Newman et al., 2016), and to compare with ethanol consumption of our $\alpha4^{Q246M}$ mouse line using the same experimental paradigm. To determine if consumption changes are specific to ethanol and not influenced by the taste of the ethanol solution, two-bottle choice tests with different concentrations of saccharin (sweet) and quinine (bitter) were conducted.

3.2 Results

3.2.1 Alcohol consumption of α4^{Q246M} mice

We assessed ethanol intake of male α4^{Q246M} wild type (α4$^{Q/Q}$) and homozygous mutant (α4$^{M/M}$) mice using the intermittent access two bottle choice protocol (*Figure 3.1 A*), also outlined in Section *2.3.2*. The animals were age-matched (days; *Figure 3.1 B; Table 3.1*), they had no difference in their starting body weight (g; *Figure 3.1 C; Table 3.1*) or their body weight change (%) during the experiment was not affected by the mutation (*Figure 3.1 D; Table 3.1*). The total fluid intake (g/kg) of animals during habituation was also unaffected by the genotype (*Figure 3.1 E; Table 3.1*). Daily individual EtOH intake (g/kg), preference (%), fluid intake (g/kg) and water intake (g/kg) values were averaged each week of the experiment. The mean ± SEM values and statistical analyses of the results for this experiment are contained in *Table 3.3* and *Table 3.4*, respectively, in *Section 3.5*.

Mutant a4^{Q26M} mice showed a significant reduction in daily ethanol intake in week 1 compared with wild type mice, however, there was no change between the genotypes in Week 2 (*Figure 3.2 A*). Two-way RM ANOVA detected a significant main effect of time on ethanol consumption, with mice consuming progressively less ethanol. Surprisingly, EtOH preference was not affected by the mutation at any time point (*Figure 3.2 B*), however, there was a trend as it decreased with time. The lack of change in preference was mainly due to α4$^{M/M}$ mice adjusting their total fluid intake (*Figure 3.2 C*). This difference in total fluid intake between genotypes was not present during the habituation period (*Figure 3.1 E*). Moreover, the reduction in fluid intake on EtOH access days (*Figure 3.2 D*) seems to be mainly driven by the reduction in EtOH intake (42 % decrease in Week 1 and 27 % decrease in Week 2 between genotypes) rather than the reduction in water intake (12 % decrease in Week 1 and 14 % decrease in Week 2 between genotypes; *Figure 3.2 F*). The decreased water intake of mutant mice on water/water days heavily contributes to the overall decrease of their fluid consumption (*Figure 3.2 E*). Therefore, we believe that the reduction in EtOH intake of homozygous mutant mice is not simply due to them consuming less total volume of fluid but rather them adjusting their intake upon EtOH exposure.

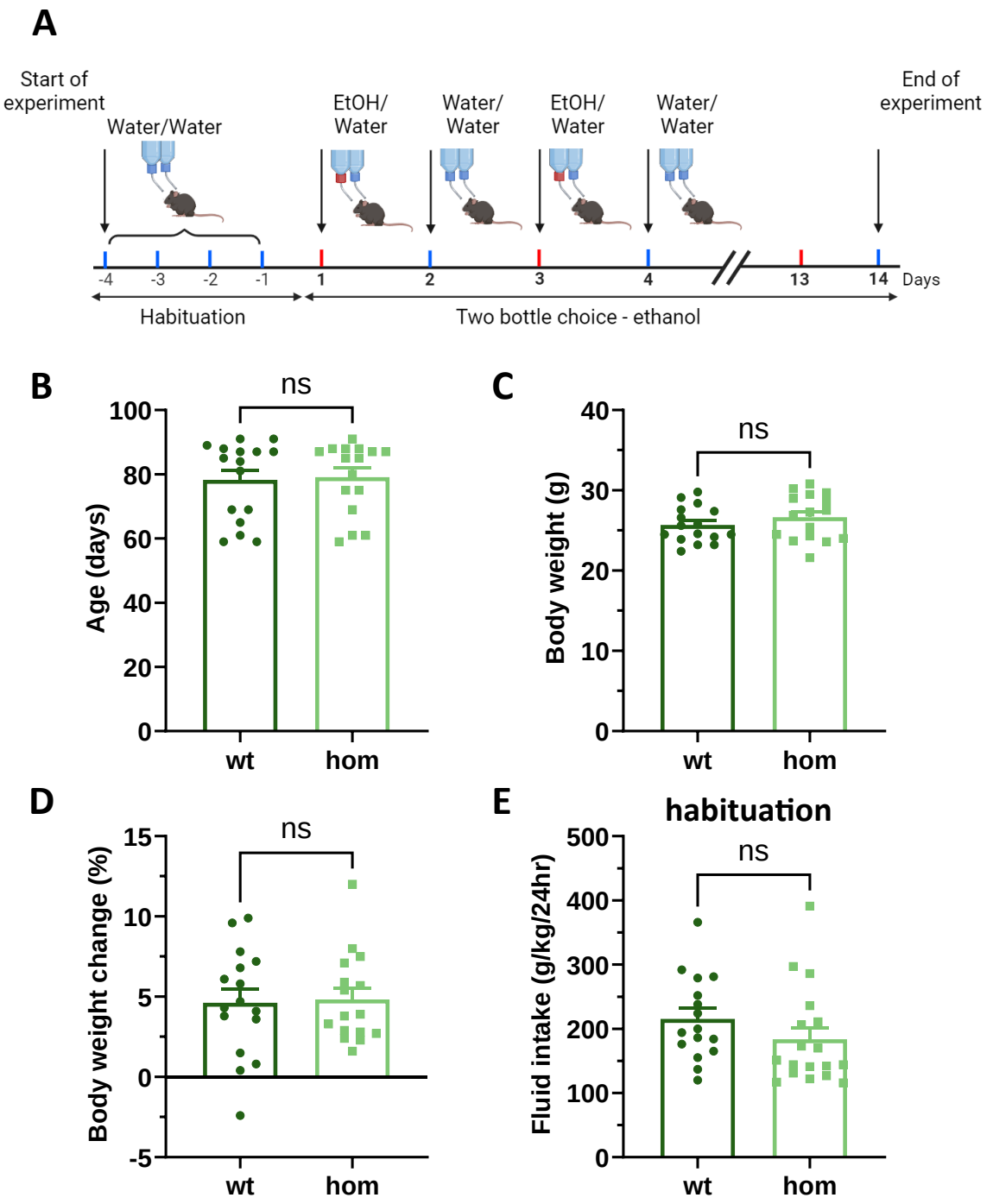


Figure 3.1 Protocol and control parameters of the alcohol two bottle choice paradigm for α4^{Q246M} wild type and homozygous mutant mice

A, Schematic diagram of the intermittent access protocol used in the experiment. ***B,*** The age (days) of animals at the start of the experiment. ***C,*** The body weight (g) of animals at the start of the experiment. ***D,*** Body weight change (%) of animals from the start to the end of the experiment. ***E,*** Fluid intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are unpaired student's t test for panels *B, C* and *E*, and Mann-Whitney test for panel *D*. Wt: n = 16; hom: n = 18.

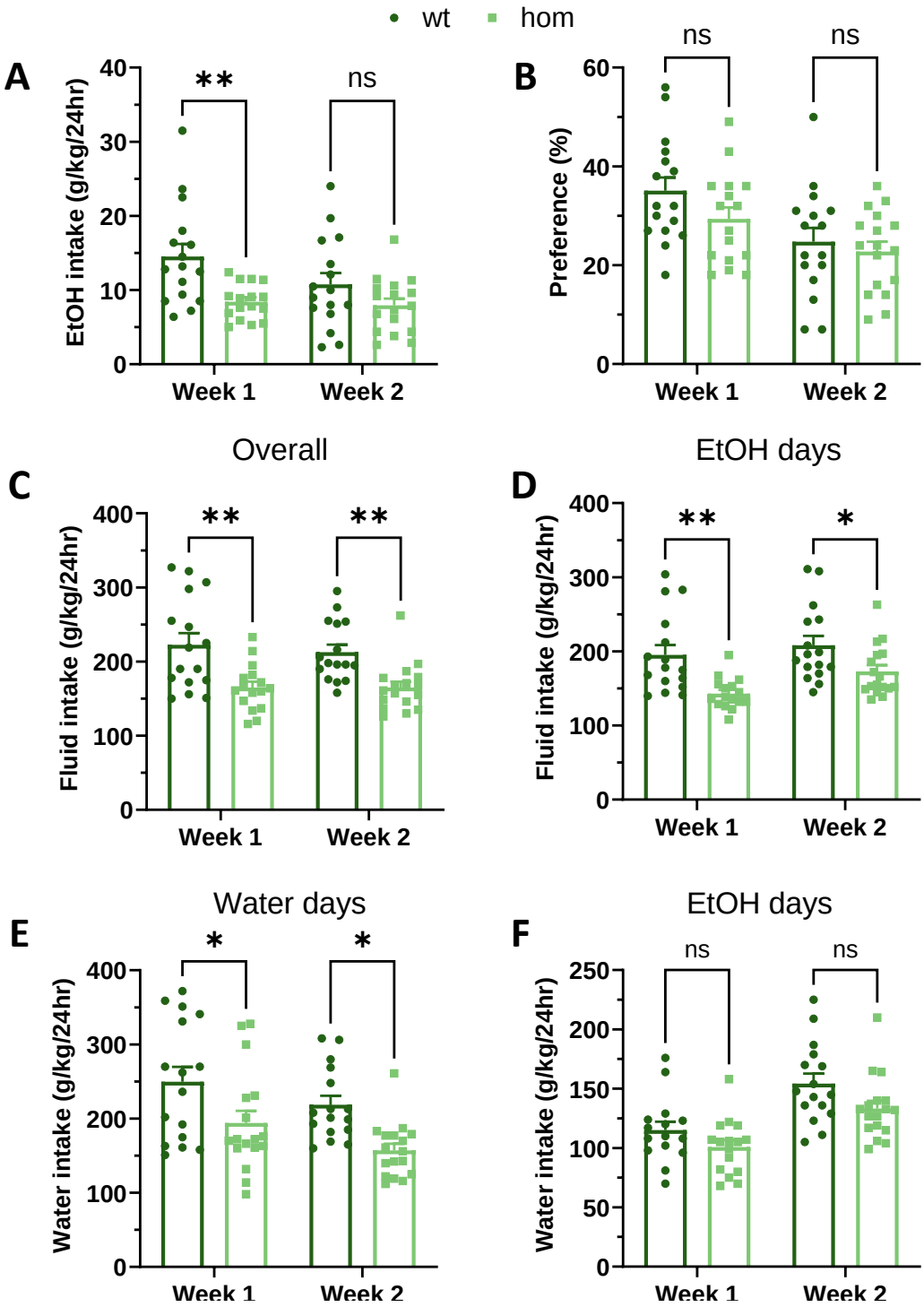


Figure 3.2 Removing neurosteroid sensitivity in α4-GABA$_A$Rs decreases ethanol intake in the intermittent access two bottle choice paradigm

A, Mean daily intake of ethanol measured in grams per kg of body weight for the two weeks mice received access to 20 % w/v EtOH. ***B***, Mean daily preference (%) for 20 % w/v EtOH. ***C***, Mean weekly fluid intake (water + EtOH) in grams per body weight (kg) during the experiment, including both EtOH/Water and Water/Water days. ***D,*** Mean daily fluid intake (water + EtOH) in grams per kg of body weight on EtOH access days (EtOH/Water). ***E***, Mean daily water intake (g) per body weight (kg) on Water/Water days. ***F***, Mean daily water intake (g) per body weight (kg) on EtOH access days (EtOH/Water). Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences). Statistical significance indicated as * $p < 0.05$, ** $p < 0.01$ compared to wild type. Wild type: n = 16; hom: n = 18.

3.2.2 Alcohol consumption of α2^{Q241M} mice

We also investigated whether rendering α2-GABA$_A$Rs insensitive to neurosteroids impacts upon ethanol consumption and preference. We used the same protocol as described above (see *Figure 3.1 A*). Wild type and mutant animals were age-matched (days; *Figure 3.3 A, Table 3.2*), had no difference in starting body weight (g; *Figure 3.3 B, Table 3.2*) and no change in their body weight over the course of the experiment (%; *Figure 3.3 C, Table 3.2*). There was no difference between fluid intake (g/kg) during the habituation period between genotypes (*Figure 3.3 D, Table 3.2*).

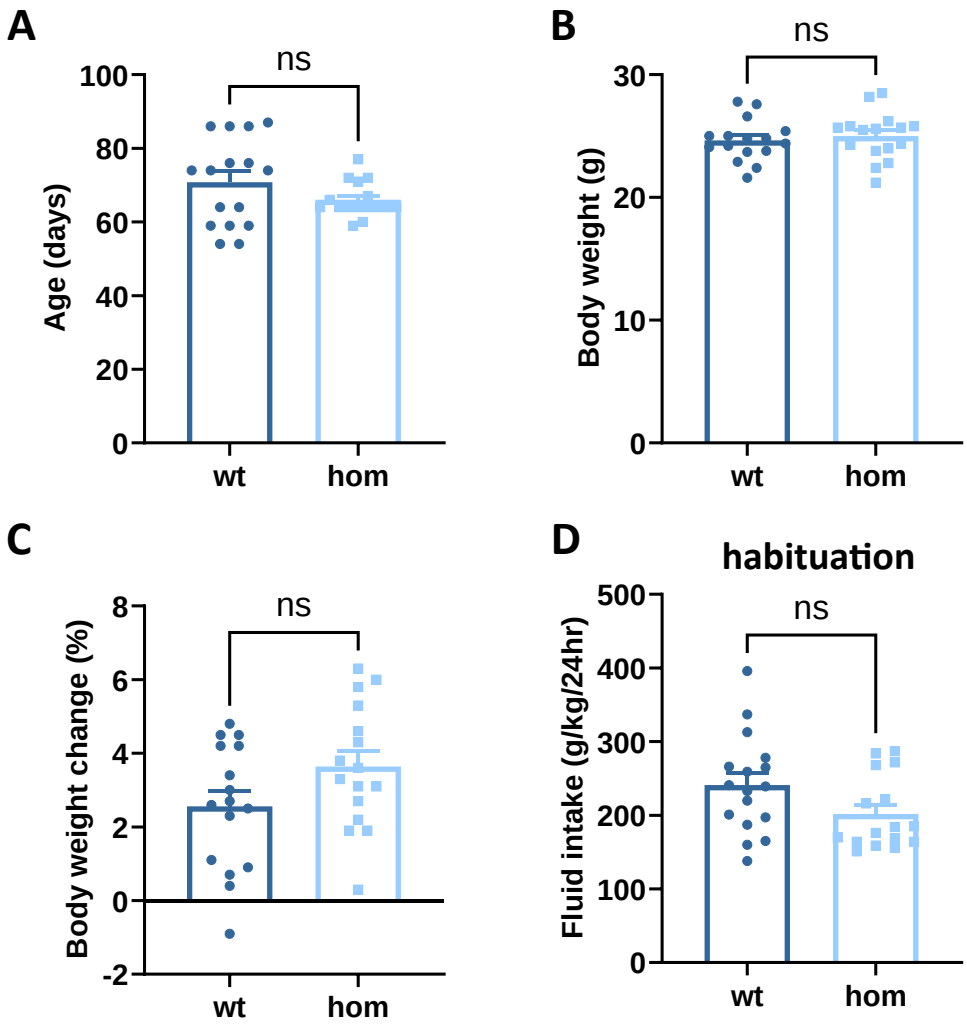


Figure 3.3 Control parameters of the alcohol two bottle choice paradigm for α2^{Q241M} wild type and homozygous mutant mice

A, The age (in days) of animals at the start of the experiment. ***B,*** The body weight (in grams) of animals at the start of the experiment. ***C,*** Body weight change (%) of animals from the start to the end of the experiment. ***D,*** Fluid intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are unpaired student's t test with Welch's correction for panel *A*, and without Welch's correction for panels *B, C* and *D*. Wt: n = 18; hom: n = 16.

The mean ± SEM values and statistical analysis results of this experiment are contained in *Table 3.3* and *Table 3.5*, respectively, in *Section 3.5*.

We observed a significant decrease in ethanol intake (g/kg; *Figure 3.4 A*) between wild type and mutant mice, which was only apparent in Week 1. Two-way RM ANOVA detected a significant interaction between time and genotype, again indicating that mutant mice consumed less ethanol in the first week of the experiment compared to wild type controls. We detected the same pattern for ethanol preference (%; *Figure 3.4 B*), with a significant reduction (20 %) between genotypes in Week 1 but no difference in Week 2. Analysis of preference data using two-way RM ANOVA also revealed a significant genotype by time interaction and a main effect of time. The difference in Week 2 disappears because wild type mice significantly reduce their EtOH intake (week 1: 15.7 ± 1.9 g/kg, week 2: 10.9 ± 1.0 g/kg, P = 0.023) and preference (week 1: 33 ± 2 %, week 2: 25 ± 2 %, P = 0.004) in the second week compared to the first, whereas EtOH consumption (week 1: 10.6 ± 1.0 g/kg , week 2: 11.6 ± 1.5 g/kg, P = 0.838) and preference (week 1: 26 ± 2 %, week 2: 25 ± 2 %, P = 0.967) remain unchanged between week 1 and week 2 in mutant mice.

Two-way RM ANOVA revealed a significant Time x Genotype interaction in overall fluid intake (g/kg; *Figure 3.4 C*) and water intake on water days (g/kg; *Figure 3.4 E*), however, post-hoc multiple comparison tests found no difference between genotypes. Furthermore, we observed no changes in fluid intake or water intake on EtOH access days (g/kg; *Figure 3.4 D* and *Figure 3.4 F,* respectively).

Taken together, our data indicates that mice possessing neurosteroid-sensitive α2-receptors exhibit an initial increase in ethanol consumption upon exposure during the first week. Conversely, this initial surge in consumption is absent in mice with neurosteroid-insensitive α2-receptors, which display a more consistent pattern of ethanol intake.

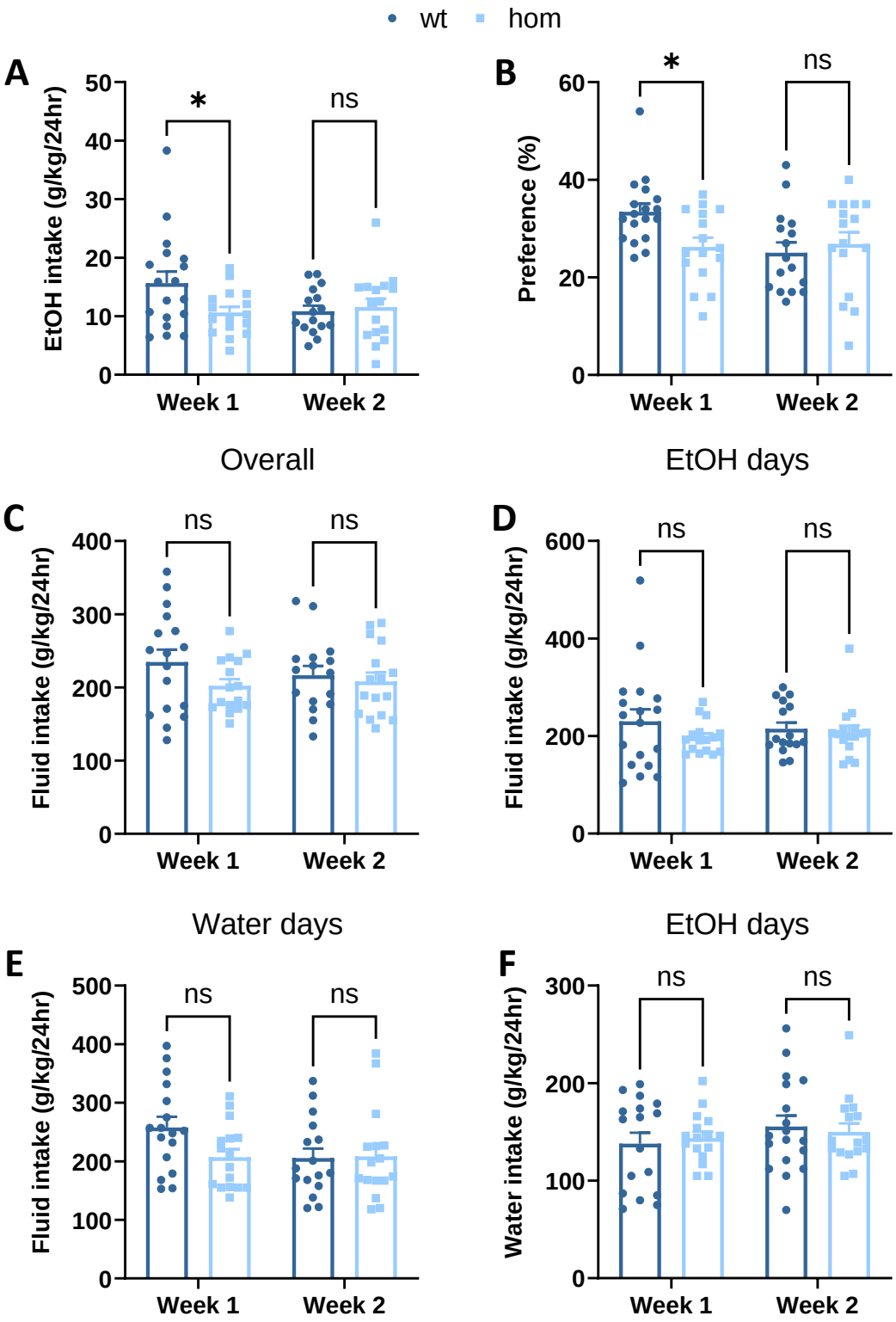


Figure 3.4 Removing neurosteroid sensitivity from α2-GABA$_A$Rs impacts upon ethanol intake and preference in the intermittent access paradigm

A, Mean daily EtOH intake (g) per body weight (kg). ***B,*** Mean daily preference (%) for 20 % w/v EtOH. ***C,*** Mean daily fluid intake (water + EtOH) in grams per kg of body weight throughout the duration of the experiment. ***D,*** Mean daily fluid intake (water + EtOH) in grams per body weight (kg) on EtOH access days. ***E,*** Mean water intake (g/kg) on Water/Water days. ***F,*** Mean daily water intake (g) per kg of body weight on EtOH access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences). Statistical significance indicated as * $p < 0.05$, ** $p < 0.01$ compared to wild type. Wild type: n = 18; hom = 16.

3.2.3 Saccharin intake of α4^{Q246M} mice

To test whether the reduction in consumption of α4$^{M/M}$ was ethanol-specific, and to confirm that the difference was not due to the taste of the ethanol solution, we assessed two bottle choice consumption of ascending concentrations of two different tastants – saccharin (sweet) and quinine (bitter). This section will cover the results of the saccharin experiments.

We chose the ascending concentration paradigm (*Figure 3.5 A*) instead of a single concentration to better resolve small differences between genotypes (Tordoff and Bachmanov, 2002). In short, mice were presented with ascending concentrations of saccharin (0.25 mM, 0.5 mM and 1 mM) in a two bottle choice with drinking water in the other bottle. Each concentration was presented for four consecutive days. The animals used for these experiments were age-matched (days; *Figure 3.5 B, Table 3.1*). Homozygous mutant mice weighed slightly more compared to wild type mice, despite being the same age, at the start of the saccharin experiment (g; *Figure 3.5 C, Table 3.1*). This difference in body weight is unlikely to affect the result of the experiment, as their fluid intake is normalised to body weight. We observed no differences between genotypes in body weight change (%; *Figure 3.5 D, Table 3.1*) and fluid intake during habituation (g/kg; *Figure 3.5 E, Table 3.1*). The mean ± SEM values and statistical analysis results of this experiment are contained in *Table 3.6, Table 3.7, Table 3.10, Table 3.11* and *Table 3.12* in *Section 3.5*.

Saccharin intake (g/kg; *Figure 3.6 A* and *B*, *Figure 3.7 A* and *B, Figure 3.8 A* and *B*), saccharin preference (%; *Figure 3.6 C* and *D, Figure 3.7 C* and *D, Figure 3.8 C* and *D*), fluid intake (g/kg; *Figure 3.6 E* and *F, Figure 3.7 E* and *F, Figure 3.8 E* and *F*) or water intake (g/kg; *Figure 3.6 G* and *H, Figure 3.7 G* and *H, Figure 3.8 G* and *H*) were unaffected by the removal of neurosteroid sensitivity at α4-GABA$_A$Rs at any of the concentrations (0.25, 0.5 and 1 mM) tested.

Two-way RM ANOVA of both saccharin intake and preference data showed a significant main effect of concentration (*Table 3.14*), with both genotypes increasing their intake and preference with higher saccharin concentrations (*Figure 3.21 A* and *B*), thereby confirming that the chosen concentrations cover an appropriate range.

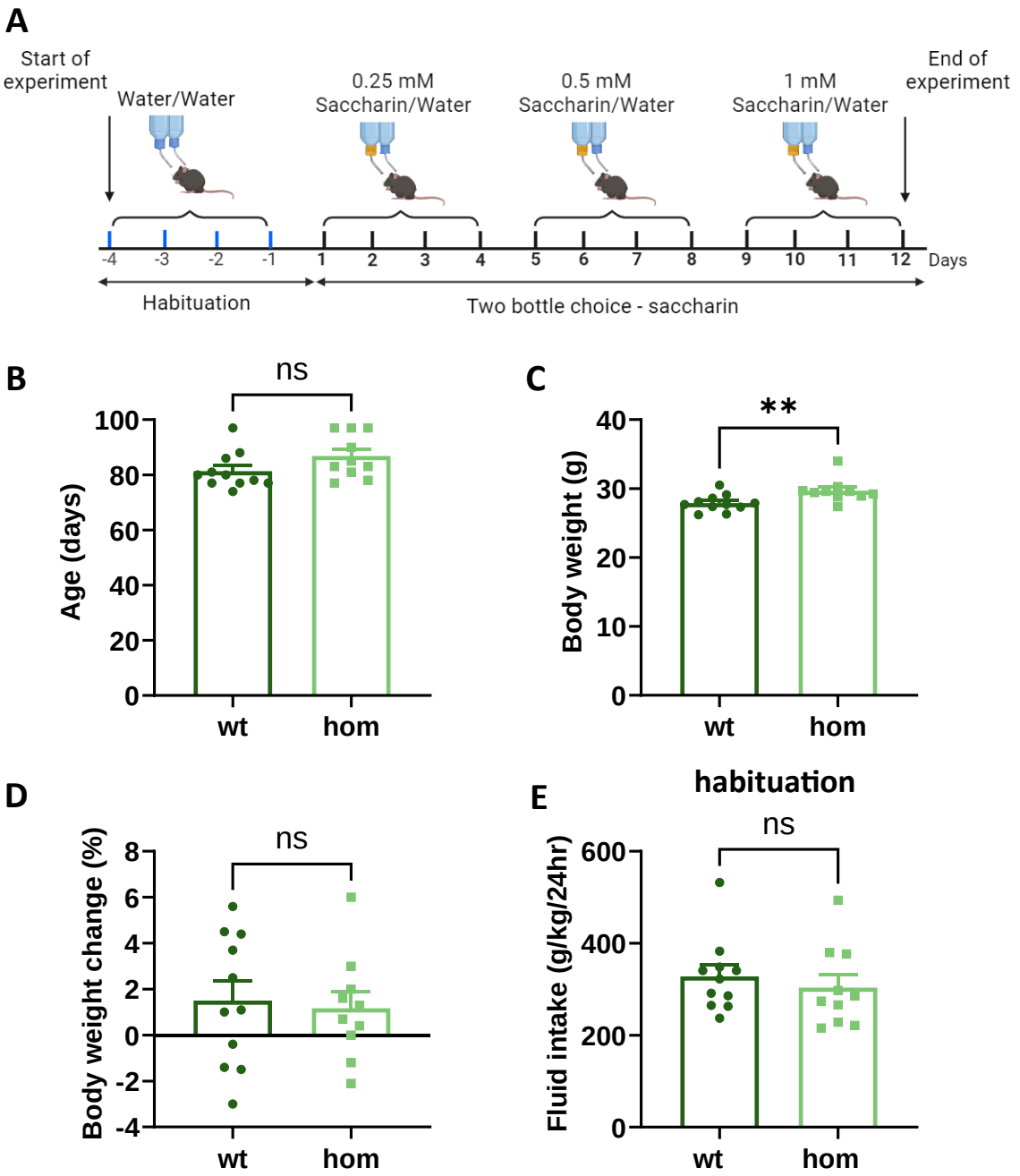


Figure 3.5 Protocol and control parameters of the saccharin two bottle choice paradigm for $\alpha4^{Q246M}$ wild type and homozygous mutant mice.

A, Schematic diagram of the tastant (saccharin) access protocol used in the experiment. ***B,*** The age (in days) of animals at the start of the experiment. ***C,*** The body weight (in grams) of animals at the start of the experiment. ***D,*** Body weight change (%) of animals from the start to the end of the experiment. ***E,*** Fluid intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are Mann-Whitney test for panels *A* and *D,* and unpaired student's t test for panels *B, C* and *E*; ** $p < 0.01$. Wt: n = 11; hom: n = 10.

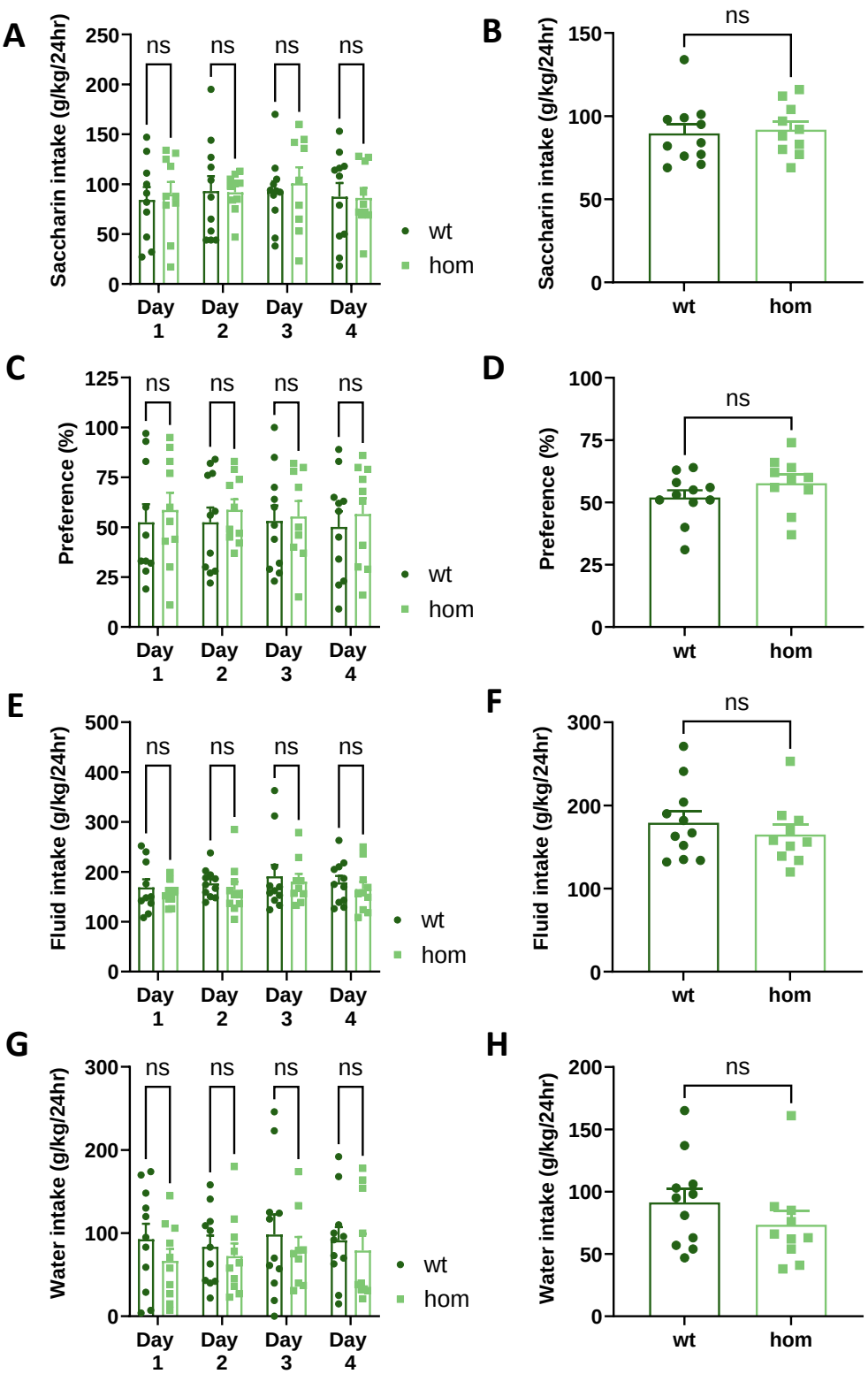


Figure 3.6 Saccharin (0.25 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α4 subunit

A, C, E and ***G***, Mean daily saccharin intake (g) per body weight (kg), mean daily saccharin preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.25 mM saccharin, respectively. ***B, D, F*** and ***H,*** Mean daily saccharin intake (g/kg), mean preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.25 mM saccharin access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, unpaired student's t test for panels B, D and F, and Mann-Whitney test for panel *H*. Wt: n = 11; hom: n = 10.

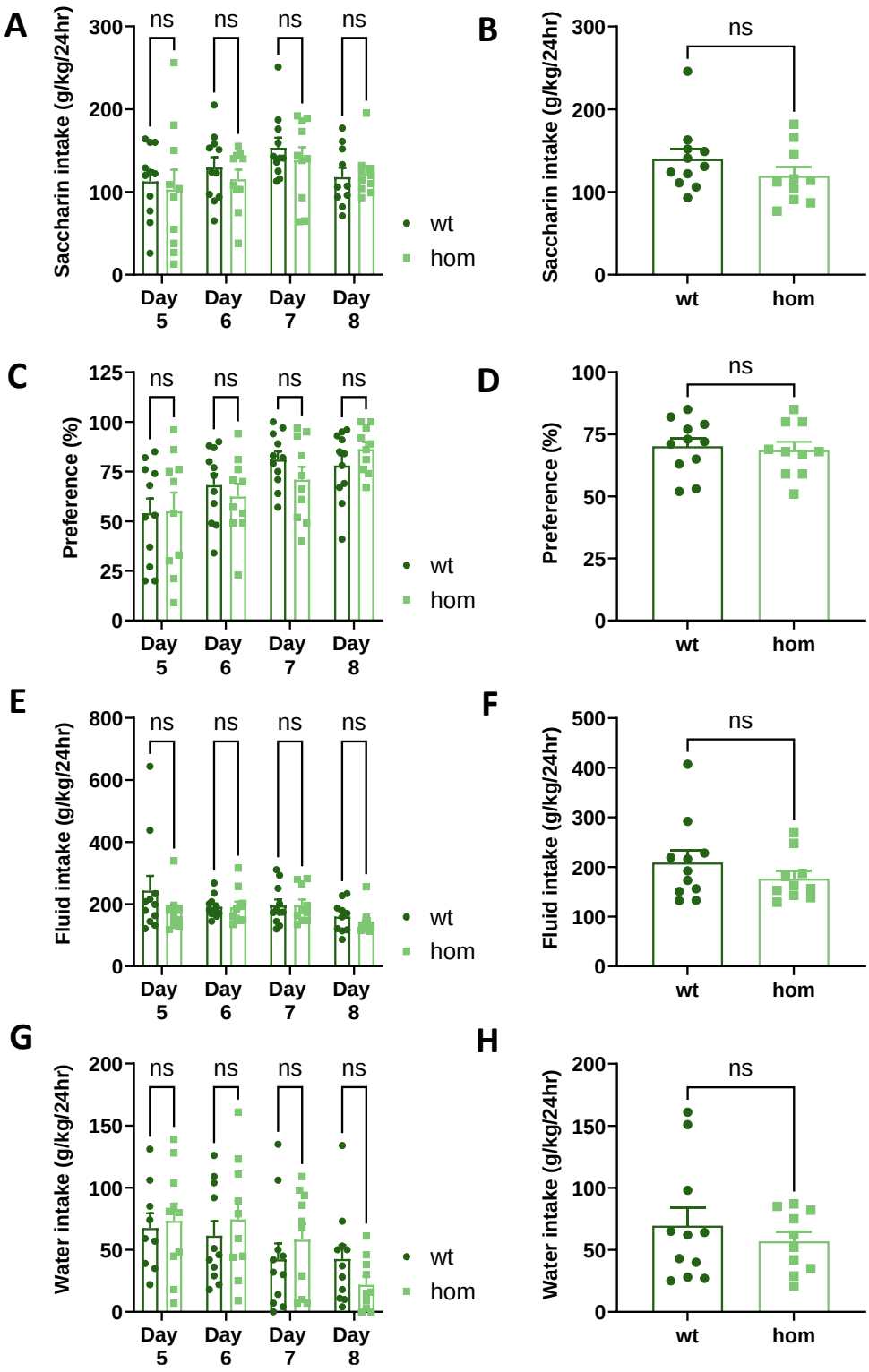


Figure 3.7 Saccharin (0.5 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α4 subunit

A, C, E and ***G***, Mean daily saccharin intake (g) per body weight (kg), mean daily saccharin preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.5 mM saccharin, respectively. ***B, D, F*** and ***H,*** Mean daily saccharin intake (g/kg), mean preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.5 mM saccharin access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, Mann-Whitney test for panels *B, F* and *H*, and unpaired student's t test for panel *D*. Wt: n = 11; hom: n = 10.

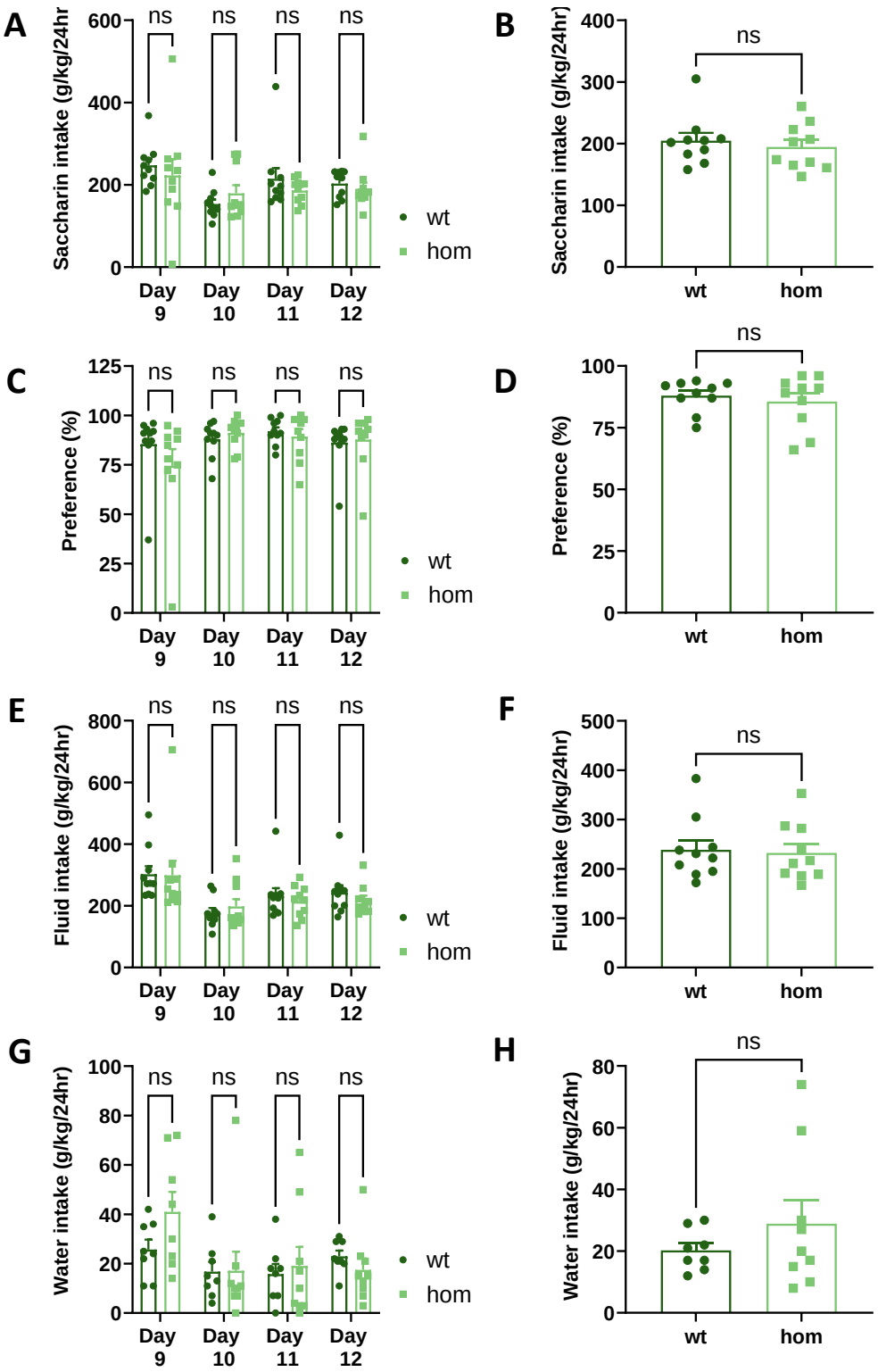


Figure 3.8 Saccharin (1 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α4 subunit

A, ***C, E*** and ***G***, Mean daily saccharin intake (g) per body weight (kg), mean daily saccharin preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 1 mM saccharin, respectively. ***B, D, F*** *and* ***H,*** Mean daily saccharin intake (g/kg), mean preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 1 mM saccharin access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, Mann-Whitney test for panels *B* and *H,* and unpaired student's t test for panels *D* and *F.* Wt: n = 11; hom: n = 10.

3.2.4 Saccharin intake of α2^{Q241M} mice

We tested the impact of the absence of the neurosteroid binding site in the α2 subunit on saccharin consumption using the ascending concentration protocol described in earlier *Section 3.2.3* (*Figure 3.5 A*). There were no differences in age (days; *Figure 3.9 A*, *Table 3.2*), body weight (g; *Figure 3.9 B*, *Table 3.2*), body weight change during the experiment (%; *Figure 3.9 C*, *Table 3.2*) or fluid intake (g/kg) during habituation (*Figure 3.9*, *Table 3.2*) between genotypes.

The mean ± SEM values and statistical analysis results of this experiment are contained in *Table 3.8, Table 3.9, Table 3.13, Table 3.14* and *Table 3.15* in *Section 3.5*.

We found no differences in saccharin intake (g/kg; *Figure 3.10 A* and *B, Figure 3.11 A* and *B, Figure 3.12 A* and *B*), saccharin preference (%; *Figure 3.10 C* and *D, Figure 3.11 C* and *D, Figure 3.12 C* and *D*), total fluid intake (g/kg; *Figure 3.10 E* and *F, Figure 3.11 E* and *F, Figure 3.12 E* and *F*) or water intake (g/kg; *Figure 3.10 G* and *H, Figure 3.11 G* and *H, Figure 3.12 G* and *H*) between wild type and homozygous mutant mice at any of the saccharin concentrations tested (0.25 mM, 0.5 mM or 1 mM).

Analysis of saccharin intake and preference of the whole experiment (12 days, *Figure 3.22 A* and *B, Table 3.17*) revealed a significant main effect of concentration, with both wild type and mutant mice increasing their saccharin intake and preference at higher concentrations, indicating that the appropriate saccharin concentration range was chosen for detecting any differences.

These results suggest that rendering α2-containing $GABA_A$ receptors insensitive to neurosteroids does not influence saccharin consumption.

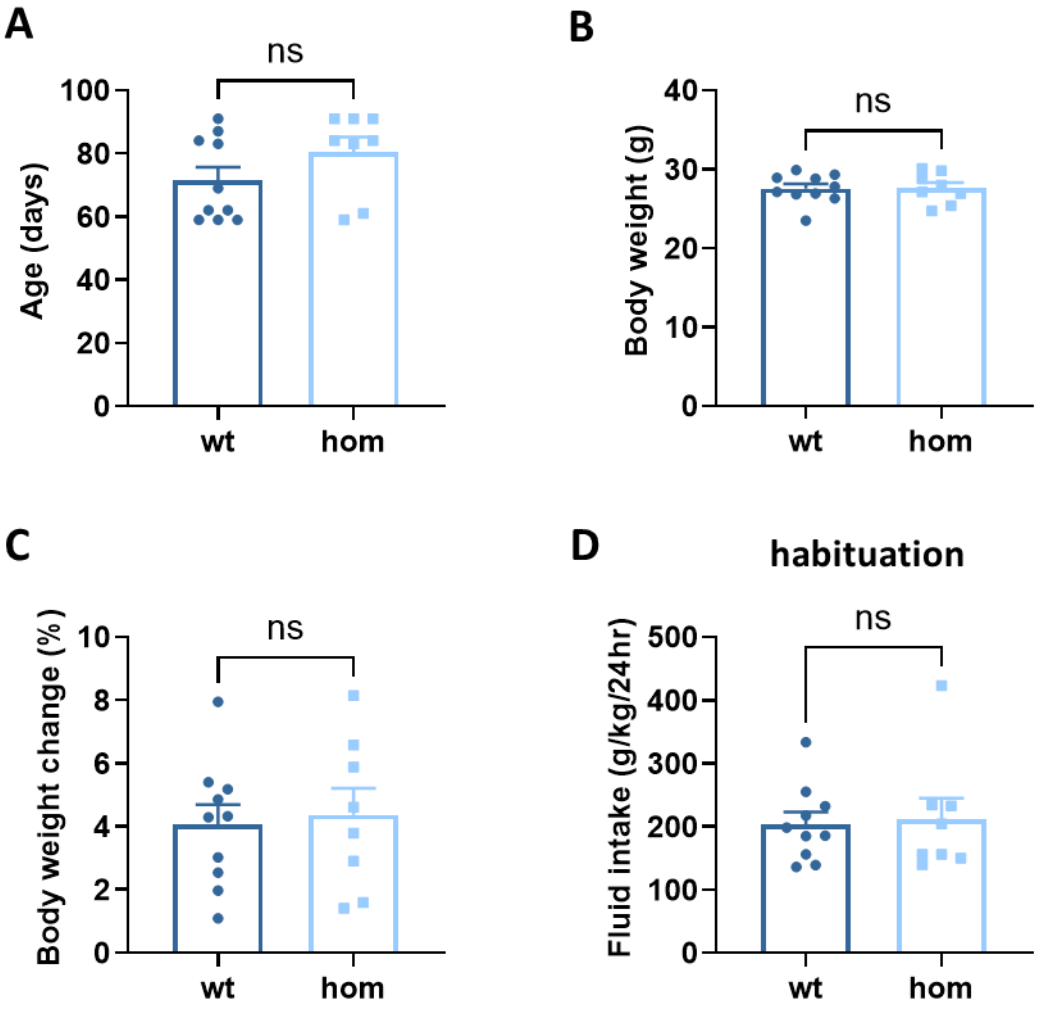


Figure 3.9 Control parameters of the tastant (saccharin) two bottle choice paradigm for $\alpha2^{Q241M}$ wild type and homozygous mutant mice

A, The age (in days) of animals at the start of the experiment. ***B,*** The body weight (in grams) of animals at the start of the experiment. ***C,*** Body weight change (%) of animals from the start to the end of the experiment. ***D,*** Fluid intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are Mann-Whitney test for panel *A*, and unpaired student's t test for panels *B, C* and *D*. Wt: n = 10; hom: n = 8.

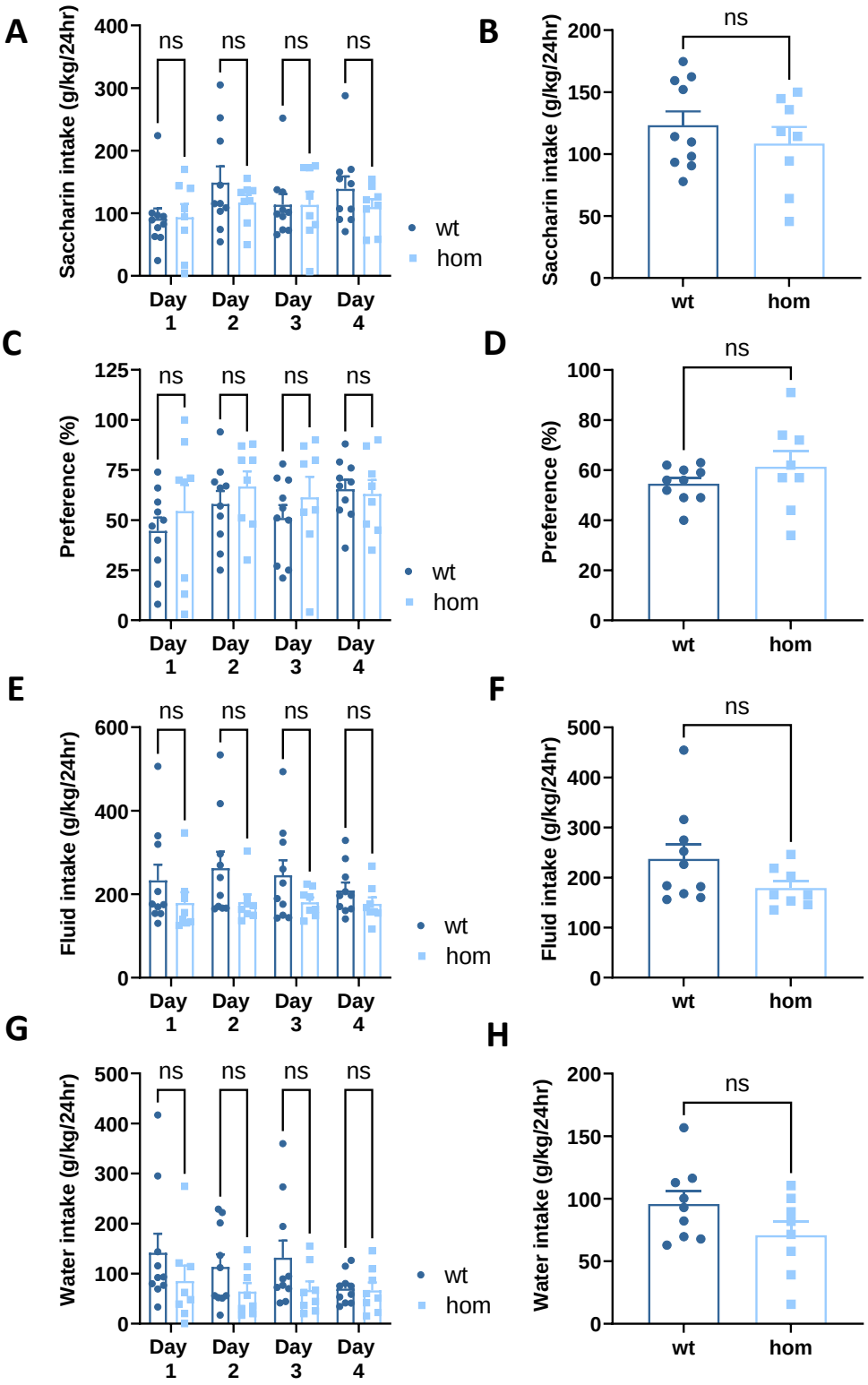


Figure 3.10 Saccharin (0.25 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α2 subunit

A, ***C, E*** and ***G***, Mean daily saccharin intake (g) per body weight (kg), mean daily saccharin preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.25 mM saccharin, respectively. ***B, D, F*** *and* ***H,*** Mean daily saccharin intake (g/kg), mean preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.25 mM saccharin access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, unpaired student's t test for panels *B* and *H*, unpaired student's t test with Welch's correction for panel *D*, and Mann-Whitney test for panel *F*. Wt: n = 10; hom: n = 8.

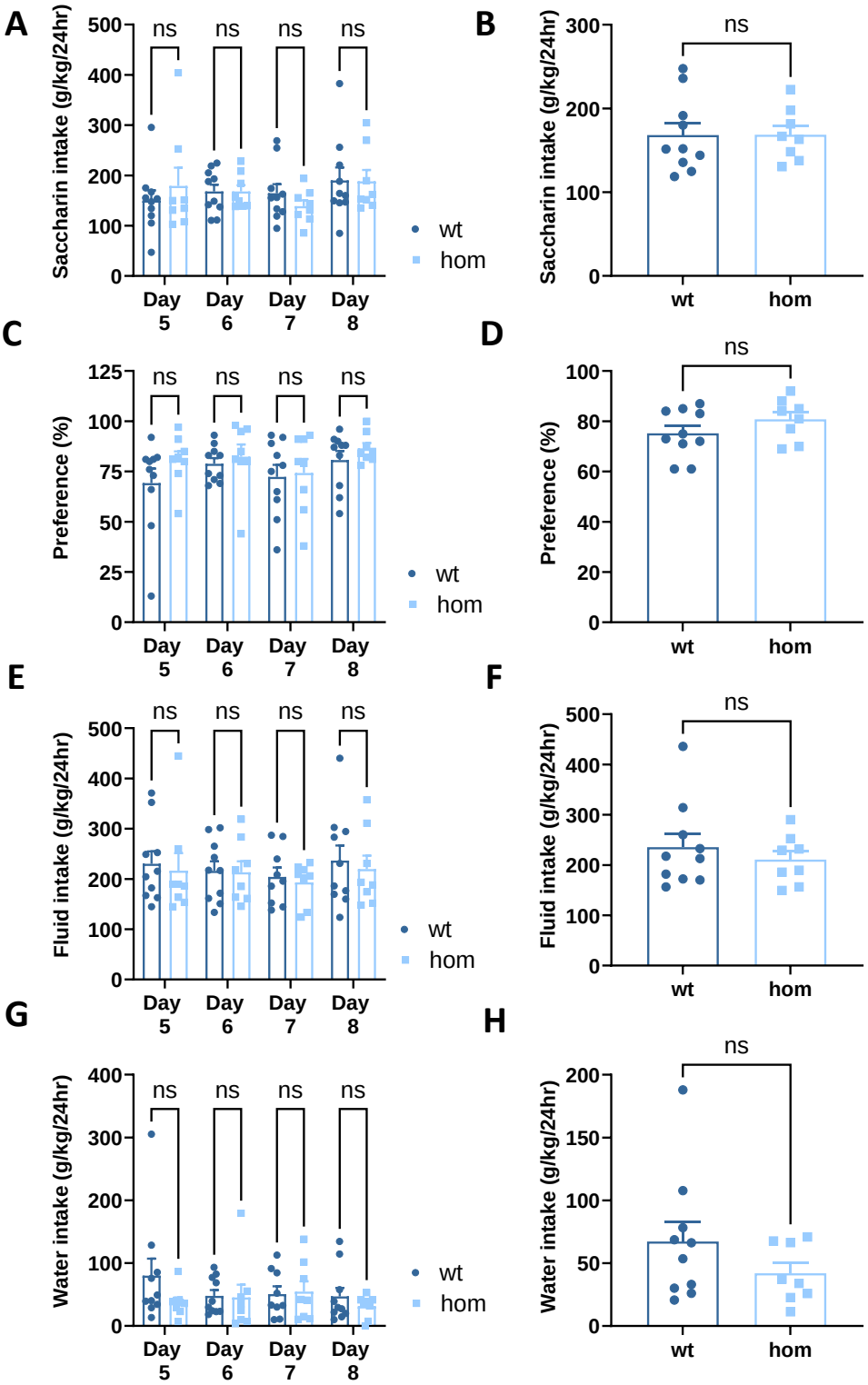


Figure 3.11 Saccharin (0.5 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α2 subunit

A, ***C, E*** and ***G***, Mean daily saccharin intake (g) per body weight (kg), mean daily saccharin preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.5 mM saccharin, respectively. ***B, D, F*** *and* ***H,*** Mean daily saccharin intake (g/kg), mean preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.5 mM saccharin access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, unpaired student's t test for panels *B* and *D*, and Mann-Whitney test for panels *F* and *H*. Wt: n = 10; hom: n = 8.

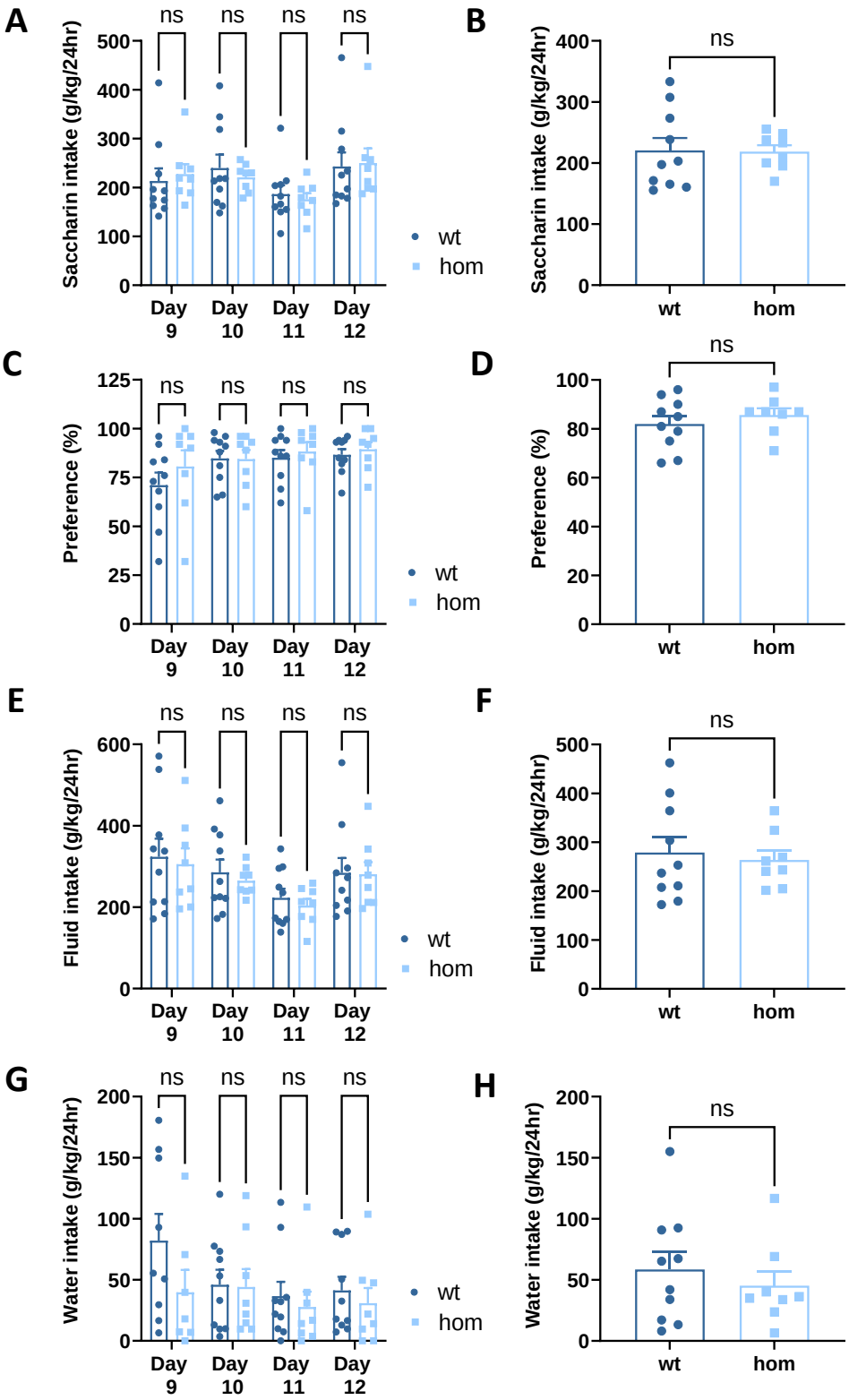


Figure 3.12 Saccharin (1 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α2 subunit

A, C, E and ***G***, Mean daily saccharin intake (g) per body weight (kg), mean daily saccharin preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 1 mM saccharin, respectively. ***B, D, F*** and ***H,*** Mean daily saccharin intake (g/kg), mean preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 1 mM saccharin access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G,* unpaired student's t test for panels *B, D* and *F*, and Mann-Whitney test for panel *H*. Wt: n = 10; hom: n = 8.

3.2.5 Quinine intake of α4^{Q246M} mice

We also assessed quinine intake (0.025 mM, 0.05 mM and 0.1 mM; (Blednov et al., 2011)) of wild type and homozygous mutant α4^{Q246M} mice using the ascending concentration paradigm (*Figure 3.13 A*). The animals were age-matched (days; *Figure 3.13 B, Table 3.1*), had no difference in their starting body weight (g; *Figure 3.13 C, Table 3.1*) and their body weight change during the experiment was not affected by the mutation (%; *Figure 3.13 D, Table 3.1*). Furthermore, we observed no differences in fluid intake (g/kg) during habituation between genotypes (*Figure 3.13 E, Table 3.1*).

The mean ± SEM values and statistical analysis results of this experiment are contained in *Table 3.6, Table 3.7, Table 3.16, Table 3.17* and *Table 3.18* in *Section 3.5*.

Experiments with quinine showed no difference in quinine intake (g/kg; *Figure 3.14 A* and *B, Figure 3.15 A* and *B, Figure 3.16 A* and *B*), quinine preference (%; *Figure 3.14 C* and *D, Figure 3.15 C* and *D, Figure 3.16 C* and *D*), overall fluid intake (g/kg; *Figure 3.14 E* and *F, Figure 3.15 E* and *F, Figure 3.16 E* and *F*) or water intake (g/kg; *Figure 3.14 G* and *H, Figure 3.15 G* and *H, Figure 3.16 G* and *H*) between wild type and homozygous mutant mice at any of the three concentrations (0.025, 0.05 and 0.1 mM) tested.

Two-way RM ANOVA of quinine intake data did not reveal any significant effect of concentration (*Figure 3.23 A*, *Table 3.20*). However, analysis of preference data detected a main effect of concentration, with both α4$^{Q/Q}$ and α4$^{M/M}$ mice having reduced preference for higher concentrations of quinine (*Figure 3.23 B*, *Table 3.20*). This can be explained by both wild type and mutant mice consuming more water at higher quinine concentrations (*Table 3.7*). Nevertheless, the differences in preference between concentrations indicate that we have likely chosen a range that could reveal differences between the genotypes.

Taken together, these results considered with those presented in *Section 3.2.3*, suggest that the removal of neurosteroid sensitivity in α4-GABA$_A$Rs is likely responsible for the reduced ethanol intake, and that the changes in total fluid intake of α4$^{M/M}$ mice during EtOH experiments is specific to ethanol.

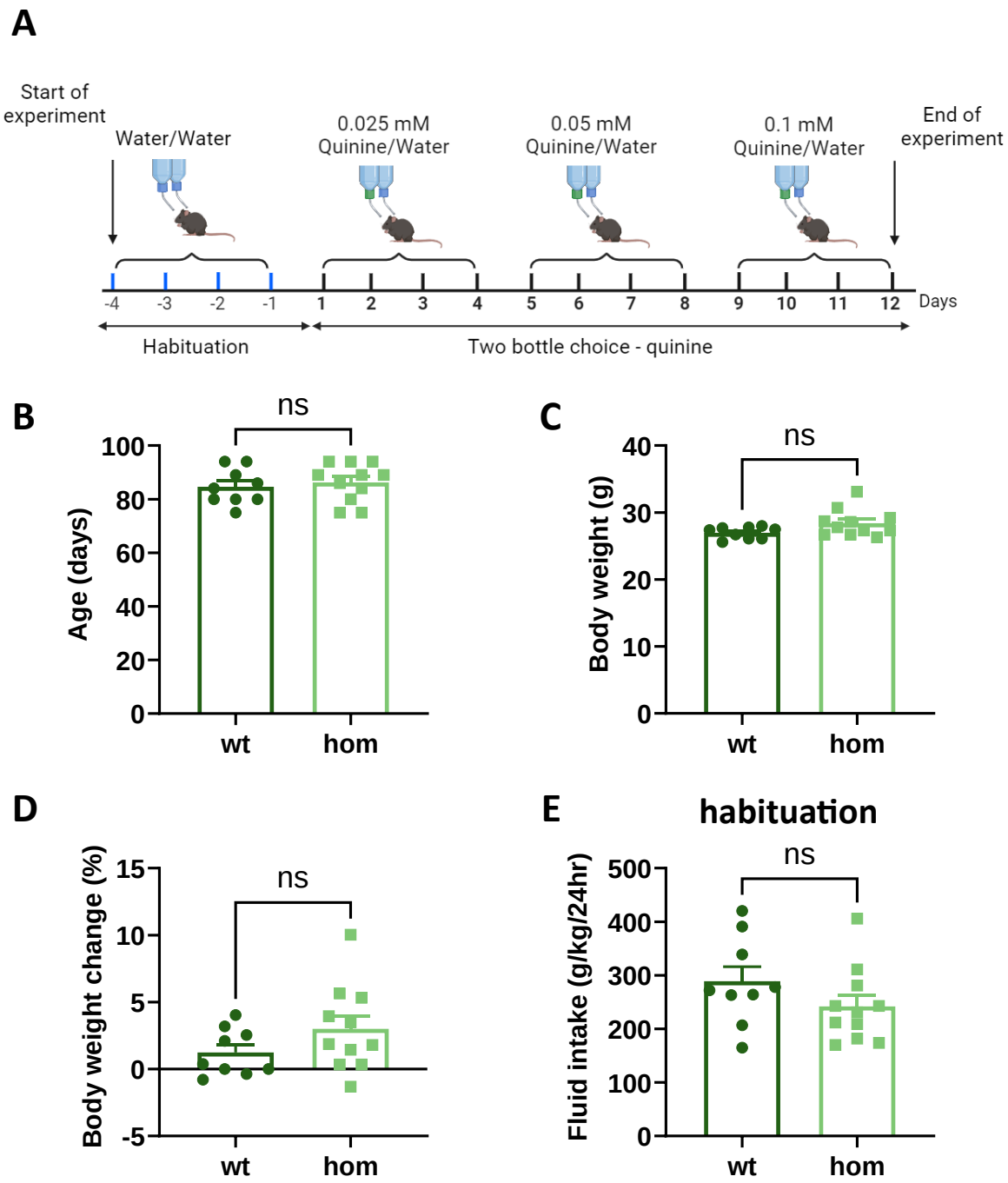


Figure 3.13 Protocol and control parameters of the quinine two bottle choice paradigm for α4^{Q246M} wild type and homozygous mutant mice

A, Schematic diagram of the tastant (quinine) access protocol used in the experiment. ***B,*** The age (in days) of animals at the start of the experiment. ***C,*** The body weight (in grams) of animals at the start of the experiment. ***D,*** Body weight change (%) of animals from the start to the end of the experiment. ***E,*** Fluid intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used unpaired student's t test for panels *B, D* and *E*, and student's t test with Welch's correction for panel *C*. Wt: n = 9; hom: n = 11.

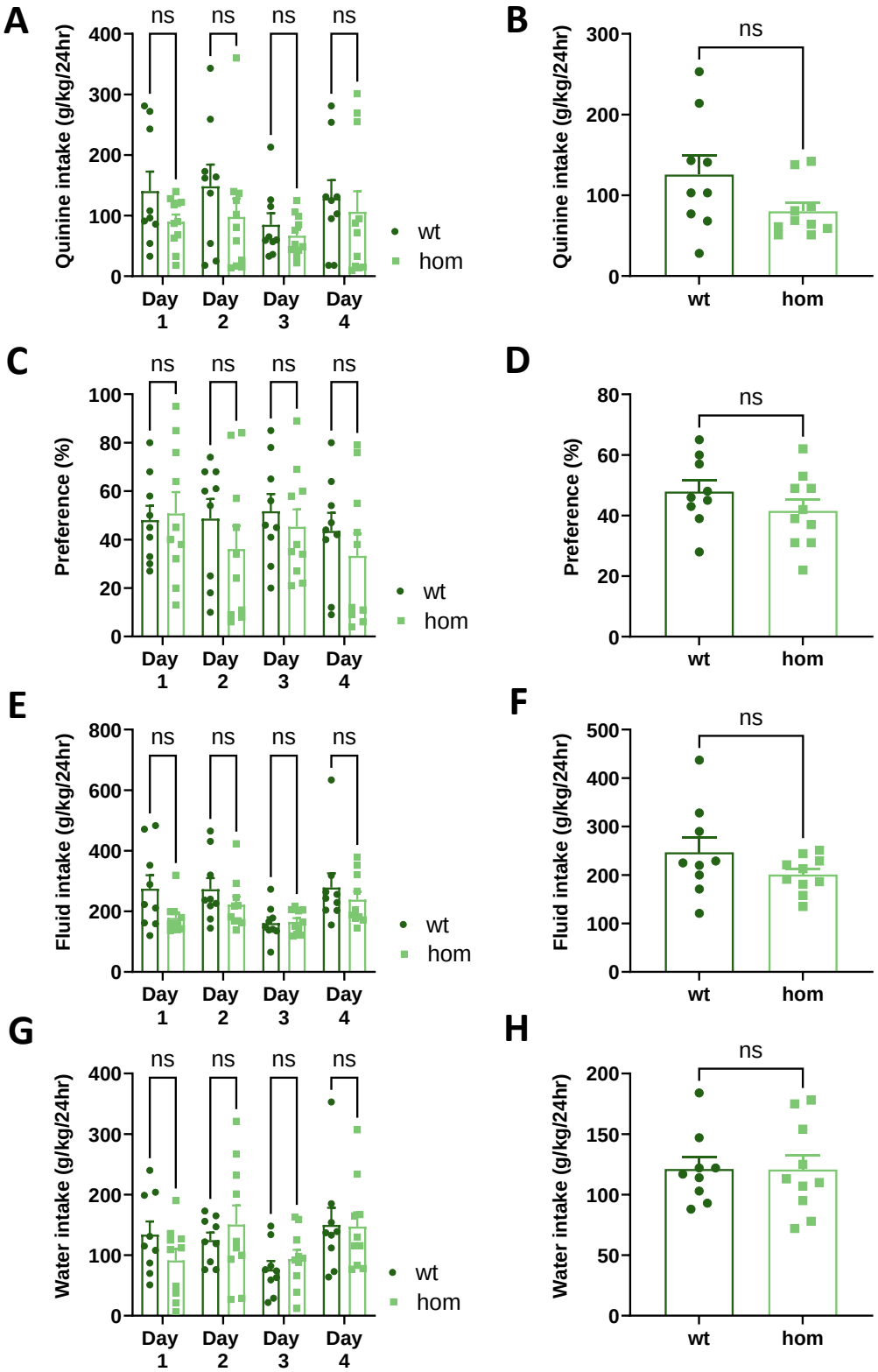


Figure 3.14 Quinine (0.025 mM) intake and preference are not affected by removing neurosteroid sensitivity from the α4 subunit

A, ***C, E*** and ***G***, Mean daily quinine intake (g) per body weight (kg), mean daily quinine preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.025 mM quinine, respectively. ***B, D, F*** *and* ***H,*** Mean daily quinine intake (g/kg), mean quinine preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.025 mM quinine access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, unpaired student's t test for panels *B, D, F* and *H*. Wt: n = 9; hom: n = 11.

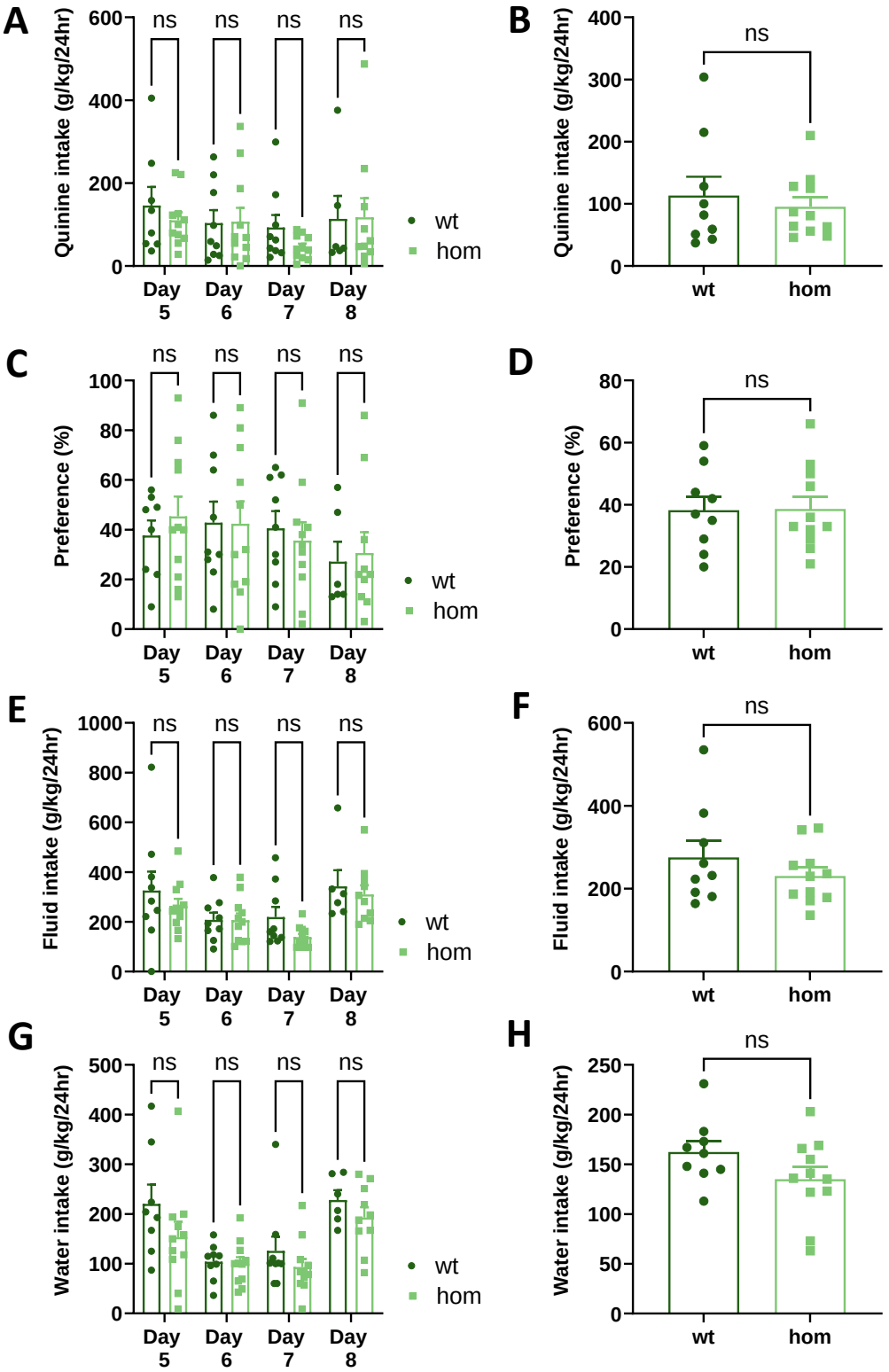


Figure 3.15 Quinine (0.05 mM) intake and preference are not affected by removing neurosteroid sensitivity from α4-GABA$_A$Rs

A, ***C, E*** and ***G***, Mean daily quinine intake (g) per body weight (kg), mean daily quinine preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.05 mM quinine, respectively. ***B, D, F*** *and* ***H,*** Mean daily quinine intake (g/kg), mean quinine preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.05 mM quinine access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, Mann-Whitney test for panel *B*, and unpaired student's t test for panels *D, F* and *H*. Wt: n = 9; hom: n = 11.

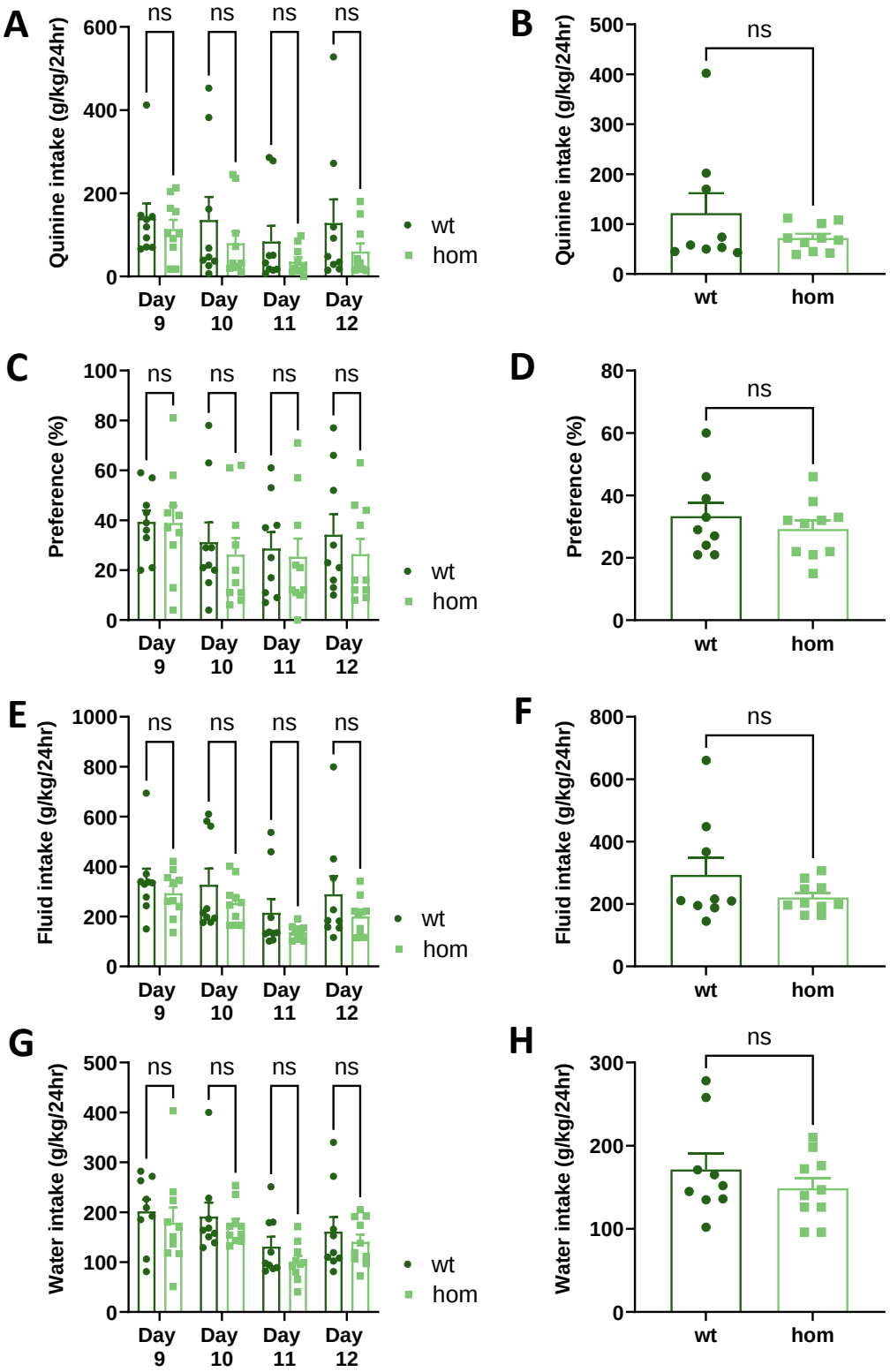


Figure 3.16 Quinine (0.1 mM) intake and preference are not affected by removing neurosteroid sensitivity from α4-GABA$_A$Rs

A, ***C, E*** and ***G***, Mean daily quinine intake (g) per body weight (kg), mean daily quinine preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.1 mM quinine, respectively. ***B, D, F*** *and* ***H,*** Mean daily quinine intake (g/kg), mean quinine preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.1 mM quinine access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, Mann-Whitney test for panels *B, D* and *H*, and unpaired student's t test for panel *F*. Wt: n = 9; hom: n = 11.

3.2.6 Quinine intake of α2^{Q241M} mice

We also explored whether removing neurosteroid sensitivity from α2-containing $GABA_A$ receptors would affect quinine consumption. We used the ascending concentration paradigm described above in *Section 3.2.5* (*Figure 3.13 A*).

The mice used in this experiment were age-matched (days; *Figure 3.17 A*). We detected no differences in body weight (g; *Figure 3.17 B*), body weight change during the experiment (%; *Figure 3.17 C*) or fluid intake (g/kg) during habituation (*Figure 3.17 D*) between wild type controls and homozygous mutant mice.

The mean ± SEM values and statistical analysis results of this experiment are contained in *Table 3.8, Table 3.9, Table 3.19, Table 3.20* and *Table 3.21* in *Section 3.5*.

There was no change in quinine intake (g/kg; *Figure 3.18 A* and *B, Figure 3.19 A* and *B, Figure 3.20 A* and *B*), quinine preference (%; *Figure 3.18 C* and *D, Figure 3.19 C* and *D, Figure 3.20 C* and *D*), total fluid intake (g/kg; *Figure 3.18 E* and *F, Figure 3.19 E* and *F, Figure 3.20 E* and *F*) or water intake (g/kg; *Figure 3.18 G* and *H, Figure 3.19 G* and *H, Figure 3.20 G* and *H*) between genotypes at any of the quinine concentrations tested (0.025 mM, 0.05 mM, 0.1 mM).

Similar to quinine experiments with α4^{Q246M} mice, analysis of quinine intake (*Figure 3.24 A, Table 3.23*) did not reveal a significant effect of concentration, however, analysis of quinine preference (*Figure 3.24 B, Table 3.23*) showed a significant main effect of concentration. Both wild type and homozygous mutant mice reduced their quinine preference by increasing their water intake at higher concentrations (*Table 3.9*). Nonetheless, the observed differences in preference across the concentrations suggest that we have likely identified a range that should be effective in uncovering genotypic differences.

Collectively, these findings, along with the results presented in *Section 3.2.4*, imply that the elimination of neurosteroid sensitivity in α2-containing $GABA_A$ receptors is likely responsible for the decreased ethanol consumption and preference, and that these reductions are not due to the taste of the ethanol solution.

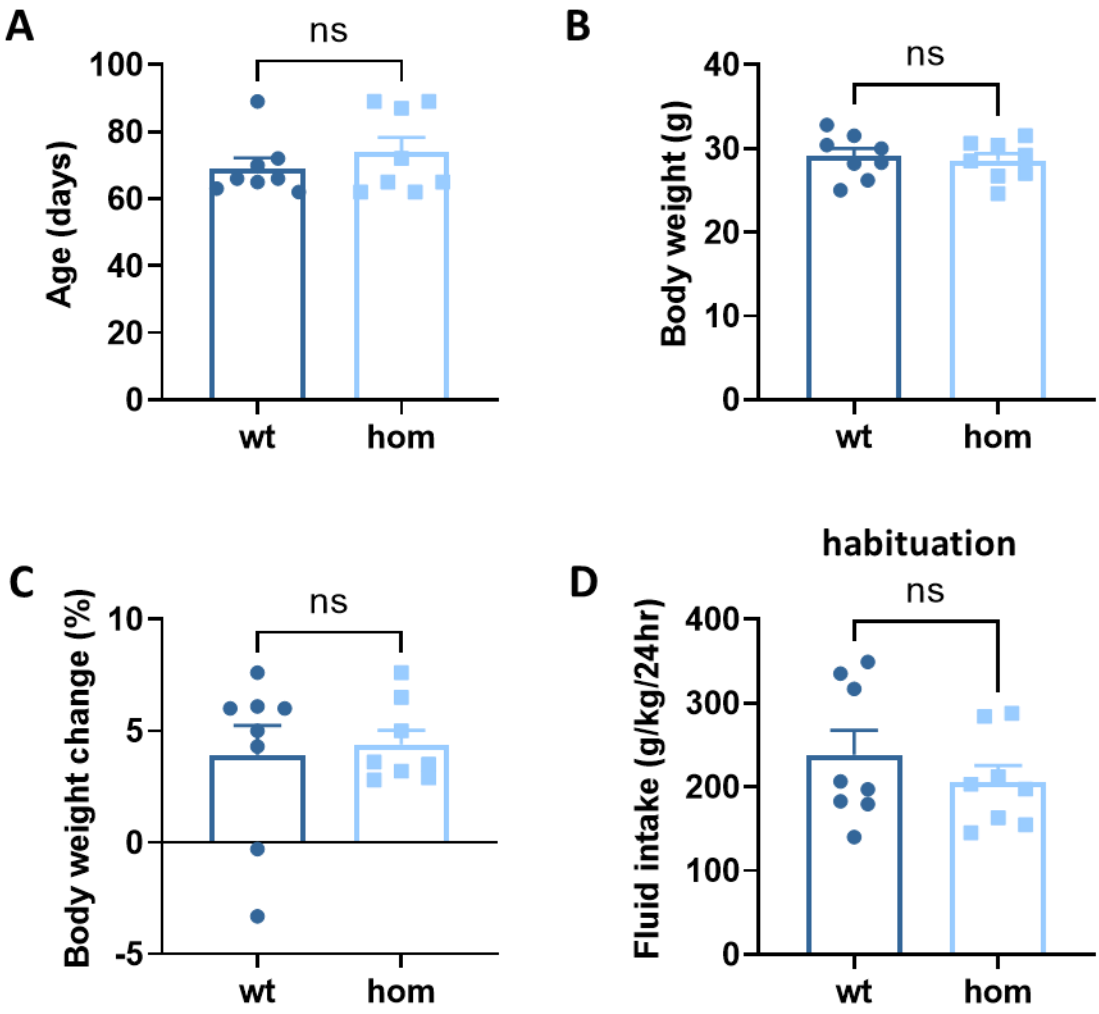


Figure 3.17 Control parameters of the tastant (quinine) two bottle choice paradigm for $\alpha2^{Q241M}$ wild type and homozygous mutant mice

A, The age (in days) of animals at the start of the experiment. ***B,*** The body weight (in grams) of animals at the start of the experiment. ***C,*** Body weight change (%) of animals from the start to the end of the experiment. ***D,*** Fluid intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are Mann-Whitney test for panel *A*, and unpaired student's t test for panels *B, C and D*. Wt: n = 8; hom: n = 8.

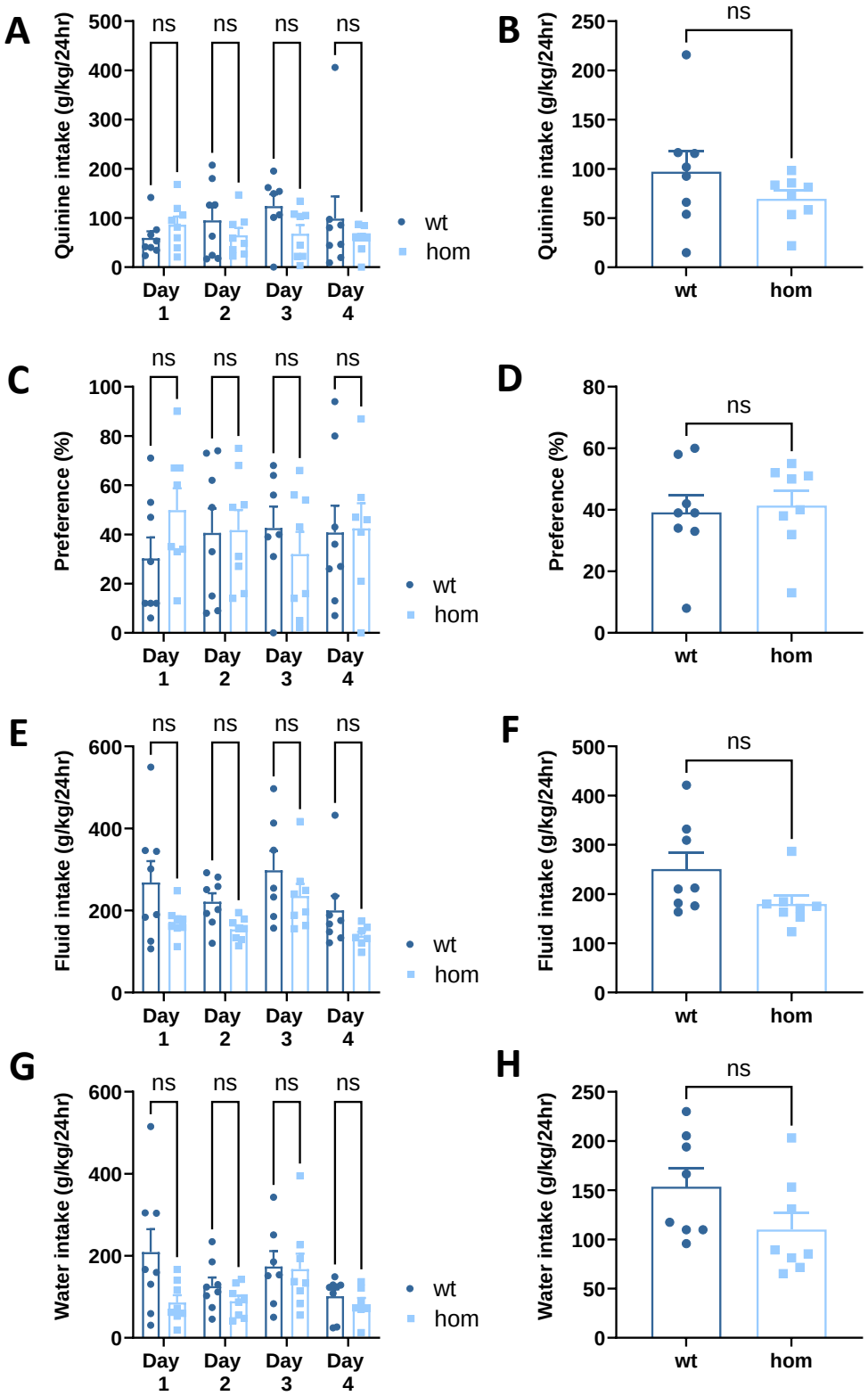


Figure 3.18 Quinine (0.025 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α2 subunit

A, ***C, E*** and ***G***, Mean daily quinine intake (g) per body weight (kg), mean daily quinine preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.025 mM quinine, respectively. ***B, D, F*** *and* ***H,*** Mean daily quinine intake (g/kg), mean quinine preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.025 mM quinine access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, unpaired student's t test with Welch's correction for panel *B*, and without Welch's correction for panels *D, F* and *H.* Wt: n = 8; hom: n = 8.

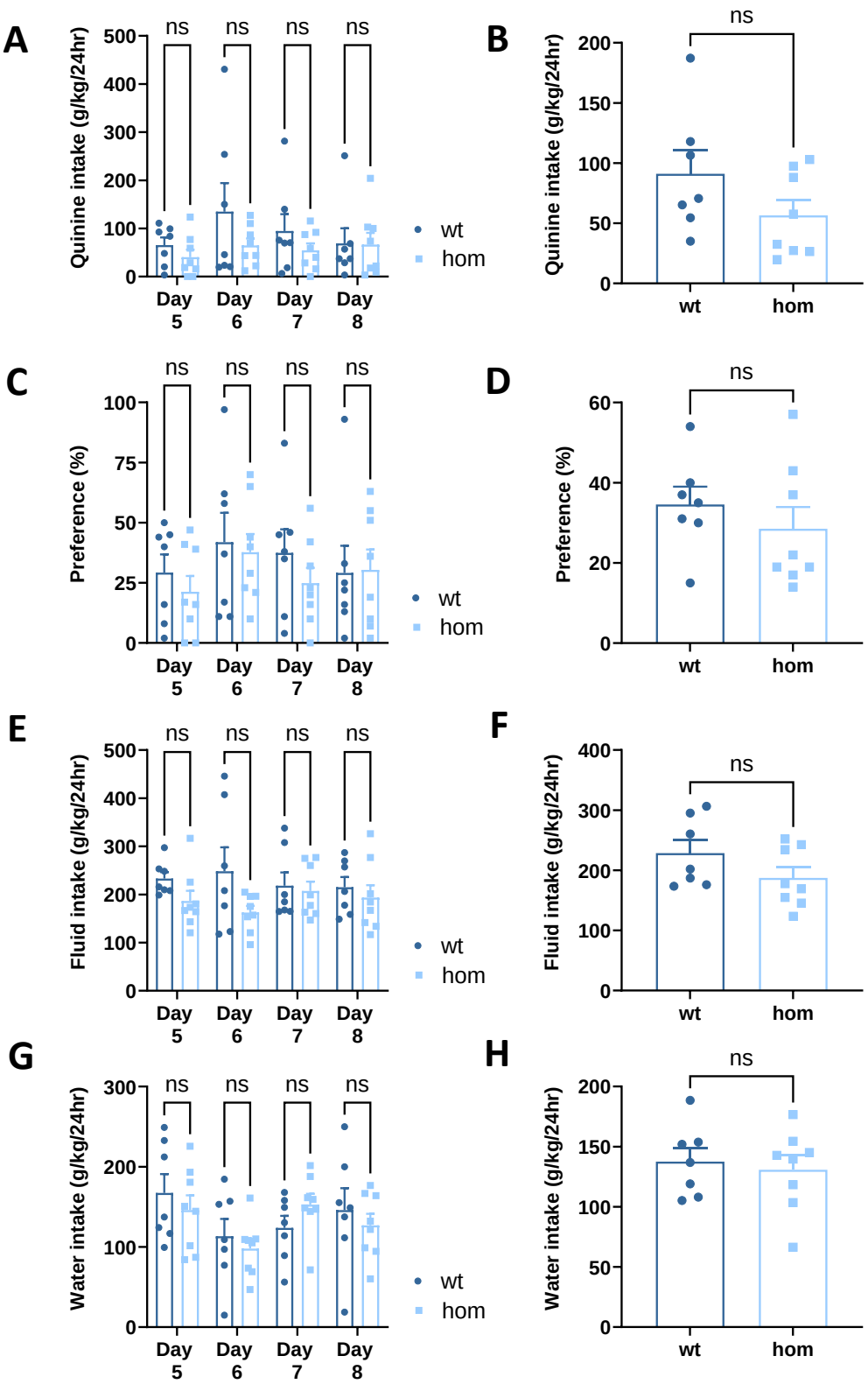


Figure 3.19 Quinine (0.05 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α2 subunit

A, ***C, E*** and ***G***, Mean daily quinine intake (g) per body weight (kg), mean daily quinine preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.05 mM quinine, respectively. ***B, D, F*** *and* ***H,*** Mean daily quinine intake (g/kg), mean quinine preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.05 mM quinine access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, unpaired student's t test for panels *B, D, F* and *H.* Wt: n = 8; hom: n = 8.

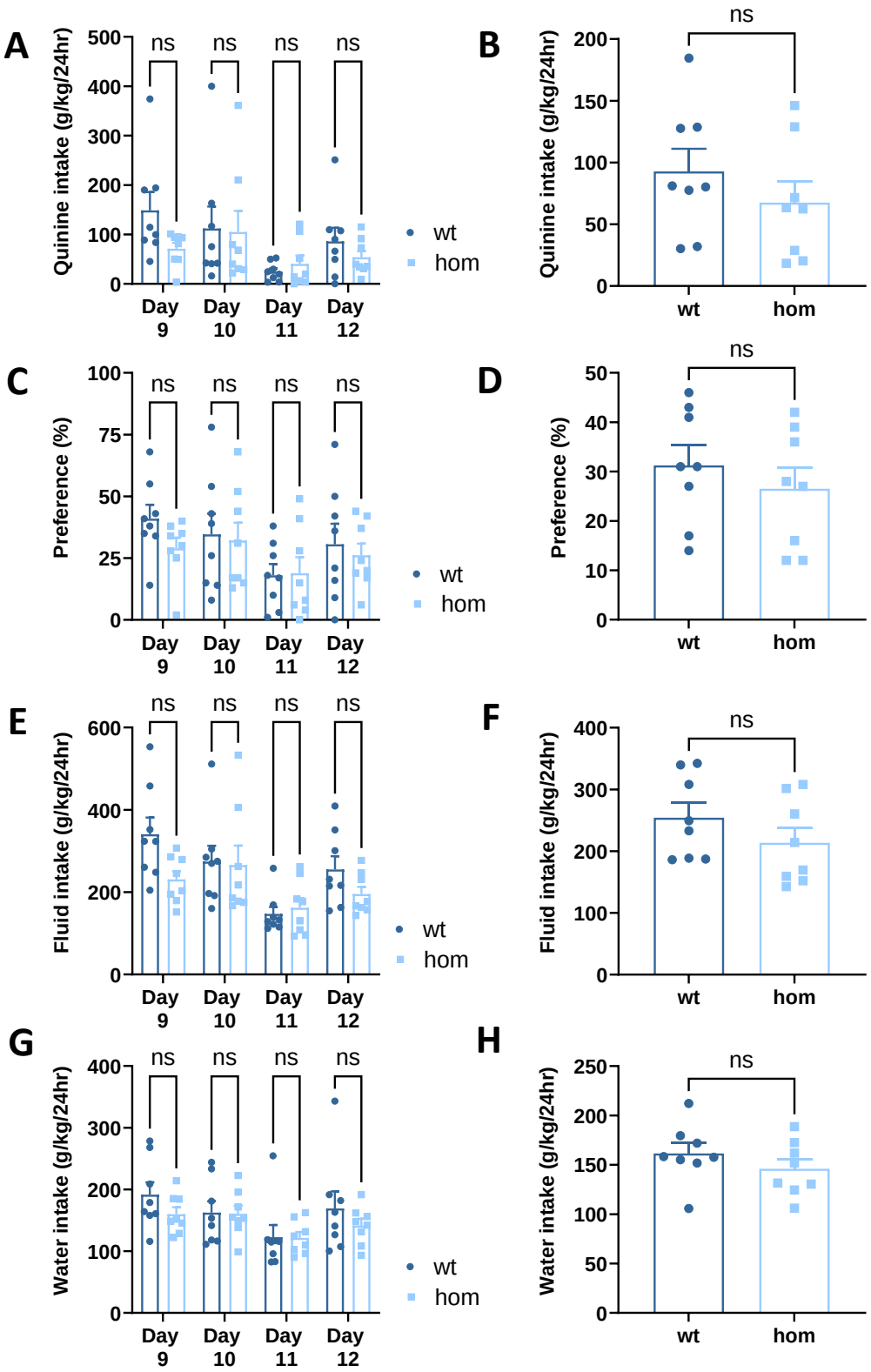


Figure 3.20 Quinine (0.1 mM) intake and preference are not influenced by the absence of the neurosteroid binding site in the α2 subunit

A, ***C, E*** and ***G***, Mean daily quinine intake (g) per body weight (kg), mean daily quinine preference (%), mean daily fluid intake (g/kg) and mean daily water intake (g/kg) when receiving 0.1 mM quinine, respectively. ***B, D, F*** *and* ***H,*** Mean daily quinine intake (g/kg), mean quinine preference (%), mean total fluid intake (g/kg) and mean total water intake (g/kg) over the course of four 0.1 mM quinine access days. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences) for panels *A, C, E* and *G*, unpaired student's t test for panels *B, D, F* and *H.* Wt: n = 8; hom: n = 8.

3.3 Discussion

3.3.1 Role of neurosteroid modulation of α4-GABA$_A$ receptors in alcohol consumption

Several studies have examined the relationship between ethanol, neurosteroids and δ-containing $GABA_A$ receptors by utilising global subunit knock-out models, regional knockdown of subunits or exogenous applications of drugs to provide indirect evidence for the role of neurosteroid modulation of $GABA_A$Rs in mediating the effects of ethanol (Mihalek et al., 2001, Nie et al., 2011, Olsen and Liang, 2017, Rewal et al., 2009). By using the novel $\alpha4^{Q246M}$ mouse line, we are able to explore the direct impact of neurosteroid modulation of extrasynaptic α4-type $GABA_A$ receptors by ethanol *in vivo*.

Using the intermittent access paradigm, we found that mutant animals harbouring the Q246M mutation in α4-$GABA_A$ receptors have reduced ethanol intake compared to their wild type littermates. Wild type mice consistently consume more alcohol, though the difference between genotypes narrows in Week 2 (*Figure 3.25 A*). In contrast, we found no differences in ethanol preference between wild type and mutant mice. This is due to mutant mice decreasing their total volumetric fluid intake upon ethanol exposure. Nevertheless, the reduction in fluid intake on ethanol access days seems to be mainly driven by the reduction in ethanol rather than water intake. Tastant solution (saccharin and quinine) intake and preference were not altered in $\alpha4^{M/M}$ mice compared to wild type controls. We found no differences in total fluid intake between wild type and mutant mice during the habituation period or during saccharin or quinine experiments, suggesting that their decreased fluid consumption is ethanol-specific.

These results suggest that α4-type $GABA_A$ receptors play an important role in alcohol drinking behaviour by mediating indirect effects of ethanol through neurosteroids. Mutant mice had a reduced ethanol intake beginning with the first day of alcohol access, indicating that neurosteroids may need to act on α4-$GABA_A$Rs for ethanol to have its reinforcing effect. Consistent with this idea, Rewal et al. (2009) found that regional knockdown of the α4 subunit in the NAc shell, an area strongly involved in the direct reinforcing effects of drugs, results in reduced alcohol consumption.

Global genetic deletion of α4 leads to increased ethanol consumption in mice (Olsen and Liang, 2017). Furthermore, α4 knockout does not affect behavioural responses to acute

administration of ethanol across various behavioural assays (Chandra et al., 2008). The mechanism behind this is unclear, however, caution should be taken when interpreting global gene deletion studies. The knockout of α4 results in compensatory increases in the expression of other $GABA_AR$ subunits, e.g. upregulation of γ2, and this change alters the pharmacological sensitivity of both synaptic and extrasynaptic $GABA_AR$ currents (Liang et al., 2008).

Both acute and chronic exposure of ethanol have been shown to alter $GABA_A$ receptor subunit expression patterns in the brain (Matthews et al., 1998, Grobin et al., 2000, Werner et al., 2016). α4 subunit expression increases in the hippocampus, cerebral cortex and thalamus after 6, but not 2, weeks of ethanol exposure (Matthews et al., 1998, Grobin et al., 2000, Werner et al., 2016). Moreover, a single high dose of ethanol transiently alters the expression of the α4 subunit in the thalamus; with an initial decrease at 2 hours post-exposure followed by an increase at 4 hours (Werner et al., 2016). Finasteride prevented this delayed increase in expression, but had no effect on basal subunit expression, suggesting that endogenous neurosteroids modulate $GABA_A$ receptor subunit expression following ethanol exposure (Werner et al., 2016). Recent findings suggest that neurosteroids might cooperate with PKC activity to control the trafficking and functioning of α4-$GABA_ARs$ (Adams et al., 2015, Abramian et al., 2014). THDOC increases α4, but not α1 or α5, subunit expression in the hippocampus (Abramian et al., 2014) by potentiating PKC-dependent phosphorylation of serine residue (S443) within the α4 subunit. Administration of pregnanolone also leads to an upregulation of both α4 and δ subunits in the CA1 region of the hippocampus (Shen et al., 2005). Further support for the involvement of progesterone metabolites in the alterations of $GABA_AR$ subunit expression is provided by observations that the fluctuations in $GABA_A$ receptors seen throughout the ovarian cycle of female mice can be replicated in male mice and ovariectomised females following progesterone treatment (Maguire and Mody, 2007). Therefore, ablating the neurosteroid binding site from α4-$GABA_A$ receptors potentially prevents this upregulation caused by ethanol, and this may contribute to the reduction in ethanol intake in homozygous mutant mice. While acute ethanol exposure results in an elevation of neurosteroid concentrations in various brain regions, chronic exposure leads to a reduction in brain neurosteroid levels (Janis et al., 1998, Snelling et al., 2014). Speculatively, a reduction in neurosteroid concentrations may in part explain why the difference in ethanol intake disappears

between wild type and mutant animals after the first week. It is particularly noteworthy as the lack of difference stems from a decrease in drinking behaviour observed in wild type mice rather than an alteration in alcohol consumption in mutant animals.

The precise mechanism through which the elimination of neurosteroid sensitivity from α4-$GABA_A$ receptors results in decreased alcohol consumption remains unclear. However, various lines of evidence indicate that neurosteroid modulation of these receptors plays a significant role in alcohol drinking behaviours.

3.3.2 Role of neurosteroid modulation of α2-$GABA_A$ receptors in alcohol consumption

We chose to study the $\alpha2^{Q241M}$ mouse line because of the link between single nucleotide polymorphisms in *GABRA2* and alcohol dependence (Bierut Laura et al., 2010, Covault et al., 2004, Edenberg et al., 2004, Ittiwut et al., 2012, Li et al., 2014). Furthermore, α2-$GABA_A$Rs are also abundantly expressed in the hippocampus, NAc and VTA, where they may significantly contribute to synaptic inhibition and also be targets for indirect modulation by ethanol (Pirker et al., 2000).

Animals rendered insensitive to neurosteroids at α2-containing $GABA_A$ receptors showed a reduction in both ethanol intake and preference in the first week of the experiment. The difference between weeks one and two is driven by the observation that whilst $\alpha2^{M/M}$ mice consumed similar amounts of alcohol in both the first and second week, wild type animals significantly decreased their intake and preference from week 1 to week 2. Newman et al. (2016) found that the difference in ethanol intake between wild type and mutant animals remained consistent for six weeks. Furthermore, they also found that both $\alpha2^{Q/Q}$ and $\alpha2^{M/M}$ animals consumed progressively more ethanol for the first three weeks of the experiment. Our findings show the opposite - mice consumed the most ethanol on the first day and their intake progressively decreased for the rest of the experiment (*Figure 3.25 C* and *D*). Our data with the $\alpha4^{Q246M}$ mouse line exhibited the same pattern – highest consumption on day one followed by a progressive reduction throughout the experiment (*Figure 3.25 A* and *B*). The reason for the discrepancy between the two studies is unclear. We replicated nearly every aspect of the protocol laid out in Newman et al. (2016). We slightly adapted the schedule of ethanol access from Monday-Wednesday-Friday to every other day, and whereas Newman et al. (2016)

pooled all wild type data together, we compared homozygous mutants only to their wild type littermates. There is evidence to suggest that lengthening the period between ethanol access sessions leads to higher intake (Holloway et al., 1984). However, the effect of increasing intermittency by one day, once a week, is unlikely to be significant. Therefore, we think that neither of the adaptations used are likely to be the source of the difference between the two studies.

Furthermore, the absolute consumption of $\alpha2^{M/M}$ mice was similar in our study to that of Newman et al. (2016). However, in their experiment wild type mice had a considerably higher ethanol intake per day than in ours (~ 20 g/kg vs 15 g/kg). Both Newman et al. (2016) and Hwa et al. (2011) report similar wild type alcohol intake values. However, a study by Crabbe et al. (2012) was also unable to replicate their findings; they did not see an escalation in consumption from week 1 to week 3. Additionally, their documented ethanol intake values for wild type mice align closely with our results. Nevertheless, rendering α2-$GABA_A$Rs insensitive to neurosteroids still affects ethanol consumption, albeit acutely rather than chronically, indicating a role for α2-type $GABA_A$ receptors in alcohol drinking behaviour.

Global deletion of the α2 subunit reduces ethanol consumption in mice (Olsen and Liang, 2017), contrary to findings from Dixon et al. (2012), where no differences were observed in acute ethanol self-administration between wild type and $\alpha2^{-/-}$ mice. This discrepancy may stem from methodological variations; Olsen and Liang examined chronic effects over weeks (reductions were apparent after 3 weeks), while Dixon et al. focused on acute ethanol impact (< 1 hour). Another study reported a decrease in ethanol intake in female α2-knockout, but not male, mice (Boehm et al., 2004). Furthermore, they showed that female null mutant mice had lower preference for quinine compared to wild types, whereas their taste sensitivity to saccharin was unaffected (Boehm et al., 2004), suggesting that the lower alcohol consumption may in part be due to the aversive taste of the ethanol solution.

The behaviour of knock-in mice ($\alpha2^{HA/HA}$) harbouring two substitutions in the α2 subunit, serine 270 to histidine (H) and leucine 277 to alanine (A), was tested in a range of alcohol-related assays (Blednov et al., 2011). These substitutions render receptors insensitive to potentiation by ethanol, while retaining normal GABA sensitivity. Male HA mutant mice exhibited a slight preference for ethanol over wild type counterparts during a one-month

period of intermittent drinking. No significant differences were observed in the quantity of ethanol consumed among male mice. In contrast, female $\alpha2^{HA/HA}$ mice exhibited higher ethanol consumption and preference compared to their wild type littermates. No differences were observed in saccharin preference between mutant and wild type mice of either sex. However, only $\alpha2^{HA/HA}$ male mice exhibited a stronger aversion to the bitter quinine solution. Moreover, knock-in female mice containing these mutant receptors are resistant to ethanol-induced conditioned place aversion, which may explain their increased ethanol consumption. α2-knockout male mice, but not other α subunit knockouts (including α1, α3, α4, α5), also show reduced conditioned taste aversion to ethanol (Blednov et al., 2013). These results together indicate this aversive property of ethanol is dependent on ethanol action at α2-containing $GABA_A$ receptors.

Our data demonstrates that tastant solution intake and preference is unchanged between $\alpha2^{Q/Q}$ and $\alpha2^{M/M}$ animals. Both wild type and mutant mice displayed the same consumption patterns – higher intake at higher saccharin concentrations and lower intake at higher quinine concentrations. These results are in agreement with Newman et al. (2016), where they also report no alterations in sensitivity to tastant solutions between genotypes. Interestingly, they also documented no changes in quinine preference between wild type and $\alpha2^{HA/HA}$ mice, a finding divergent from that reported by Blednov et al. (2011). Collectively, these findings suggest that the aversive property of alcohol is not mediated via neurosteroid modulation of α2-$GABA_A$ receptors and that the decreased consumption of ethanol of $\alpha2^{M/M}$ mice is presumably not related to an increased aversion to the bitter taste.

Several lines of evidence suggest that α2-$GABA_A$ receptors are involved in the neural circuits that mediate ethanol's positive reinforcing effects (Lindemeyer et al., 2017, Liu et al., 2011, Koob, 2004, Roh et al., 2011). In this study, $\alpha2^{Q241M}$ mutants exhibited reduced alcohol intake starting from their first day of access (*Figure 3.25 C*), implying that neurosteroids may need to target α2-containing $GABA_A$ receptors for alcohol to produce its immediate rewarding effects. Future studies should compare alcohol-reinforced responses between these mutants and wild type controls to directly address whether ethanol's rewarding value is mediated via neurosteroid modulation of α2-$GABA_A$ receptors.

Similar to effects on α4 subunit levels, acute and chronic ethanol exposure also alters α2 subunit expression. Lindemeyer et al. (2017) showed a downregulation of α1 and δ subunits and an upregulation of α4, α2, γ1, and γ2 subunits, resulting in decreased α4βδ- and α1βγ2-containing $GABA_A$ receptors and increased postsynaptic α4βγ2- and α2β1γ1-$GABA_A$Rs in hippocampal neurons. These changes were also evident in other brain regions, including the basolateral amygdala (Diaz et al., 2011, Lindemeyer et al., 2014) and nucleus accumbens (Liang et al., 2014). The increase in α2 subtypes correlates with ethanol-enhanced synaptic currents, which were also present in α4-knockout mice (Liang et al., 2008, Suryanarayanan et al., 2011). The α4 subunit cannot be responsible for these enhanced currents in α4-knockout mice. α2 subunits colocalise with gephyrin and presynaptic glutamic acid decarboxylase in hippocampal neurons, and these are upregulated in α4 knockout mice. Taken together, these studies demonstrate the plasticity of α2-containing $GABA_A$ receptors following ethanol exposure.

Phosphorylation can alter the neurosteroid sensitivity of $GABA_A$ receptors at synapses (Harney et al., 2003, Hodge et al., 2002, Brussaard et al., 2000, Fáncsik et al., 2000). Magnocellular oxytocin neurons in the supraoptic nucleus of the hypothalamus exclusively express the α1, α2, β2/3, and γ2 subunits of the $GABA_A$ receptor. The expression of these subunit mRNAs changes at different reproductive stages in female rats (Fenelon et al., 1995). Specifically, α1 subunit expression is elevated during pregnancy and decreases at parturition (Fenelon and Herbison, 1996). This suggests that magnocellular oxytocin neurons adjust their $GABA_A$ receptor subunit expression in response to physiological changes. Brussaard et al. (1997) demonstrated significant $GABA_A$ receptor plasticity in these neurons, showing that ion channel kinetics and neurosteroid sensitivity are influenced by the relative expression of α1 and α2 subunits (decrease in α1, increase in α2). During parturition, $GABA_A$ receptors become insensitive to modulation by allopregnanolone due to a change in the balance between endogenous phosphatase and PKC activity (Koksma et al., 2003). After parturition, a sustained endogenous oxytocin tone within the supraoptic nucleus decreases $GABA_A$ receptor neurosteroid sensitivity through PKC activation. Following parturition, the insensitivity of synaptic $GABA_A$ receptors to allopregnanolone can also be reversed by stimulating phosphatase activity or inhibiting PKC (Koksma et al., 2003).

In contrast, phosphorylation in the hippocampus appears to enhance the interaction between neurosteroids and $GABA_A$ receptors (Harney et al., 2003). In dentate gyrus granule cells, PKC activation increases the prolongation of IPSCs by neurosteroids, while in CA1 neurons, PKC inhibition reduces the neurosteroid effect (Harney et al., 2003). Thus, the differing sensitivity of DGGCs and CA1 neurons to neurosteroids is at least partially dependent on phosphorylation.

Ethanol has consistently been shown to enhance PKC activity (Messing et al., 1991), therefore it is possible that the plasticity that occurs between week 1 and week 2 in our experiments is in part due to ethanol's action on PKC. Speculatively, by elevating PKC levels, ethanol could alter the phosphorylation state of synaptic receptors, which in turn could modify the receptor's sensitivity to neurosteroids. Given that α2 subunit expression is upregulated, ethanol could have a more pronounced effect in wild types leading to higher alcohol consumption.

The exact mechanism by which removing neurosteroid sensitivity from α2-$GABA_A$ receptors leads to reduced alcohol consumption is not fully understood. Nevertheless, multiple pieces of evidence suggest that neurosteroid modulation of these receptors is key in alcohol drinking behaviours.

3.3.3 Limitations

A potential limitation of the two bottle choice experiments is the impact of social isolation, as the animals were singly housed for three weeks. Isolation of this duration may lead to various effects, such as increased stress, anxiety or depression (Ieraci et al., 2016), though no such signs were evident, potentially affecting neurosteroid levels. Indeed, previous studies have reported reductions in neurosteroid levels following 30 days of isolation (Serra et al., 2006). However, research also indicates that mice aged 3-12 weeks (the age of our experimental subjects) exhibit minimal behavioural effects from social isolation, showing no significant changes in anxiety- or depression-like behaviours (Magalhães et al., 2024). Therefore, we concluded that social isolation was a minor confounding factor, particularly given that both genotypes—wild type and mutant animals—underwent the same housing conditions. A potential improvement for future

studies could involve using HM2 cages, which allow group housing while enabling individual monitoring of fluid intake in a minimally disruptive manner.

Another limitation of the intermittent ethanol access paradigm is the testing of only a single ethanol concentration (20 %). Future experiments should incorporate a range of ethanol concentrations (1-10 %), as previous studies indicate that ethanol intake and preference in C57BL/6J mice increase with concentration, reaching a peak at 10 % (Bachmanov et al., 1996). Testing a broader range of concentrations may thus uncover differences between our wild type and mutant animals that were not observable at the initial concentration tested.

3.4 Conclusions

1. Neurosteroid modulation of α4-containing $GABA_A$ receptors plays a role in alcohol consumption, suggested by the reduced intake of homozygous mutant $\alpha4^{Q246M}$ mice

2. Removing neurosteroid sensitivity from α4-$GABA_A$ receptors does not affect saccharin or quinine consumption

3. Rendering α2-$GABA_A$ receptors insensitive to modulation by neurosteroids impacts upon ethanol intake of mice, leading to a reduction in intake, indicating that neurosteroid modulation of these receptors participates in alcohol drinking behaviours

4. Ablating the neurosteroid binding site from α2-$GABA_A$ receptors does not influence saccharine or quinine intake

3.5 Appendix

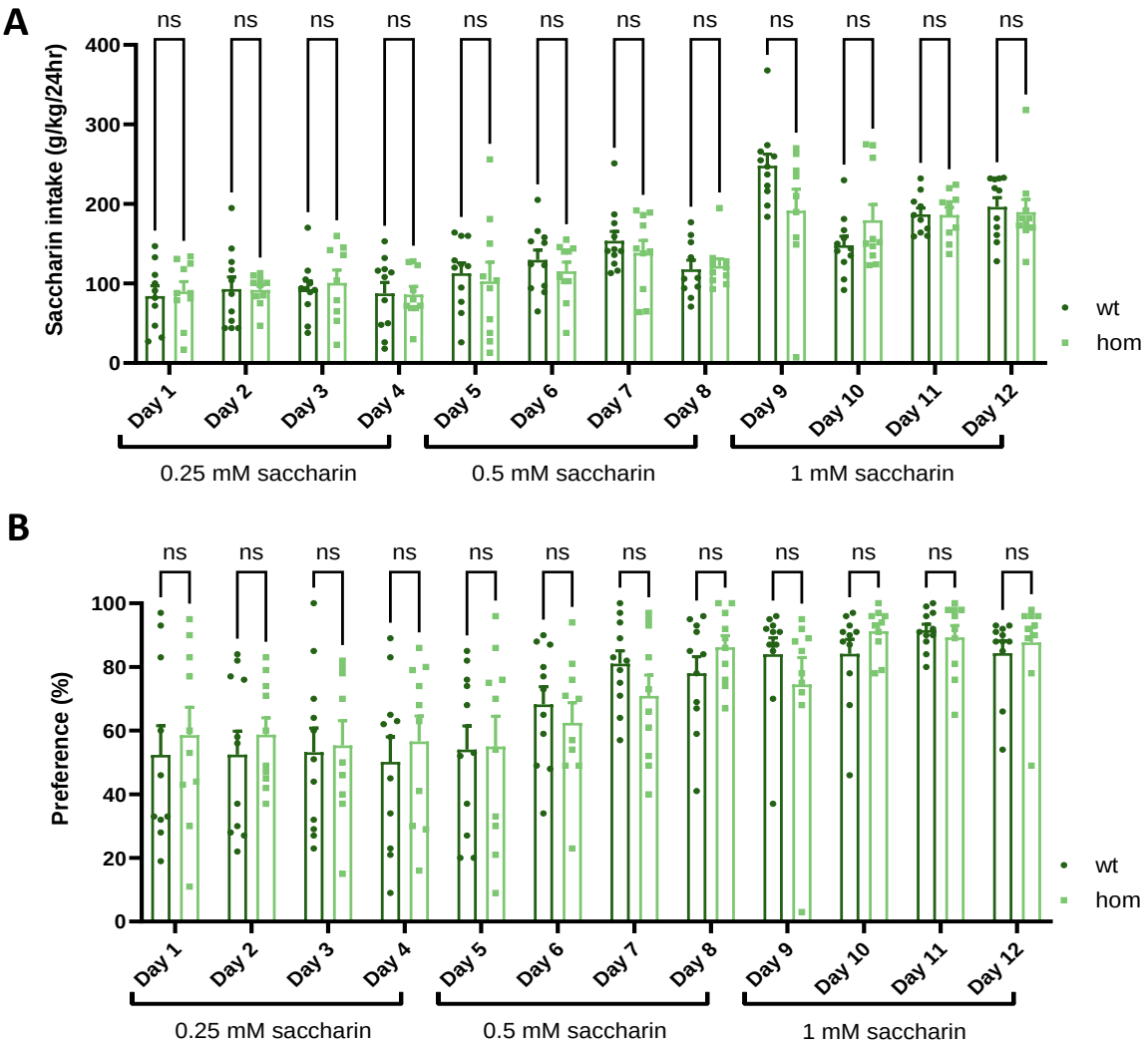


Figure 3.21 Saccharin intake and preference do not differ between wild type and homozygous mutant α4^{Q246M} mice

A, Mean daily saccharin intake (g) per body weight (kg) and ***B,*** mean daily saccharin preference (%) over the course of the saccharin ascending concentration paradigm. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences). Wt: n = 11; hom: n = 10.

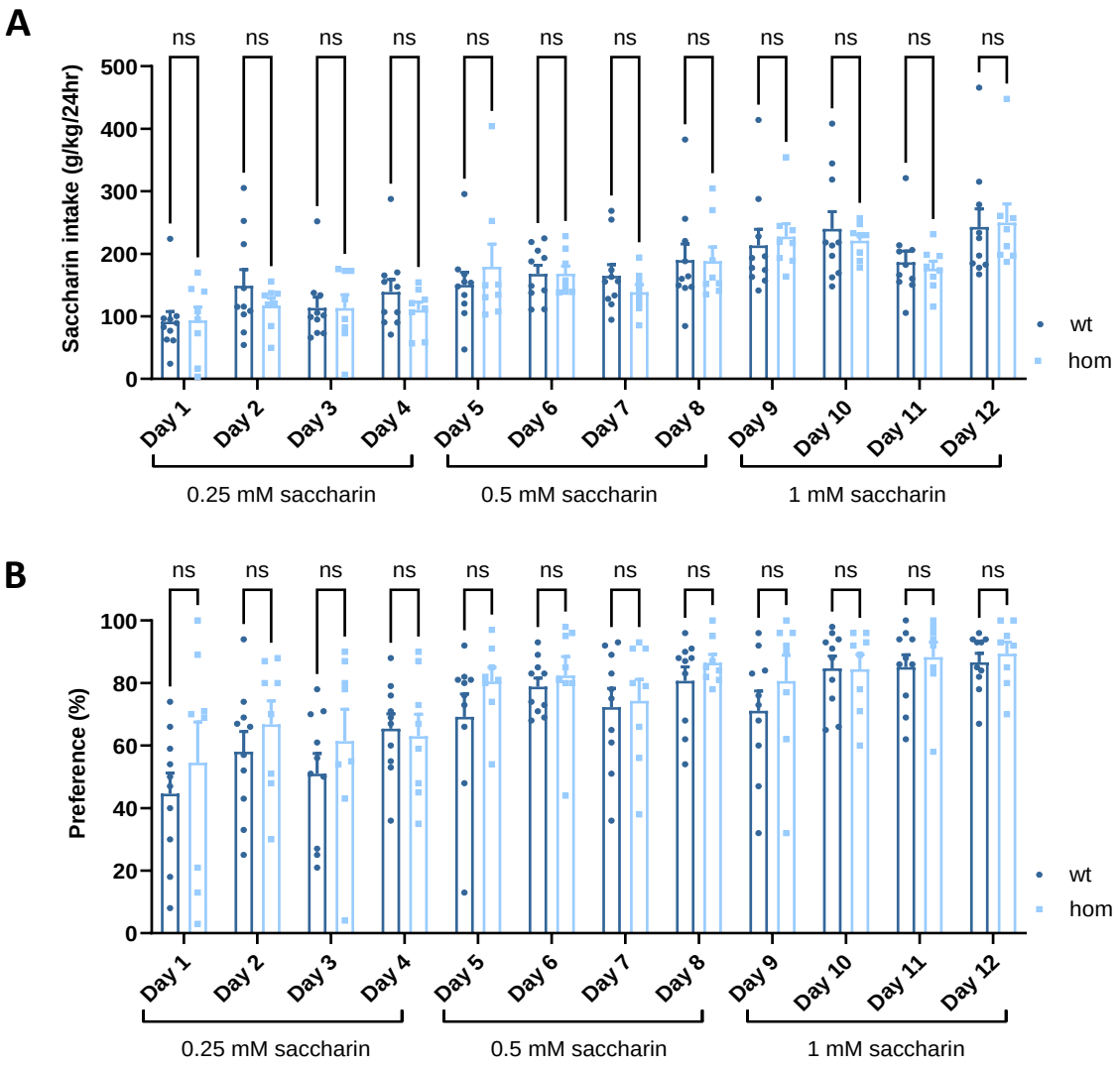


Figure 3.22 Saccharin intake and preference do not differ between wild type and homozygous mutant α2^{Q241M} mice

A, Mean daily saccharin intake (g) per body weight (kg) and ***B,*** mean daily saccharin preference (%) over the course of the saccharin ascending concentration paradigm. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences). Wt: n = 10; hom: n = 8.

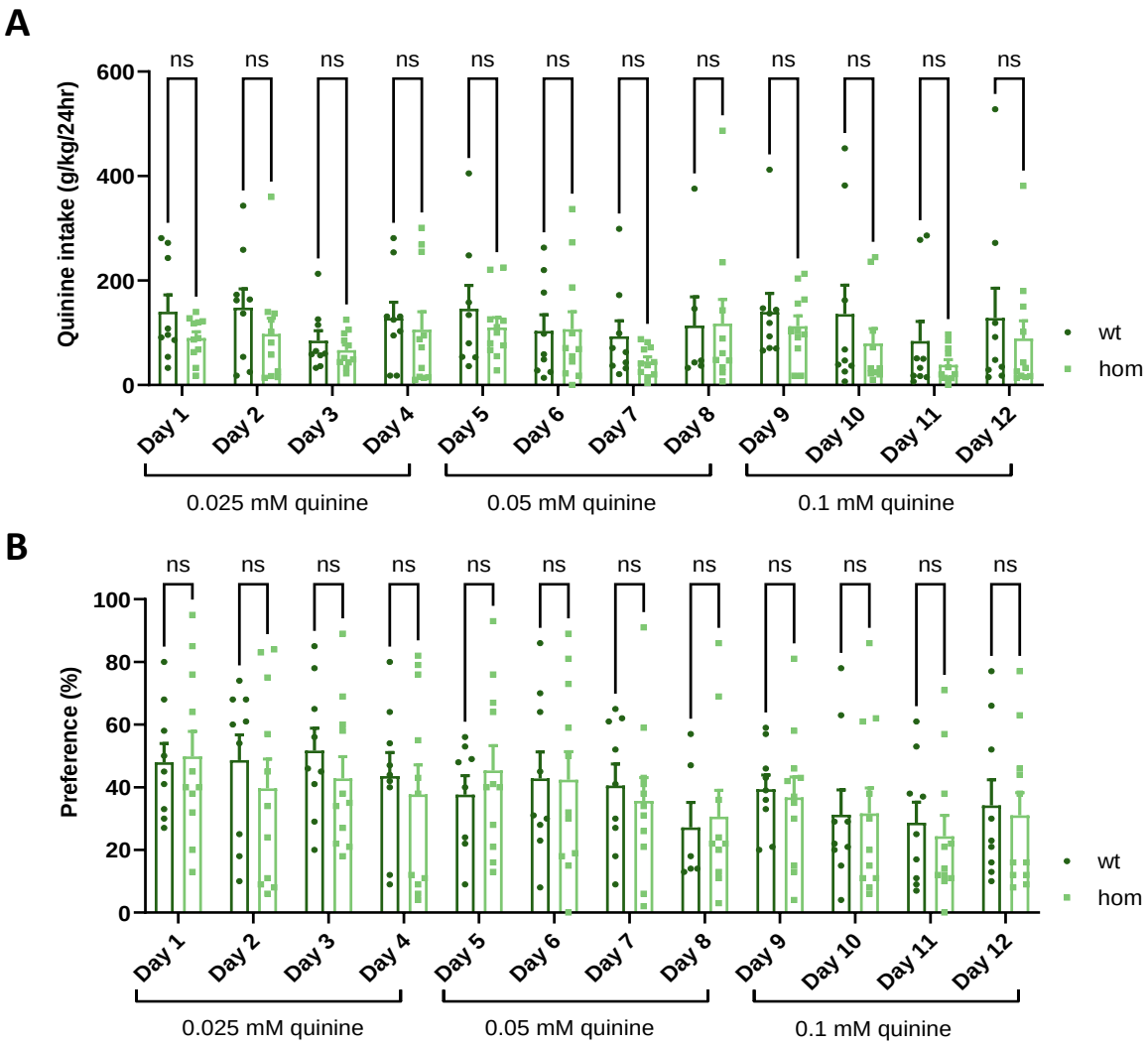


Figure 3.23 Quinine intake and preference do not vary between wild type and homozygous mutant α4^{Q246M} mice

A, Mean daily quinine intake (g) per body weight (kg) and ***B,*** mean daily quinine preference (%) over the course of the quinine ascending concentration paradigm. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences). Wt: n = 9; hom: n = 11.

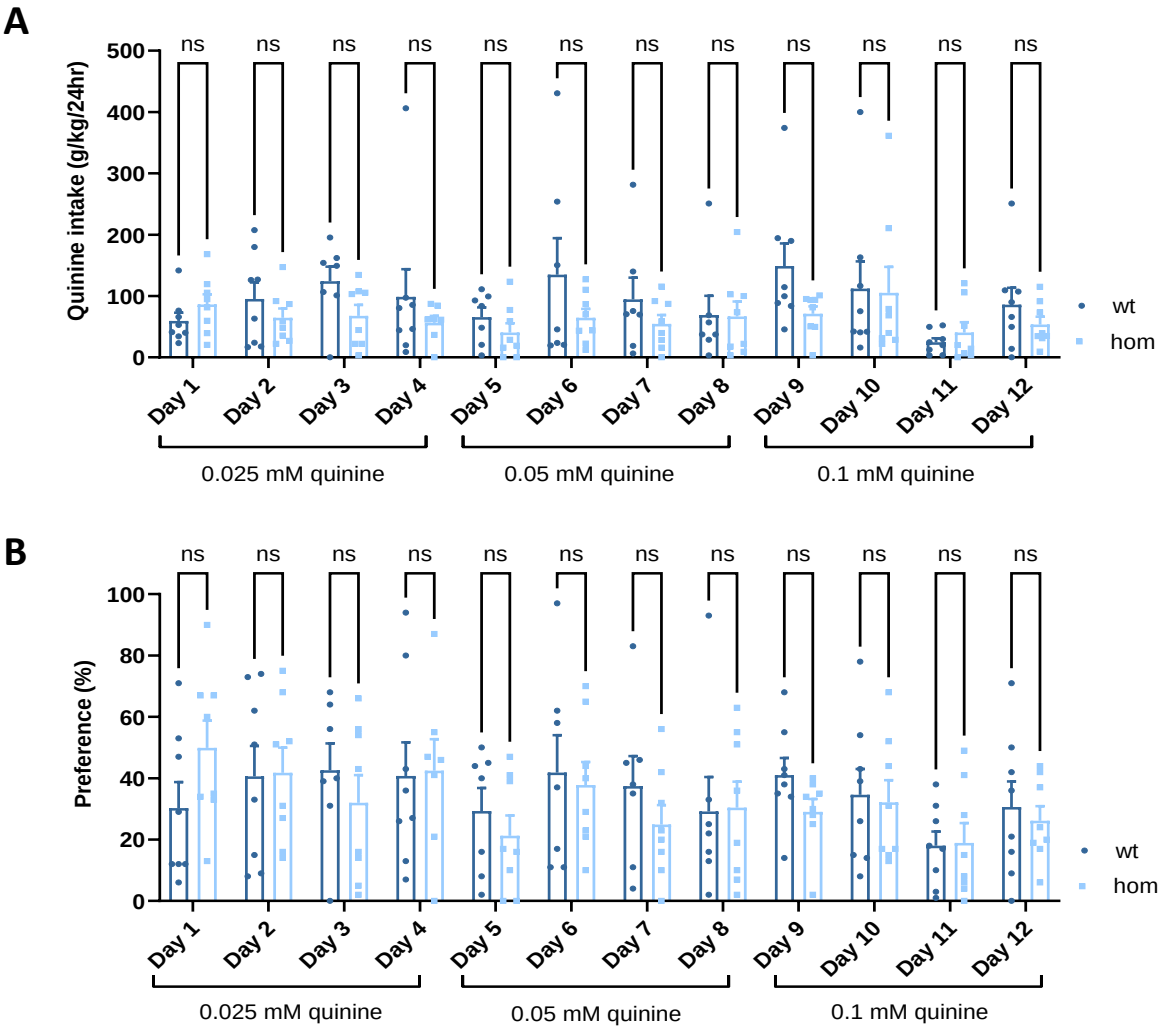


Figure 3.24 Quinine intake and preference do not vary between wild type and homozygous mutant α2^{Q241M} mice

A, Mean daily quinine intake (g) per body weight (kg) and ***B,*** mean daily quinine preference (%) over the course of the quinine ascending concentration paradigm. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences). Wt: n = 8; hom: n = 8.

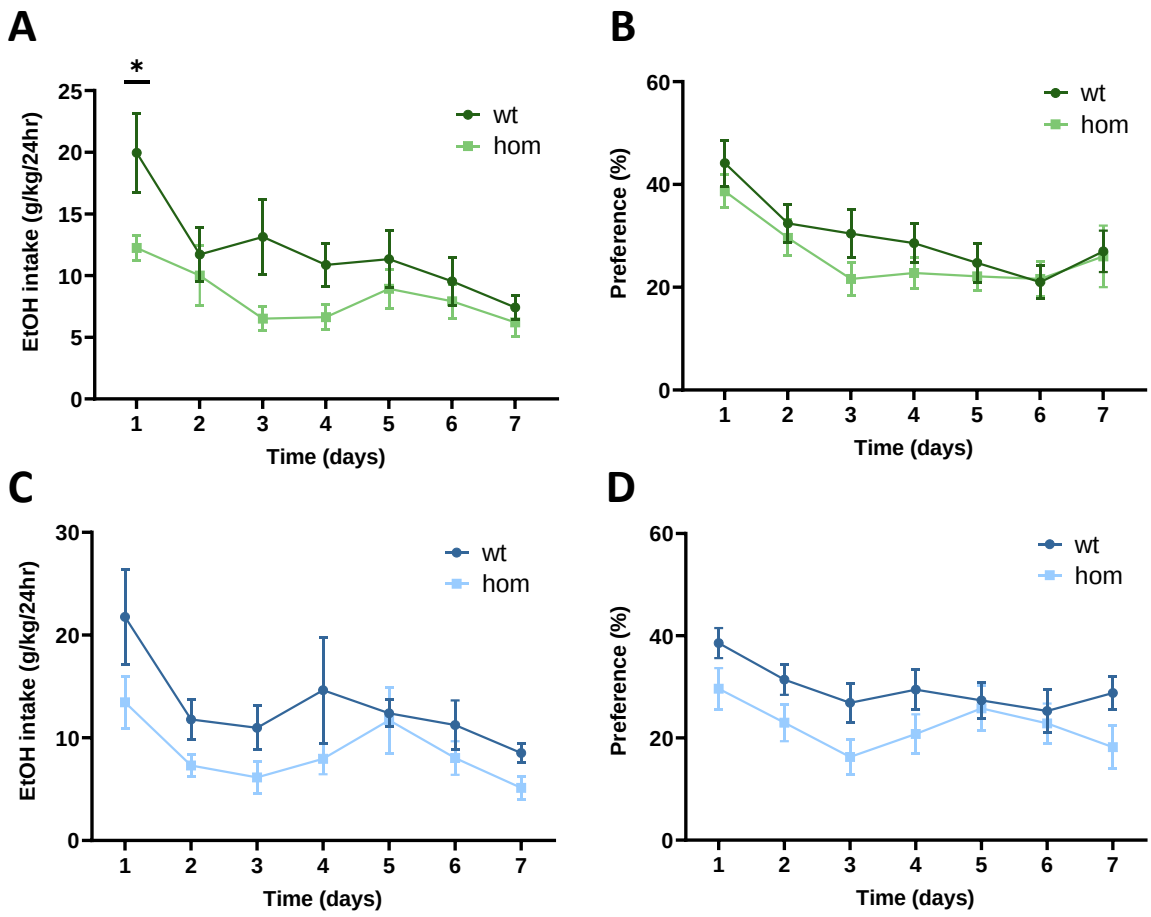


Figure 3.25 Daily ethanol intake and preference of wild type and mutant α4^{Q246M} and α2^{Q241M} mice

A, Mean daily ethanol intake (g/kg) and ***B***, mean daily preference (%) values for α4^{Q246M} wild type and mutant animals over 7 days during the intermittent access paradigm. ***C***, Mean daily ethanol intake (g/kg) and ***D***, mean daily preference (%) values for α2$^{Q/Q}$ and α2$^{M/M}$ mice over the course of the ethanol intermittent access protocol. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences). Statistical significance indicated as * $p < 0.05$ compared to wild type. α4^{Q246M}: wt n = 16; hom n = 18. α2^{Q241M}: wt n = 18, hom n = 16. Note: Time (days) refer to the seven ethanol access days within the two-week intermittent access paradigm, during which mice were provided with ethanol bottles every other day.

Table 3.1 $\alpha4^{Q246M}$ - control parameters for two bottle choice experiments

	Age (days)			Fluid intake – habituation (g/kg/24hr)		
	Mean ± SEM		P value	Mean ± SEM		P value
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
$\alpha4^{Q246M}$ EtOH	78 ± 3	79 ± 3	0.833	215.6 ± 16.4	171.4 ± 13.8	0.200
$\alpha4^{Q246M}$ saccharin	81 ± 2	87 ± 3	0.079	328.0 ± 24.3	303.8 ± 27.9	0.519
$\alpha4^{Q246M}$ quinine	85 ± 2	86 ± 2	0.610	288.8 ± 27.4	242.0 ± 21.0	0.185
	Body weight at start (g)			Body weight change (%)		
	Mean ± SEM		P value	Mean ± SEM		P value
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
$\alpha4^{Q246M}$ EtOH	25.7 ± 0.6	26.7 ± 0.7	0.283	4.6 ± 0.8	4.8 ± 0.7	0.846
$\alpha4^{Q246M}$ saccharin	27.9 ± 0.4	29.7 ± 0.5	0.007	1.5 ± 0.9	1.2 ± 0.7	0.774
$\alpha4^{Q246M}$ quinine	27.0 ± 0.3	28.4 ± 0.6	0.056	1.2 ± 0.6	3.0 ± 1.0	0.155

Table 3.2 $\alpha2^{Q241M}$ - control parameters for two bottle choice experiments

	Age (days)			Fluid intake – habituation (g/kg/24hr)		
	Mean ± SEM		P value	Mean ± SEM		P value
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
$\alpha2^{Q241M}$ EtOH	71 ± 3	66 ± 1	0.153	240.9 ± 16.2	201.7 ± 12.4	0.066
$\alpha2^{Q241M}$ saccharin	72 ± 4	81 ± 5	0.207	203.8 ± 18.9	212.0 ± 33.0	0.824
$\alpha2^{Q241M}$ quinine	69 ± 3	74 ± 4	0.814	238.4 ± 28.9	205.9 ± 19.4	0.367
	Body weight at start (g)			Body weight change (%)		
	Mean ± SEM		P value	Mean ± SEM		P value
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
$\alpha2^{Q241M}$ EtOH	24.6 ± 0.4	25.0 ± 0.5	0.577	2.6 ± 0.4	3.6 ± 0.4	0.081
$\alpha2^{Q241M}$ saccharin	27.5 ± 0.6	27.6 ± 0.7	0.939	4.1 ± 0.6	4.4 ± 0.9	0.775
$\alpha2^{Q241M}$ quinine	29.1 ± 0.9	28.6 ± 0.8	0.701	3.9 ± 1.3	4.4 ± 0.6	0.758

Table 3.3 Ethanol intermittent access two bottle choice experiment results

$\alpha4^{Q246M}$	Week 1			Week 2		
	Mean ± SEM		P value	Mean ± SEM		P value
	Wt (n = 16)	Hom (n = 18)	Wt vs Hom	Wt (n = 16)	Hom (n = 18)	Wt vs Hom
EtOH intake (g/kg/24hr)	14.5 ± 1.7	8.4 ± 0.6	0.002	10.8 ± 1.5	7.9 ± 0.9	0.215
EtOH preference (%)	35 ± 3	29 ± 2	0.204	25 ± 3	23 ± 2	0.812
Total fluid intake (g/kg/24hr)	222.6 ± 15.7	164.9 ± 7.9	0.002	212.8 ± 10.1	165.1 ± 7.8	0.006
Fluid intake on EtOH days (g/kg/24hr)	195.1 ± 13.4	142.9 ± 5.2	0.003	208.1 ± 12.7	172.9 ± 8.4	0.040
Water intake on Water days (g/kg/24hr)	249.6 ± 20.2	193.9 ± 16.5	0.032	218.7 ± 12.2	157.1 ± 9.0	0.028
Water intake on EtOH days (g/kg/24hr)	115.1 ± 7.1	100.9 ± 5.8	0.381	154.3 ± 8.4	133.4 ± 6.5	0.077
$\alpha2^{Q241M}$	Week 1			Week 2		
	Mean ± SEM		P value	Mean ± SEM		P value
	Wt (n = 18)	Hom (n = 16)	Wt vs Hom	Wt (n = 18)	Hom (n = 16)	Wt vs Hom
EtOH intake (g/kg/24hr)	15.7 ± 1.9	10.6 ± 1.0	0.030	10.9 ± 1.0	11.6 ± 1.5	0.975
EtOH preference (%)	33 ± 2	26 ± 2	0.004	25 ± 2	27 ± 2	0.967
Total fluid intake (g/kg/24hr)	234.6 ± 17.0	202.1 ± 9.1	0.163	216.8 ± 12.7	208.1 ± 12.1	0.947
Fluid intake on EtOH days (g/kg/24hr)	230.0 ± 24.8	197.1 ± 8.2	0.355	214.9 ± 12.9	207.7 ± 13.7	0.726
Water intake on Water days (g/kg/24hr)	257.9 ± 18.2	207.1 ± 13.8	0.080	205.4 ± 16.5	208.4 ± 19.5	0.994
Water intake on EtOH days (g/kg/24hr)	151.6 ± 17.3	143.8 ± 6.4	0.878	155.4 ± 11.3	149.9 ± 8.8	0.937

Table 3.4 $\alpha4^{Q246M}$ - Ethanol intermittent access two bottle choice experiment statistics

Intermittent EtOH protocol				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.2 A	Time	0.018	*	F (1, 30) = 6.259
	Genotype	0.0076	**	F (1, 31) = 8.153
	Time x Genotype	0.0746	ns	F (1, 30) = 3.412
Figure 3.2 B	Time	<0.0001	****	F (1, 30) = 22.71
	Genotype	0.2065	ns	F (1, 31) = 1.665
	Time x Genotype	0.3066	ns	F (1, 30) = 1.082
Figure 3.2 C	Time	0.2311	ns	F (1, 30) = 1.494
	Genotype	0.0014	**	F (1, 31) = 12.39
	Time x Genotype	0.6309	ns	F (1, 30) = 0.2357
Figure 3.2 D	Time	0.007	**	F (1, 30) = 8.381
	Genotype	0.0032	**	F (1, 31) = 10.24
	Time x Genotype	0.3273	ns	F (1, 30) = 0.9916
Figure 3.2 E	Time	0.0053	**	F (1, 30) = 9.042
	Genotype	0.0046	**	F (1, 32) = 9.306
	Time x Genotype	0.8178	ns	F (1, 30) = 0.05401
Figure 3.2 F	Time	<0.0001	****	F (1, 29) = 47.58
	Genotype	0.0634	ns	F (1, 31) = 3.708
	Time x Genotype	0.4149	ns	F (1, 29) = 0.6841

Table 3.5 $\alpha2^{Q241M}$ - Ethanol intermittent access two bottle choice experiment statistics

Intermittent EtOH protocol				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.4 A	Time	0.1441	ns	F (1, 30) = 2.249
	Genotype	0.1699	ns	F (1, 32) = 1.972
	Time x Genotype	0.031	*	F (1, 30) = 5.121
Figure 3.4 B	Time	0.0363	*	F (1, 30) = 4.801
	Genotype	0.2141	ns	F (1, 32) = 1.607
	Time x Genotype	0.0172	*	F (1, 30) = 6.356
Figure 3.4 C	Time	0.2271	ns	F (1, 30) = 1.521
	Genotype	0.289	ns	F (1, 31) = 1.164
	Time x Genotype	0.0343	*	F (1, 30) = 4.915
Figure 3.4 D	Time	0.7128	ns	F (1, 30) = 0.1381
	Genotype	0.2896	ns	F (1, 32) = 1.160
	Time x Genotype	0.4178	ns	F (1, 30) = 0.6749
Figure 3.4 E	Time	0.0162	*	F (1, 30) = 6.488
	Genotype	0.2499	ns	F (1, 31) = 1.375
	Time x Genotype	0.0113	*	F (1, 30) = 7.294
Figure 3.4 F	Time x Genotype	0.9031	ns	F (1, 32) = 0.01505
	Time	0.5944	ns	F (1, 32) = 0.2892
	Genotype	0.6459	ns	F (1, 32) = 0.2151

Table 3.6 $\alpha4^{Q246M}$ - Mean intake and preference values for the ascending concentration protocol with tastants

	Intake (g/kg)			Preference (%)		
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
Saccharin	**Mean ± SEM**		**P value**	**Mean ± SEM**		**P value**
0.25 mM	89.7 ± 5.6	91.8 ± 4.9	0.777	52 ± 3	58 ± 3	0.213
0.5 mM	139.8 ± 12.4	119.5 ± 11.0	0.223	70 ± 3	69 ± 3	0.742
1 mM	204.7 ± 12.7	194.6 ± 11.7	0.739	88 ± 2	86 ± 3	0.552
Quinine						
0.025 mM	125.6 ± 23.8	80.2 ± 10.6	0.090	48 ± 4	42 ± 4	0.251
0.05 mM	113.2 ± 30.2	95.2 ± 15.2	0.957	38 ± 4	39 ± 4	0.946
0.1 mM	121.9 ± 40.1	72.2 ± 8.5	0.675	33 ± 4	29 ± 3	0.432

Table 3.7 $\alpha4^{Q246M}$ - Mean fluid and water intake values for the ascending concentration protocol with tastants

	Fluid intake (g/kg)			Water intake (g/kg)		
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
Saccharin	**Mean ± SEM**		**P value**	**Mean ± SEM**		**P value**
0.25 mM	179.2 ± 13.6	165.1 ± 11.8	0.448	91.5 ± 11.0	73.4 ± 11.1	0.191
0.5 mM	209.0 ± 24.5	176.7 ± 14.8	0.397	69.5 ± 14.5	57.0 ± 7.8	0.877
1 mM	238.7 ± 19.8	232.2 ± 18.5	0.813	20.3 ± 2.3	28.9 ± 7.6	0.835
Quinine						
0.025 mM	246.8 ± 31.2	200.9 ± 11.8	0.170	121.1 ± 9.8	120.7 ± 11.8	0.979
0.05 mM	275.6 ± 39.7	230.5 ± 20.4	0.301	162.4 ± 11.0	135.1 ± 12.3	0.121
0.1 mM	293.3 ± 56.1	220.9 ± 15.5	0.534	171.3 ± 19.5	148.7 ± 12.5	0.534

Table 3.8 $\alpha2^{Q241M}$ - Mean intake and preference values for the ascending concentration protocol with tastants

	Intake (g/kg)			Preference (%)		
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
Saccharin	**Mean ± SEM**		**P value**	**Mean ± SEM**		**P value**
0.25 mM	123.2 ± 11.2	108.4 ± 13.4	0.405	55 ± 2	61 ± 6	0.340
0.5 mM	168.2 ± 14.2	168.6 ± 11.0	0.986	75 ± 3	81 ± 3	0.210
1 mM	220.5 ± 20.4	218.6 ± 10.4	0.938	82 ± 3	87 ± 3	0.426
Quinine						
0.025 mM	97.2 ± 20.9	69.6 ± 8.6	0.252	39 ± 6	41 ± 5	0.771
0.05 mM	91.0 ± 19.4	56.6 ± 12.3	0.147	35 ± 5	29 ± 6	0.412
0.1 mM	92.8 ± 18.5	67.5 ± 17.0	0.332	31 ± 4	27 ± 4	0.439

Table 3.9 $\alpha2^{Q241M}$ - Mean fluid and water intake values for the ascending concentration protocol with tastants

	Fluid intake (g/kg)			Water intake (g/kg)		
	Wt	Hom	Wt vs Hom	Wt	Hom	Wt vs Hom
Saccharin	**Mean ± SEM**		**P value**	**Mean ± SEM**		**P value**
0.25 mM	237.4 ± 30.0	179.2 ± 13.8	0.083	95.8 ± 10.0	70.8 ± 11.3	0.117
0.5 mM	235.4 ± 26.9	210.7 ± 17.2	0.762	67.2 ± 16.0	42.0 ± 8.1	0.360
1 mM	279.1 ± 31.6	263.8 ± 19.9	0.704	58.6 ± 14.5	45.2 ± 11.9	0.633
Quinine						
0.025 mM	250.7 ± 32.8	179.6 ± 16.8	0.074	153.6 ± 18.3	110.0 ± 17.1	0.104
0.05 mM	228.6 ± 21.7	187.4 ± 17.3	0.157	137.6 ± 11.3	130.8 ± 12.1	0.691
0.1 mM	254.3 ± 23.9	213.5 ± 24.1	0.248	161.5 ± 10.6	145.9 ± 9.8	0.297

Table 3.10 $\alpha4^{Q246M}$ - Mean daily saccharin intake and preference during the ascending concentration protocol with saccharin

	Saccharin intake (g/kg/24hr)			Preference (%)		
	Wt (n=11)	Hom (n=10)	Wt vs Hom	Wt (n=11)	Hom (n=10)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	84.2 ± 12.9	89.9 ± 12.4	0.996	52 ± 9	59 ± 9	0.981
Day 2	93.1 ± 14.9	91.9 ± 6.3	0.999	53 ± 7	59 ± 5	0.938
Day 3	92.5 ± 10.6	100.8 ± 16.0	0.988	53. ± 8	55 ± 8	0.999
Day 4	87.5 ± 13.7	86.2 ± 10.0	0.999	50 ± 8	57 ± 8	0.967
Day 5	112.7 ± 13.2	102.7 ± 24.1	0.994	54 ± 8	55 ± 10	0.999
Day 6	129.5 ± 12.4	115.2 ± 11.8	0.882	68 ± 6	62 ± 6	0.940
Day 7	153.5 ± 12.0	138.4 ± 15.6	0.911	81 ± 4	71 ± 7	0.608
Day 8	117.9 ± 11.3	121.9 ± 9.1	0.872	78 ± 5	86 ± 4	0.621
Day 9	248.0 ± 14.8	191.6 ± 27.1	0.969	84 ± 5	75 ± 8	0.754
Day 10	148.1 ± 11.2	179.4 ± 19.9	0.725	84 ± 5	91 ± 2	0.850
Day 11	186.8 ± 8.0	186.2 ± 9.4	0.792	92 ± 2	89 ± 4	0.958
Day 12	196.3 ± 11.6	189.8 ± 15.8	0.934	84 ± 4	88 ± 5	0.999

Table 3.11 $\alpha4^{Q246M}$ - Mean daily fluid and water intake values during the ascending concentration protocol with saccharin

	Fluid intake intake (g/kg/24hr)			Water intake (g/kg/24hr)		
	Wt (n=11)	Hom (n=10)	Wt vs Hom	Wt (n=11)	Hom (n=10)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	168.9 ± 16.2	156.6 ± 7.4	0.939	92.6 ± 18.7	66.6 ± 14.5	0.738
Day 2	176.8 ± 8.7	164.1 ± 15.9	0.935	83.5 ± 13.6	72.3 ± 15.3	0.972
Day 3	190.9 ± 23.1	180.4 ± 15.5	0.993	98.4 ± 23.8	79.8 ± 15.7	0.948
Day 4	178.6 ± 13.0	165.3 ± 14.9	0.942	91.3 ± 16.0	79.1 ± 20.1	0.984
Day 5	203.5 ± 28.6	175.7 ± 20.1	0.614	92.6 ± 27.2	73.3 ± 13.9	0.683
Day 6	191.2 ± 10.7	189.8 ± 18.1	0.999	61.4 ± 11.7	74.5 ± 15.0	0.937
Day 7	195.6 ± 19.1	196.8 ± 18.1	0.999	42.3 ± 12.9	58.1 ± 12.9	0.867
Day 8	159.4 ± 15.7	143.6 ± 13.3	0.668	42.5 ± 11.2	21.8 ± 6.7	0.432
Day 9	307.5 ± 24.5	253.6 ± 13.8	0.999	59.4 ± 26.4	75.8 ± 24.2	0.463
Day 10	179.6 ± 13.7	197.9 ± 23.6	0.928	31.5 ± 10.0	18.6 ± 7.1	0.999
Day 11	227.6 ± 22.9	213.5 ± 16.0	0.954	18.0 ± 4.2	27.5 ± 10.8	0.992
Day 12	238.5 ± 21.5	218.4 ± 14.8	0.858	42.0 ± 16.0	28.7 ± 12.0	0.760

Table 3.12 $\alpha4^{Q246M}$ – Statistical results of the ascending protocol with saccharin

0.25 mM saccharin				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.6 A	Time	0.653	ns	F (1.261, 31.11) = 0.2820
	Genotype	0.744	ns	F (1, 74) = 0.1079
	Time x Genotype	0.973	ns	F (3, 74) = 0.07623
Figure 3.6 C	Time	0.888	ns	F (1.199, 29.59) = 0.03671
	Genotype	0.345	ns	F (1, 74) = 0.9037
	Time x Genotype	0.991	ns	F (3, 74) = 0.03440
Figure 3.6 E	Time	0.178	ns	F (2.335, 42.81) = 1.765
	Genotype	0.476	ns	F (1, 19) = 0.5281
	Time x Genotype	1.000	ns	F (3, 55) = 0.004914
Figure 3.6 G	Time	0.773	ns	F (1.500, 28.00) = 0.1809
	Genotype	0.280	ns	F (1, 19) = 1.238
	Time x Genotype	0.961	ns	F (3, 56) = 0.09850
0.5 mM saccharin				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.7 A	Time	0.258	ns	F (1.787, 33.96) = 1.406
	Genotype	0.241	ns	F (1, 19) = 1.468
	Time x Genotype	0.879	ns	F (3, 57) = 0.2239
Figure 3.7 C	Time	0.001	**	F (2.114, 40.17) = 7.898
	Genotype	0.729	ns	F (1, 19) = 0.1237
	Time x Genotype	0.468	ns	F (3, 57) = 0.8580
Figure 3.7 E	Time	0.440	ns	F (1.521, 28.90) = 0.7691
	Genotype	0.283	ns	F (1, 19) = 1.220
	Time x Genotype	0.287	ns	F (3, 57) = 1.288
Figure 3.7 G	Time	0.023	*	F (1.504, 28.58) = 4.877
	Genotype	0.474	ns	F (1, 19) = 0.5333
	Time x Genotype	0.193	ns	F (3, 57) = 1.629
1 mM saccharin				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.8 A	Time	0.030	*	F (1.731, 31.16) = 4.158
	Genotype	0.567	ns	F (1, 18) = 0.3395
	Time x Genotype	0.497	ns	F (3, 54) = 0.8046
Figure 3.8 C	Time	0.110	ns	F (1.366, 24.60) = 2.604
	Genotype	0.587	ns	F (1, 18) = 0.3067
	Time x Genotype	0.345	ns	F (3, 54) = 1.131
Figure 3.8 E	Time	0.001	***	F (1.655, 29.79) = 11.27
	Genotype	0.810	ns	F (1, 18) = 0.05930
	Time x Genotype	0.680	ns	F (3, 54) = 0.5061
Figure 3.8 G	Time	0.052	ns	F (1.161, 17.41) = 4.173
	Genotype	0.323	ns	F (1, 15) = 1.045
	Time x Genotype	0.100	ns	F (3, 45) = 2.208
Whole experiment				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.21 A	Time	<0.0001	****	F (4.204, 77.96) = 25.38
	Genotype	0.623	ns	F (1, 19) = 0.2492
	Time x Genotype	0.239	ns	F (11, 204) = 1.279
Figure 3.21 B	Time	<0.0001	****	F (2.787, 52.44) = 12.50
	Genotype	0.772	ns	F (1, 19) = 0.08617
	Time x Genotype	0.821	ns	F (11, 207) = 0.6077
Figure 3.21 C	Time	0.094	ns	F (4.123, 78.34) = 2.049
	Genotype	0.009	**	F (1, 19) = 8.586
	Time x Genotype	0.696	ns	F (11, 209) = 0.7438

Table 3.13 $\alpha2^{Q241M}$ - Mean daily saccharin intake and preference during the ascending concentration protocol with saccharin

	Saccharin intake (g/kg/24hr)			Preference (%)		
	Wt (n=10)	Hom (n=8)	Wt vs Hom	Wt (n=10)	Hom (n=8)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	91.3 ± 16.4	93.6 ± 21.2	0.999	45 ± 7	55 ± 13	0.943
Day 2	148.9 ± 25.8	117.0 ± 12.0	0.736	58 ± 6	67 ± 8	0.864
Day 3	113.6 ± 17.1	113.2 ± 21.3	0.999	51 ± 6	61 ± 10	0.876
Day 4	139.0 ± 19.9	109.9 ± 12.7	0.662	65 ± 5	63 ± 7	0.998
Day 5	150.2 ± 20.0	179.2 ± 36.1	0.935	69 ± 7	81 ± 5	0.606
Day 6	168.0 ± 13.6	168.1 ± 12.3	0.999	79 ± 3	82 ± 6	0.976
Day 7	164.8 ± 17.8	138.7 ± 11.8	0.670	72 ± 6	74 ± 7	0.999
Day 8	189.9 ± 25.9	188.3 ± 22.6	0.999	81 ± 4	87 ± 3	0.735
Day 9	213.2 ± 25.9	227.5 ± 20.5	0.988	71 ± 6	81 ± 8	0.848
Day 10	239.7 ± 27.6	220.8 ± 9.9	0.951	85 ± 4	84 ± 5	0.999
Day 11	186.4 ± 18.1	176.0 ± 12.3	0.984	85 ± 4	88 ± 5	0.979
Day 12	242.8 ± 29.0	250.0 ± 29.9	0.997	87 ± 3	89 ± 4	0.964

Table 3.14 $\alpha2^{Q241M}$ - Mean daily fluid and water intake values during the ascending concentration protocol with saccharin

	Fluid intake intake (g/kg/24hr)			Water intake (g/kg/24hr)		
	Wt (n=10)	Hom (n=8)	Wt vs Hom	Wt (n=10)	Hom (n=8)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	233.0 ± 37.7	178.7 ± 26.2	0.692	141.7 ± 38.0	85.1 ± 30.9	0.707
Day 2	262.3 ± 39.4	181.2 ± 18.5	0.302	113.3 ± 25.2	64.2 ± 17.1	0.419
Day 3	245.3 ± 36.0	180.4 ± 11.6	0.386	131.7 ± 34.2	67.3 ± 17.4	0.392
Day 4	209.1 ± 18.8	176.6 ± 16.0	0.604	70.1 ± 9.8	66.7 ± 15.8	0.999
Day 5	230.2 ± 24.6	216.5 ± 34.9	0.996	80.1 ± 27.2	37.3 ± 8.1	0.504
Day 6	216.0 ± 19.2	213.3 ± 21.9	0.999	47.9 ± 9.2	45.3 ± 20.3	0.999
Day 7	258.8 ± 57.4	193.2 ± 14.6	0.751	94.0 ± 44.6	54.5 ± 16.5	0.889
Day 8	236.7 ± 29.9	219.5 ± 27.0	0.989	46.9 ± 13.7	31.2 ± 6.4	0.784
Day 9	323.6 ± 44.6	305.7 ± 38.7	0.997	110.3 ± 34.3	78.2 ± 41.6	0.963
Day 10	285.7 ± 31.4	264.8 ± 12.5	0.959	46.0 ± 12.2	44.1 ± 14.6	0.999
Day 11	223.0 ± 22.6	203.7 ± 16.8	0.940	36.6 ± 11.8	27.7 ± 12.6	0.978
Day 12	284.2 ± 36.6	280.8 ± 29.9	0.999	41.4 ± 11.1	30.8 ± 12.4	0.953

Table 3.15 α2^{Q241M} – Statistical results of the ascending protocol with saccharin

0.25 mM saccharin				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.10 A	Time	0.161	ns	F (1.525, 24.40) = 2.028
	Genotype	0.405	ns	F (1, 16) = 0.7313
	Time x Genotype	0.651	ns	F (3, 48) = 0.5501
Figure 3.10 C	Time	0.230	ns	F (1.648, 26.36) = 1.559
	Genotype	0.292	ns	F (1, 16) = 1.187
	Time x Genotype	0.806	ns	F (3, 48) = 0.3263
Figure 3.10 E	Time	0.416	ns	F (2.108, 33.73) = 0.9119
	Genotype	0.120	ns	F (1, 16) = 2.699
	Time x Genotype	0.595	ns	F (3, 48) = 0.6366
Figure 3.10 G	Time	0.265	ns	F (1.702, 27.24) = 1.385
	Genotype	0.103	ns	F (1, 16) = 2.985
	Time x Genotype	0.545	ns	F (3, 48) = 0.7193
0.5 mM saccharin				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.11 A	Time	0.289	ns	F (1.602, 25.63) = 1.281
	Genotype	0.984	ns	F (1, 16) = 0.0004333
	Time x Genotype	0.570	ns	F (3, 48) = 0.6783
Figure 3.11 C	Time	0.198	ns	F (1.797, 28.75) = 1.728
	Genotype	0.205	ns	F (1, 16) = 1.748
	Time x Genotype	0.820	ns	F (3, 48) = 0.3070
Figure 3.11 E	Time	0.881	ns	F (1.790, 28.64) = 0.1049
	Genotype	0.475	ns	F (1, 16) = 0.5342
	Time x Genotype	0.630	ns	F (3, 48) = 0.5813
Figure 3.11 G	Time	0.389	ns	F (2.011, 32.17) = 0.9732
	Genotype	0.212	ns	F (1, 16) = 1.689
	Time x Genotype	0.767	ns	F (3, 48) = 0.3806
1 mM saccharin				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.12 A	Time	0.032	*	F (1.471, 23.54) = 4.458
	Genotype	0.937	ns	F (1, 16) = 0.006485
	Time x Genotype	0.796	ns	F (3, 48) = 0.3410
Figure 3.12 C	Time	0.089	ns	F (1.415, 22.64) = 2.927
	Genotype	0.404	ns	F (1, 16) = 0.7336
	Time x Genotype	0.740	ns	F (3, 48) = 0.4199
Figure 3.12 E	Time	<0.0001	****	F (2.543, 40.69) = 10.51
	Genotype	0.704	ns	F (1, 16) = 0.1497
	Time x Genotype	0.962	ns	F (3, 48) = 0.09522
Figure 3.12 G	Time	0.042	*	F (1.268, 20.28) = 4.338
	Genotype	0.501	ns	F (1, 16) = 0.4753
	Time x Genotype	0.879	ns	F (3, 48) = 0.2237
Whole experiment				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.22 A	Time	<0.0001	****	F (3.053, 48.85) = 14.22
	Genotype	0.769	ns	F (1, 16) = 0.08912
	Time x Genotype	0.885	ns	F (11, 176) = 0.5252
Figure 3.22 B	Time	<0.0001	****	F (3.219, 51.50) = 9.389
	Genotype	0.185	ns	F (1, 16) = 1.920
	Time x Genotype	0.984	ns	F (11, 176) = 0.3075
Figure 3.22 C	Time	<0.0001	****	F (4.998, 79.96) = 25.11
	Genotype	0.796	ns	F (1, 16) = 0.06933
	Time x Genotype	0.948	ns	F (11, 176) = 0.4159

Table 3.16 $\alpha4^{Q246M}$ - Mean daily quinine intake and preference during the ascending concentration protocol with quinine

	Quinine intake (g/kg/24hr)			Preference (%)		
	Wt (n=9)	Hom (n=11)	Wt vs Hom	Wt (n=9)	Hom (n=11)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	140.4 ± 32.3	89.7 ± 12.2	0.529	48 ± 6	50 ± 8	0.998
Day 2	148.3 ± 35.8	97.7 ± 30.2	0.754	49 ± 8	40 ± 9	0.798
Day 3	84.9 ± 19.2	66.7 ± 10.1	0.886	52 ± 7	43 ± 7	0.955
Day 4	128.4 ± 30.1	106.2 ± 34.1	0.981	44 ± 8	38 ± 9	0.871
Day 5	146.0 ± 44.6	110.2 ± 19.0	0.926	38 ± 6	45 ± 8	0.908
Day 6	103.7 ± 31.0	106.9 ± 33.4	0.999	43 ± 8	42 ± 9	0.999
Day 7	93.0 ± 30.0	45.2 ± 8.6	0.498	41 ± 7	36 ± 7	0.982
Day 8	113.7 ± 55.3	117.5 ± 46.3	0.999	27 ± 8	31 ± 8	0.997
Day 9	139.9 ± 35.7	112.5 ± 19.9	0.958	39 ± 5	37 ± 7	0.999
Day 10	135.7 ± 55.5	79.7 ± 28.2	0.859	31 ± 8	32 ± 8	0.982
Day 11	83.9 ± 37.8	38.6 ± 10.1	0.680	29 ± 7	24 ± 7	0.996
Day 12	128.4 ± 56.7	89.0 ± 34.1	0.731	34 ± 8	31 ± 7	0.914

Table 3.17 $\alpha4^{Q246M}$ - Mean daily fluid and water intake values during the ascending concentration protocol with quinine

	Fluid intake intake (g/kg/24hr)			Water intake (g/kg/24hr)		
	Wt (n=9)	Hom (n=11)	Wt vs Hom	Wt (n=9)	Hom (n=11)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	274.4 ± 44.9	189.4 ± 19.1	0.260	133.9 ± 21.9	99.5 ± 18.8	0.501
Day 2	272.9 ± 36.9	245.3 ± 33.7	0.730	124.8 ± 12.6	147.7 ± 28.7	0.918
Day 3	161.3 ± 19.0	161.3 ± 11.8	0.999	76.3 ± 14.0	94.5 ± 13.7	0.880
Day 4	278.4 ± 47.0	245.0 ± 25.0	0.924	150.0 ± 28.3	138.9 ± 22.9	0.999
Day 5	366.3 ± 73.4	263.5 ± 29.3	0.918	220.3 ± 38.9	153.3 ± 31.1	0.589
Day 6	208.1 ± 28.9	206.6 ± 27.6	0.999	104.0 ± 12.0	99.7 ± 13.3	0.999
Day 7	219.0 ± 41.1	138.6 ± 12.9	0.323	125.8 ± 28.5	93.2 ± 16.5	0.811
Day 8	342.3 ± 65.1	310.5 ± 36.5	0.990	228.2 ± 19.7	192.9 ± 20.8	0.666
Day 9	341.9 ± 49.4	326.4 ± 42.2	0.878	201.8 ± 23.5	214.0 ± 44.3	0.964
Day 10	327.1 ± 64.8	292.8 ± 46.1	0.784	191.6 ± 27.8	166.5 ± 13.7	0.967
Day 11	215.0 ± 54.6	168.1 ± 33.0	0.571	131.1 ± 19.6	129.7 ± 30.7	0.610
Day 12	289.3 ± 72.3	226.8 ± 34.2	0.715	161.2 ± 29.2	137.9 ± 13.6	0.955

Table 3.18 $\alpha4^{Q246M}$ – Statistical results of the ascending protocol with quinine

0.025 mM QUININE				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.14 A	Time	0.171	ns	F (1.963, 35.34) = 1.864
	Genotype	0.195	ns	F (1, 18) = 1.811
	Time x Genotype	0.817	ns	F (3, 54) = 0.3116
Figure 3.14 C	Time	0.429	ns	F (1.460, 24.82) = 0.7866
	Genotype	0.236	ns	F (1, 17) = 1.510
	Time x Genotype	0.802	ns	F (3, 51) = 0.3331
Figure 3.14 E	Time	0.001	**	F (2.666, 45.32) = 6.573
	Genotype	0.170	ns	F (1, 17) = 2.055
	Time x Genotype	0.220	ns	F (3, 51) = 1.523
Figure 3.14 G	Time	0.049	*	F (1.941, 33.00) = 3.343
	Genotype	0.974	ns	F (1, 17) = 0.001136
	Time x Genotype	0.417	ns	F (3, 51) = 0.9638
0.05 mM QUININE				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.15 A	Time	0.195	ns	F (1.945, 31.76) = 1.727
	Genotype	0.568	ns	F (1, 18) = 0.3392
	Time x Genotype	0.746	ns	F (3, 49) = 0.4114
Figure 3.15 C	Time	0.342	ns	F (1.457, 23.80) = 1.059
	Genotype	0.824	ns	F (1, 18) = 0.05089
	Time x Genotype	0.866	ns	F (3, 49) = 0.2427
Figure 3.15 E	Time	<0.0001	****	F (2.249, 37.48) = 12.94
	Genotype	0.332	ns	F (1, 18) = 0.9954
	Time x Genotype	0.510	ns	F (3, 50) = 0.7806
Figure 3.15 G	Time	0.000	***	F (2.160, 35.27) = 10.23
	Genotype	0.072	ns	F (1, 18) = 3.668
	Time x Genotype	0.611	ns	F (3, 49) = 0.6114
0.1 mM QUININE				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.16 A	Time	0.111	ns	F (1.783, 30.30) = 2.426
	Genotype	0.219	ns	F (1, 17) = 1.632
	Time x Genotype	0.866	ns	F (3, 51) = 0.2433
Figure 3.16 C	Time	0.289	ns	F (1.745, 29.66) = 1.280
	Genotype	0.431	ns	F (1, 17) = 0.6498
	Time x Genotype	0.956	ns	F (3, 51) = 0.1061
Figure 3.16 E	Time	<0.0001	****	F (2.221, 37.75) = 13.56
	Genotype	0.209	ns	F (1, 17) = 1.708
	Time x Genotype	0.855	ns	F (3, 51) = 0.2582
Figure 3.16 G	Time	0.005	**	F (1.488, 25.29) = 7.525
	Genotype	0.329	ns	F (1, 17) = 1.008
	Time x Genotype	0.987	ns	F (3, 51) = 0.04656
WHOLE EXPERIMENT				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.23 A	Time	0.169	ns	F (3.592, 62.70) = 1.692
	Genotype	0.289	ns	F (1, 18) = 1.193
	Time x Genotype	0.995	ns	F (11, 192) = 0.2342
Figure 3.23 B	Time	0.038	*	F (2.258, 37.56) = 3.418
	Genotype	0.511	ns	F (1, 18) = 0.4490
	Time x Genotype	0.991	ns	F (11, 183) = 0.2644
Figure 3.23 C	Time	<0.0001	****	F (4.089, 73.60) = 8.734
	Genotype	0.026	*	F (1, 18) = 5.880
	Time x Genotype	0.026	*	F (11, 198) = 2.051

Table 3.19 $\alpha2^{Q241M}$ - Mean daily quinine intake and preference during the ascending concentration protocol with quinine

	Quinine intake (g/kg/24hr)			Preference (%)		
	Wt (n=8)	Hom (n=8)	Wt vs Hom	Wt (n=8)	Hom (n=8)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	59.5 ± 13.2	86.2 ± 16.6	0.649	30 ± 9	50 ± 9	0.434
Day 2	95.3 ± 26.7	64.7 ± 14.9	0.810	41 ± 10	42 ± 8	0.999
Day 3	124.0 ± 24.0	67.8 ± 17.7	0.301	43 ± 9	32 ± 9	0.884
Day 4	98.5 ± 45.3	56.3 ± 11.3	0.864	41 ± 11	42 ± 10	0.999
Day 5	65.6 ± 15.8	40.5 ± 15.3	0.722	29 ± 8	21 ± 7	0.900
Day 6	134.9 ± 59.5	64.6 ± 14.6	0.747	42 ± 12	38 ± 8	0.998
Day 7	94.6 ± 35.2	54.7 ± 14.3	0.791	37 ± 10	25 ± 6	0.768
Day 8	69.0 ± 31.3	66.6 ± 24.3	0.999	29 ± 11	30 ±8	0.999
Day 9	148.9 ± 37.0	71.0 ± 12.0	0.279	41 ± 6	29 ± 4	0.376
Day 10	111.9 ± 44.5	105.1 ± 42.5	0.999	35 ± 8	32 ± 7	0.999
Day 11	24.5 ± 6.7	40.3 ± 17.0	0.876	18 ± 5	19 ± 6	0.999
Day 12	85.9 ± 27.5	53.7 ± 12.6	0.777	31 ± 8	26 ± 5	0.985

Table 3.20 $\alpha2^{Q241M}$ - Mean daily fluid and water intake values during the ascending concentration protocol with quinine

	Fluid intake intake (g/kg/24hr)			Water intake (g/kg/24hr)		
	Wt (n=8)	Hom (n=8)	Wt vs Hom	Wt (n=8)	Hom (n=8)	Wt vs Hom
	Mean ± SEM		P value	Mean ± SEM		P value
Day 1	268.3 ± 52.1	172.3 ± 13.5	0.380	208.9 ± 56.2	86.1 ± 17.6	0.249
Day 2	221.2 ± 21.0	154.0 ± 9.7	0.063	125.9 ± 21.2	89.3 ± 13.6	0.529
Day 3	298.0 ± 47.3	235.2 ± 29.8	0.742	173.9 ± 37.5	167.4 ± 37.9	0.999
Day 4	200.1 ± 35.4	138.2 ± 9.6	0.429	101.5 ± 17.1	82.0 ± 15.2	0.878
Day 5	233.0 ± 13.3	186.4 ± 21.4	0.316	167.4 ± 23.4	145.9 ± 18.4	0.929
Day 6	248.2 ± 49.8	162.4 ± 13.7	0.456	113.3 ± 21.7	97.9 ± 12.3	0.959
Day 7	218.2 ± 27.6	207.4 ± 19.4	0.996	123.6 ± 15.2	152.7 ± 13.7	0.546
Day 8	215.0 ± 21.3	193.5 ± 25.9	0.952	146.0 ± 27.3	126.9 ± 14.5	0.960
Day 9	340.5 ± 40.7	231.0 ± 20.0	0.137	191.6 ± 20.0	160.0 ± 11.1	0.579
Day 10	274.3 ± 38.5	265.7 ± 47.3	0.999	162.3 ± 18.6	160.6 ± 13.1	0.999
Day 11	147.2 ± 17.1	161.9 ± 23.3	0.979	122.8 ± 19.6	121.6 ± 9.5	0.999
Day 12	255.3 ± 32.0	195.2 ± 17.4	0.422	169.4 ± 27.4	141.6 ± 10.9	0.843

Table 3.21 $\alpha2^{Q241M}$ – Statistical results of the ascending protocol with quinine

0.025 mM QUININE				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.18 A	Time	0.498	ns	F (1.744, 23.25) = 0.6790
	Genotype	0.239	ns	F (1, 14) = 1.514
	Time x Genotype	0.166	ns	F (3, 40) = 1.783
Figure 3.18 C	Time	0.853	ns	F (1.320, 17.60) = 0.07425
	Genotype	0.728	ns	F (1, 14) = 0.1256
	Time x Genotype	0.379	ns	F (3, 40) = 1.055
Figure 3.18 E	Time	0.001	***	F (2.179, 29.05) = 9.369
	Genotype	0.067	ns	F (1, 14) = 3.948
	Time x Genotype	0.711	ns	F (3, 40) = 0.4618
Figure 3.18 G	Time	0.084	ns	F (1.676, 22.34) = 2.898
	Genotype	0.075	ns	F (1, 14) = 3.691
	Time x Genotype	0.207	ns	F (3, 40) = 1.589
0.05 mM QUININE				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.19 A	Time	0.378	ns	F (1.727, 22.45) = 0.9833
	Genotype	0.147	ns	F (1, 13) = 2.374
	Time x Genotype	0.669	ns	F (3, 39) = 0.5236
Figure 3.19 C	Time	0.350	ns	F (1.842, 23.94) = 1.084
	Genotype	0.429	ns	F (1, 13) = 0.6666
	Time x Genotype	0.860	ns	F (3, 39) = 0.2513
Figure 3.19 E	Time	0.930	ns	F (2.125, 27.63) = 0.08252
	Genotype	0.157	ns	F (1, 13) = 2.256
	Time x Genotype	0.243	ns	F (3, 39) = 1.450
Figure 3.19 G	Time	0.075	ns	F (1.399, 18.19) = 3.278
	Genotype	0.692	ns	F (1, 13) = 0.1644
	Time x Genotype	0.380	ns	F (3, 39) = 1.054
0.1 mM QUININE				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.20 A	Time	0.042	*	F (1.536, 21.50) = 4.039
	Genotype	0.331	ns	F (1, 14) = 1.015
	Time x Genotype	0.322	ns	F (3, 42) = 1.199
Figure 3.20 C	Time	0.049	*	F (1.743, 24.40) = 3.571
	Genotype	0.458	ns	F (1, 14) = 0.5831
	Time x Genotype	0.700	ns	F (3, 42) = 0.4767
Figure 3.20 E	Time	0.000	***	F (1.848, 25.87) = 12.40
	Genotype	0.248	ns	F (1, 14) = 1.451
	Time x Genotype	0.054	ns	F (3, 42) = 2.756
Figure 3.20 G	Time	0.029	*	F (2.080, 29.12) = 3.962
	Genotype	0.297	ns	F (1, 14) = 1.174
	Time x Genotype	0.673	ns	F (3, 42) = 0.5165
WHOLE EXPERIMENT				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Figure 3.24 A	Time	0.152	ns	F (2.423, 32.59) = 1.940
	Genotype	0.188	ns	F (1, 14) = 1.917
	Time x Genotype	0.439	ns	F (11, 148) = 1.012
Figure 3.24 B	Time	0.047	*	F (3.330, 41.78) = 2.785
	Genotype	0.677	ns	F (1, 14) = 0.1809
	Time x Genotype	0.917	ns	F (11, 138) = 0.4728
Figure 3.24 C	Time	<0.0001	****	F (2.182, 30.55) = 15.11
	Genotype	0.803	ns	F (1, 14) = 0.06452
	Time x Genotype	0.502	ns	F (11, 154) = 0.9425

Chapter 4 Functional effects of ethanol in $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ animals

4.1 Introduction

To investigate whether specific neuronal populations of α2- and/or α4-$GABA_A$ receptors underpin the behavioural consequences of removing neurosteroid sensitivity reported in our experiments described in *Chapter 3*, we used electrophysiological recordings in *ex vivo* acute brain slices from these animals to examine GABA-mediated inhibitory transmission.

4.1.1 GABAergic neurotransmission in the dentate gyrus

The hippocampal formation, comprising the hippocampus proper, the dentate gyrus and the subiculum, is vital for cognitive processes (Schultz and Engelhardt, 2014). The dentate gyrus, as the initial processing station in the hippocampus, forms the first synapse in the 'tri-synaptic pathway.' In this sequence, signals move from the associative cortices to the dentate gyrus granule cells, then to the CA3 region, and finally to CA1 (Basu and Siegelbaum, 2015). It is believed that the dentate gyrus converts cortical signals into a sparse code for memory encoding, highlighting its significant role in hippocampal memory and learning functions (Amaral et al., 2007).

The dentate gyrus is composed of three layers: molecular, granule cell, and polymorphic, with the granule cell layer being the principal one (Amaral et al., 2007). Dentate gyrus granule cells are under extensive GABAergic control to maintain normal function (Klausberger, 2009).

In situ hybridisation and immunocytochemical studies have reported that dentate gyrus granule cells express a variety of $GABA_A$ receptor subunits (Pirker et al., 2000, Sperk et al., 1997, Wisden et al., 1992). Synaptic $GABA_A$ receptors in dentate granule cells show high expression of α1 subunits, although other α subunits, such as α2 and α4, may also influence synaptic responses (Coulter and Carlson, 2007). The majority (but not all) of the tonic current in the dentate gyrus is thought to be mediated by α4βδ receptors (Herd

et al., 2007, Stell et al., 2003). Global genetic knock-out of either the α4 or δ subunit leads to a significant reduction in tonic current (Chandra et al., 2006, Spigelman et al., 2003, Wei et al., 2003).

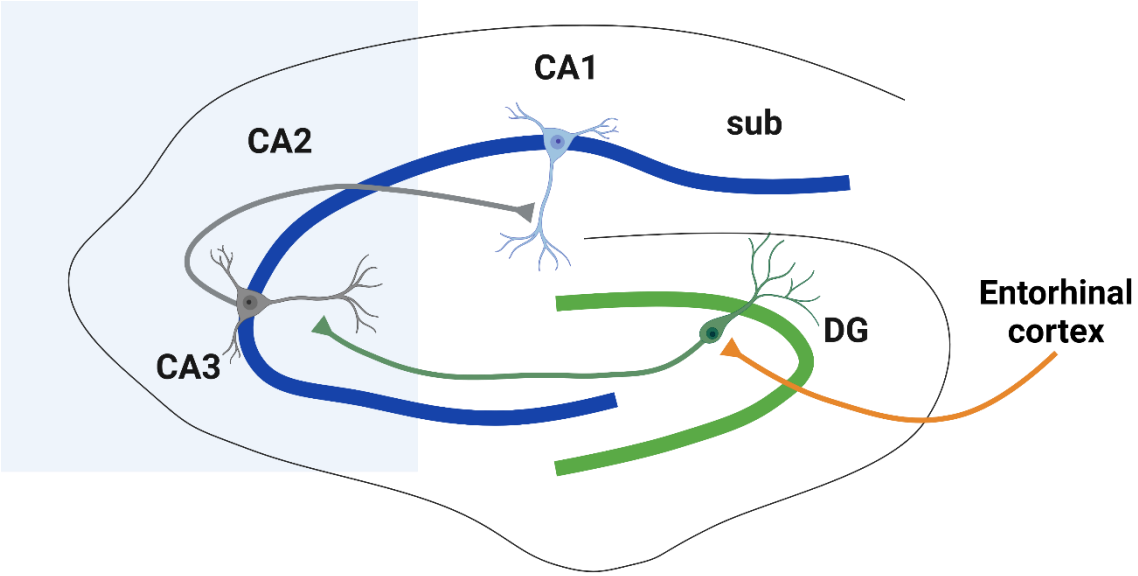


Figure 4.1 Hippocampal tri-synaptic circuit

Schematic diagram showing the hippocampal formation and the classical tri-synaptic circuit. The hippocampal formation consists of the *cornu ammonis* (CA1-3, blue) regions, the dentate gyrus (DG, green) and the subiculum (sub, blue). The DG receives input from the entorhinal cortex. These signals are then propagated downstream to CA3, and finally to CA1.

The dentate gyrus is heavily implicated in alcohol and substance abuse. Chronic alcohol intake results in increased neuronal loss and decreased adult neurogenesis (Anderson et al., 2012, Herrera et al., 2003). The total count of granule cells in both rodent models and individuals diagnosed with AUD is significantly reduced (Leasure and Nixon, 2010, Cadete-Leite et al., 1988, Dhanabalan et al., 2018). Manipulations that disrupt adult neurogenesis in the dentate gyrus enhance drug consumption and the motivation to seek drugs (Deroche-Gamonet et al., 2019, Noonan et al., 2010). These findings suggest that compromised dentate gyrus connectivity contributes to addiction-related behaviours.

4.1.2 Neurosteroid modulation of $GABA_A$ receptors in the dentate gyrus

Potentiating neurosteroids, such as allopregnanolone and THDOC, enhance GABA currents by prolonging the decay of IPSCs (Lu et al., 2020) and/or increasing the size of tonic currents (Stell et al., 2003) in dentate gyrus granule cells. Removing neurosteroid sensitivity from α2-$GABA_A$ receptors results in faster decay kinetics in DGGCs (Durkin et al., 2018), while ablating neurosteroid sensitivity from α4-containing $GABA_A$ receptors has no effect on IPSC kinetics in the same neurons (Minère, 2019). Nevertheless, experiments with $\alpha4^{Q246M}$ mice showed that the α4 subunit is crucial for neurosteroid modulation of tonic inhibition. In neurosteroid-insensitive mice, the enhancement of tonic currents by THDOC was significantly diminished compared to wild type mice (Minère, 2019).

4.1.3 Functional effects of ethanol in the hippocampus

Ethanol potentiation of $GABA_A$ receptor function has been extensively studied. There is evidence to suggest that ethanol potentiates the function of both synaptic and extrasynaptic receptors in the hippocampus.

Acute effects of ethanol

Several studies using hippocampal brain slices found inconsistent effects of ethanol on postsynaptic $GABA_A$ receptors. Ethanol (10–100 mM) showed no significant impact on sIPSC or mIPSC amplitude or kinetics in CA1 pyramidal neurons (Carta et al., 2003, Spigelman et al., 2003, Wei et al., 2004). However, Sanna et al. (2004) reported biphasic modulation of mIPSCs by ethanol in CA1 neurons. In the early phase (3 min) of ethanol application, there is an increase in mIPSC amplitude, which partially diminishes after 10 minutes of continuous ethanol exposure. However, after 30 minutes of continuous ethanol exposure, a delayed effect is observed where mIPSC amplitude increases again, and the decay is prolonged. The effects seen after 30 minutes are blocked by finasteride. Ethanol's effects may involve an increase in neurosteroid synthesis. Furthermore, Wu et al. (2005) demonstrated ethanol's direct modulation of $GABA_A$ receptors by increasing somatic evoked IPSCs in CA1 neurons, which was unaffected by $GABA_B$ receptor

blockade. However, ethanol did not enhance currents at distal dendrites under control conditions, except with $GABA_B$ receptor inhibition. These findings imply modulation of postsynaptic $GABA_A$ receptors by ethanol, with distal receptor sensitivity regulated by $GABA_B$ receptors.

Wei et al. (2004) investigated ethanol's acute effects on tonic inhibition mediated by α4- and δ-subunit-containing $GABA_A$ receptors in dentate gyrus. They found that 30 mM ethanol increased tonic current by approximately 80 %. This effect was absent in DGGCs lacking the δ-subunit or in CA1 pyramidal neurons with α5- and γ-subunit-mediated tonic currents. Ethanol at 30 mM reduced excitability in dentate gyrus but not CA1 neurons. However, another study reported no effect of 30 mM ethanol on similar currents in younger mice, suggesting factors beyond receptor subunits influence ethanol sensitivity (Borghese et al., 2006). Spigelman et al. (2004) also showed ethanol (100 mM) potentiating tonic currents in rat CA1 neurons, suggesting α5- and γ-subunit-containing receptors may be ethanol targets, especially at higher concentrations.

Research also suggests that ethanol enhances GABA release from presynaptic terminals, contributing to increased synaptic inhibition. Analysis of GABAergic IPSCs helps determine if synaptic event frequency (a presynaptic change) or amplitude (usually a postsynaptic change) is affected. Several studies have demonstrated increased sIPSC and/or mIPSC frequencies by ethanol in various brain regions, including the hippocampus (Ariwodola and Weiner, 2004, Sanna et al., 2004). Electrophysiological recordings also demonstrated no differences in ethanol modulation of excitatory and inhibitory transmission in the hippocampus between monkeys and rats, providing evidence for the use of rodent brain slice preparations in elucidating synaptic mechanisms of ethanol action in the primate central nervous system (Ariwodola et al., 2003).

Chronic effects of ethanol

Studies on GABAergic synaptic adaptation following chronic ethanol exposure were conducted using a chronic intermittent ethanol (CIE) treatment paradigm. This protocol involves administering ethanol every other day for 60 treatments over 120 days (Olsen and Spigelman, 2010). It was found that CIE protocol in rodents results in decreased

GABAergic neurotransmission in the hippocampus that lasts for over a month after withdrawal of CIE treatment (Kang et al., 1996).

Immunocytochemical and electrophysiological studies revealed altered $GABA_A$ receptor subunit expression in CIE-treated rats (Cagetti et al., 2003). Specifically, there was an increase in α4- and γ2-subunits and a decrease in α1- and δ-subunits in hippocampal homogenates. Analysis of mIPSCs from CA1 neurons showed decreased amplitude and decay, along with reduced frequency, suggesting potential postsynaptic and presynaptic alterations in GABAergic transmission due to chronic ethanol exposure (Cagetti et al., 2003).

Later on, it was shown that CIE treatment and subsequent withdrawal result, in CA1 neurons and DGGCs, in a reduction of ethanol-enhanced δ-mediated tonic currents, associated with the down-regulation of the δ subunit (Liang et al., 2007). Concurrently, an increase in ethanol sensitivity of mIPSCs was observed, which is correlated with the presence of hippocampal α4βγ2 subtypes, including upregulated α4, and their synaptic localization as demonstrated by immunogold labelling electron microscopy (Liang et al., 2006).

Subsequently, using Western blotting and cross-linking, an upregulation of α2β1γ1 receptors was also reported in CA1 and dentate gyrus neurons following chronic ethanol exposure (Lindemeyer et al., 2017). Analysis of mIPSC kinetics corroborated these findings – receptors with different subunit compositions are recognisable based on their decay (α1 < α2 < α4) and rise times (γ2 < γ1) (Olsen and Liang, 2017).

Effects of ethanol on neurosteroids and neurosteroid modulation of $GABA_A$ receptors

Several lines of evidence indicate that allopregnanolone and THDOC contribute to ethanol's pharmacological effects (Morrow, 2007). Acute ethanol exposure leads to an elevation of neurosteroid levels in the brain. Previously it was believed that this increase depended on the activation of the HPA axis. However, numerous studies (both *in vitro* and *in vivo*) have shown that ethanol produces local brain synthesis of allopregnanolone (Cook et al., 2014a, Cook et al., 2014b, Cook et al., 2014c, Sanna et al., 2004, Tokuda et al., 2011). On the contrary, chronic ethanol exposure leads to a decrease in neurosteroid concentrations (Snelling et al., 2014).

Furthermore, as previously described in *Section 1.3.2*, Sanna et al. (2004) demonstrated that ethanol exposure leads to an increase in mIPSC amplitude and a prolongation of mIPSC decay, which was not present in animals pre-treated with finasteride, suggesting a link between ethanol, neurosteroids and $GABA_A$ receptors.

The aim of this chapter is to explore the functional effect(s) of ethanol on neurosteroid insensitive α2- and α4-$GABA_A$ receptors.

To achieve this, we used electrophysiological recordings in dentate gyrus granule cells from these animals to examine inhibitory transmission, both phasic and tonic currents. We chose to study the dentate gyrus because of its implication in alcohol dependence and because both α2 and α4 subunits are highly expressed in that region. We hypothesised that α2-$GABA_A$Rs would contribute more to phasic currents, whereas α4-$GABA_A$Rs would participate in tonic currents. However, given the subunit switch and differential localisation of receptors induced by ethanol exposure described earlier, we assessed both types of GABA currents in both animal lines. Slices were treated with 100 mM ethanol to evaluate the effect of the knock-in mutation on sensitivity to acute exposure to ethanol. Chronic effects of ethanol on inhibitory neurotransmission were also investigated by recording from animals that took part in the intermittent two bottle choice paradigm.

4.2 Results

4.2.1 Acute ethanol effects in $\alpha4^{Q246M}$ brain slices

We examined the effects of ethanol on $GABA_AR$-mediated mIPSCs in dentate gyrus granule cells in ethanol-naïve wild type ($\alpha4^{Q/Q}$) and mutant homozygous ($\alpha4^{M/M}$) mice. Miniature IPSCs were recorded in the presence of 500 nM tetrodotoxin (to block network activity) and 2 mM kynurenic acid (to block excitatory transmission) at a holding potential of -60 mV. During paired (control, drug) recordings, mIPSCs were recorded for 10-20 minutes before (baseline/control conditions), for 30 min during, and for 10-15 minutes after bath application of 100 mM ethanol (*Figure 4.2 A* and *B*). If recordings were stable, 50 µM bicuculline was applied at the end of the experiment.

The mean ± SEM values and statistical analysis results of paired experiments of this section are contained in *Table 4.3* (amplitude), *Table 4.4* (frequency), *Table 4.5* (weighted decay time), *Table 4.6* (rise time), *Table 4.7* (charge transfer), *Table 4.8* (% changes in mIPSC parameters), *Table 4.9* (RMS noise), *Table 4.10* (% RMS noise change), *Table 4.11* (RMS noise statistics) and *Table 4.13* (mIPSC parameter statistics) in *Section 4.5*.

Two-way RM ANOVA detected a significant main effect of treatment; ethanol increased mIPSC amplitude (pA) in a time-dependent manner across both genotypes (*Figure 4.2 C* and *D, Table 4.13,* page 199). Dividing the 30-minute ethanol application into three 10-minute-long epochs, revealed that there was no increase in amplitude in the first 10 minutes of ethanol application for either wild type or homozygous mutant mice. However, ethanol induced a significant increase between 10-20 and 20-30 minutes in both genotypes. The ethanol-induced increase in mIPSC amplitude was reversed 10 minutes after drug washout (wt: control vs wash: 1.0 ± 5.0 %, P = 0.936, n = 4; hom: control vs wash: 3.5 ± 5.3 %, P = 0.997, n = 6). There was no observable difference in mIPSC amplitude between wild type and mutant cells at any time point.

Application of ethanol did not affect mIPSC frequency (Hz) of mutant cells (*Figure 4.2 E* and *F, Table 4.4*, page 190). However, in wild type cells, it led to an increase in frequency between 20-30 minutes of ethanol exposure (*Figure 4.2 E* and *F, Table 4.4*, page 190). This effect disappeared after 10 minutes of wash (wt: control vs wash: 8.3 ± 13.9 %, P = 0.964, n = 4). However, post-hoc tests suggested that there were no significant differences in mIPSC frequency between $\alpha4^{Q/Q}$ and $\alpha4^{M/M}$ DGGCs at any time point.

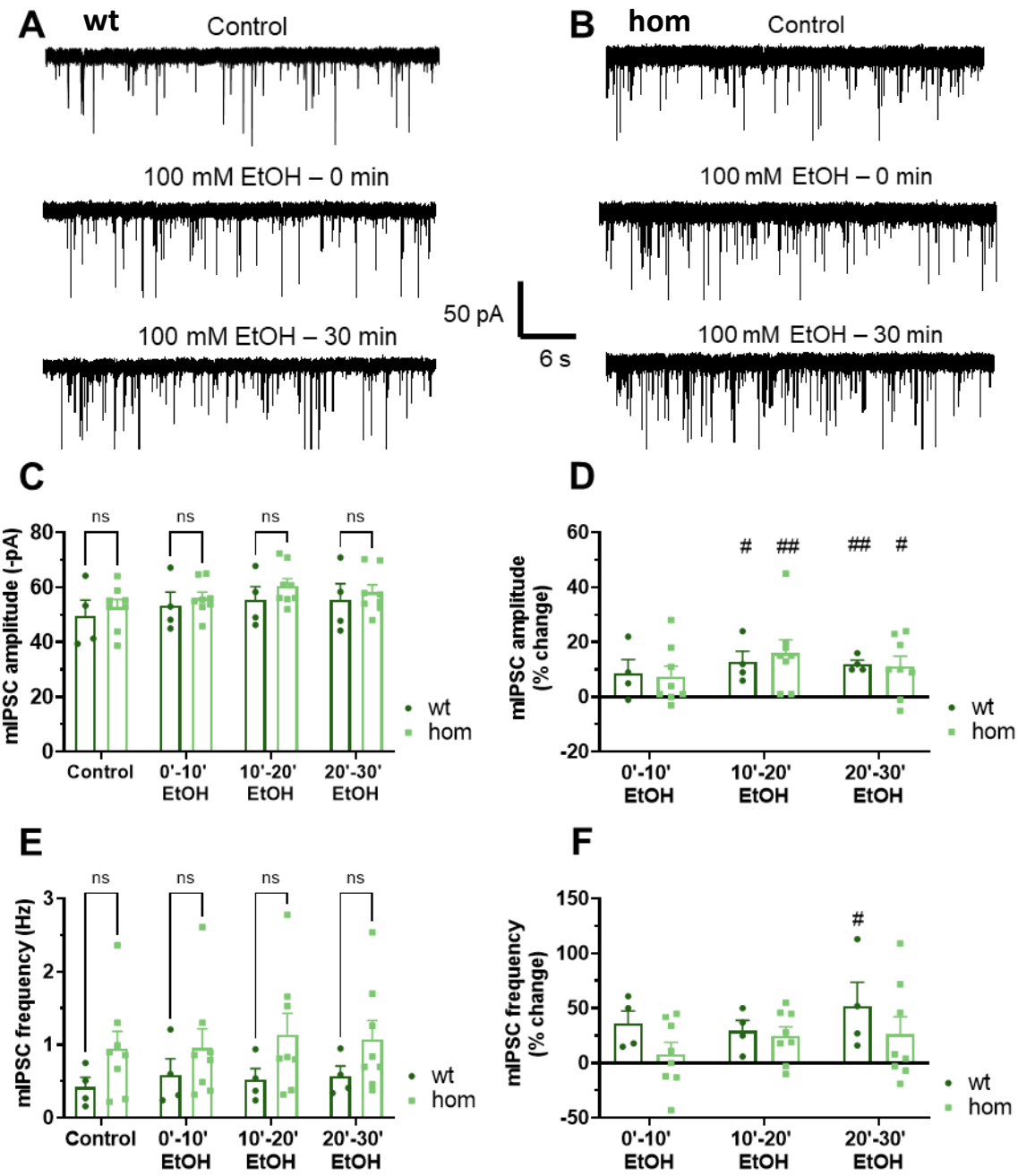


Figure 4.2 Modulation of $GABA_A$R-mediated mIPSC amplitude and frequency by ethanol in ethanol naïve wild type and homozygous α4^{Q246M} mutant animals

A and ***B***, Representative mIPSC recordings from wild type and homozygous mutant α4^{Q246M} mice before (control), during the initial 3 min (time 0) and at the end of a 30 min bath application of 100 mM ethanol (30 min), respectively. ***C,*** Mean mIPSC amplitudes recorded during bath application of 100 mM ethanol. ***D***, Percentage change in mean mIPSC amplitude induced in the presence of 100 mM ethanol. ***E,*** Mean mIPSC frequencies recorded at various times during bath application of 100 mM ethanol. ***F,*** Percentage change in mean mIPSC frequency upon exposure to 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C* and *E*) and with comparisons using Dunnett's test (to compare the effect of ethanol treatment: *D* and *F*; # *p < 0.05, ## p < 0.01;* where # is missing it indicates non-significance). Wild type: n = 4; hom: n = 8.

Two-way RM ANOVA of mIPSC weighted decay times (τ_w; ms) revealed a significant main effect of treatment, with τ_w increasing upon ethanol exposure across both wild type and mutant mice (*Figure 4.3 A, B, C* and *D, Table 4.13,* page 199). The increase occurred during the initial minutes of perfusion and stayed constant throughout the duration of the whole experiment for mutant animals, whereas the increase almost disappeared by the end of drug application for wild type mice. There were no detectable differences between the two genotypes.

Ethanol treatment did not influence mIPSC rise times (ms) in either wild type or mutant cells (*Figure 4.3 E and F*). Moreover, rise times were unchanged between $\alpha4^{Q/Q}$ and $\alpha4^{M/M}$ animals.

Two-way RM ANOVA detected a significant effect of treatment on mIPSC charge transfer. (*Figure 4.3 G* and *H, Table 4.13*, page 199). Both genotypes showed an increase in charge transfer during drug application, however, statistically it was significant only in the initial 10 minutes for mutant, and last 10 minutes for wild type cells. An increase in charge transfer upon ethanol application is not surprising, given the increase in other mIPSC parameters, such as amplitude, frequency and decay. All of these factors together lead to increased inhibition. However, we did not observe any differences between wild type and mutant mice.

Variations in root mean square (RMS) noise have been utilised to indicate changes in tonic $GABA_A$ receptor-mediated conductance. We measured RMS noise under control conditions, during ethanol application, drug wash-out and upon bicuculline treatment to examine whether continuous application of ethanol increases tonic currents (*Figure 4.4 A, B* and *C*). Ethanol did not influence RMS noise in either $\alpha4^{Q/Q}$ or $\alpha4^{M/M}$ cells compared to control. Bicuculline significantly decreased RMS noise in both genotypes compared to control, indicating the presence of GABAergic tonic currents. Two-way RM ANOVA showed a significant treatment by genotype interaction, however, post-hoc analysis did not detect any observable changes between genotypes.

These results show that ethanol treatment leads to an increase in mIPSC amplitude, weighted decay times and charge transfer, however, the lack of difference between genotypes suggests that these effects are likely not mediated by neurosteroids acting via α4-$GABA_A$Rs in DGGCs. The effect on mIPSC frequency potentially indicates the involvement of neurosteroid modulation of α4-type receptors.

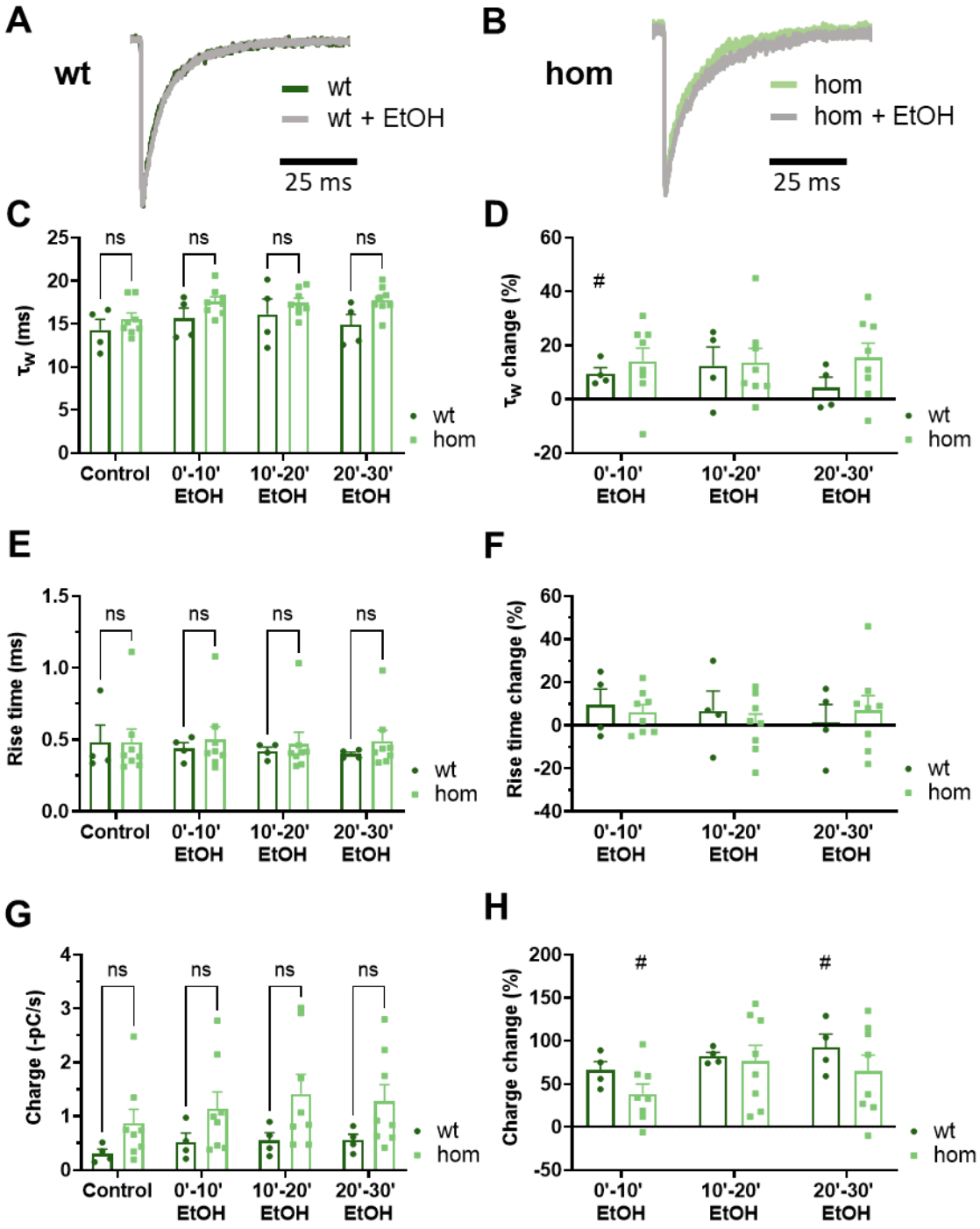


Figure 4.3 Modulation of $GABA_AR$-mediated mIPSC kinetics by ethanol in ethanol naive wild type and homozygous $\alpha4^{Q246M}$ mutant animals

A and ***B***, Representative mean mIPSC waveform from wild type and homozygous mutant $\alpha4^{Q246M}$ mice before (control) and at the end of a 30 min bath application of 100 mM ethanol (30 min), respectively. ***C, E***, and ***G***, Mean mIPSC weighted tau decay (ms; ***C***), mean mIPSC rise time (ms; ***E***) and mean mIPSC charge transfer (-pC/s; ***G***) recorded during bath application of 100 mM ethanol, respectively. ***D***, ***F*** and ***H***, Percentage change in mean mIPSC weighted tau (***D***), mean mIPSC rise time (***F***) and mean mIPSC charge transfer (***H***) induced in the presence of 100 mM ethanol, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C, E* and *G*) and with comparisons using Dunnett's test (to compare the effect of ethanol treatment: *D, F* and *H*; *# $p < 0.05$;* where # is missing it indicates non-significance). Wild type: n = 4; hom: n = 8.

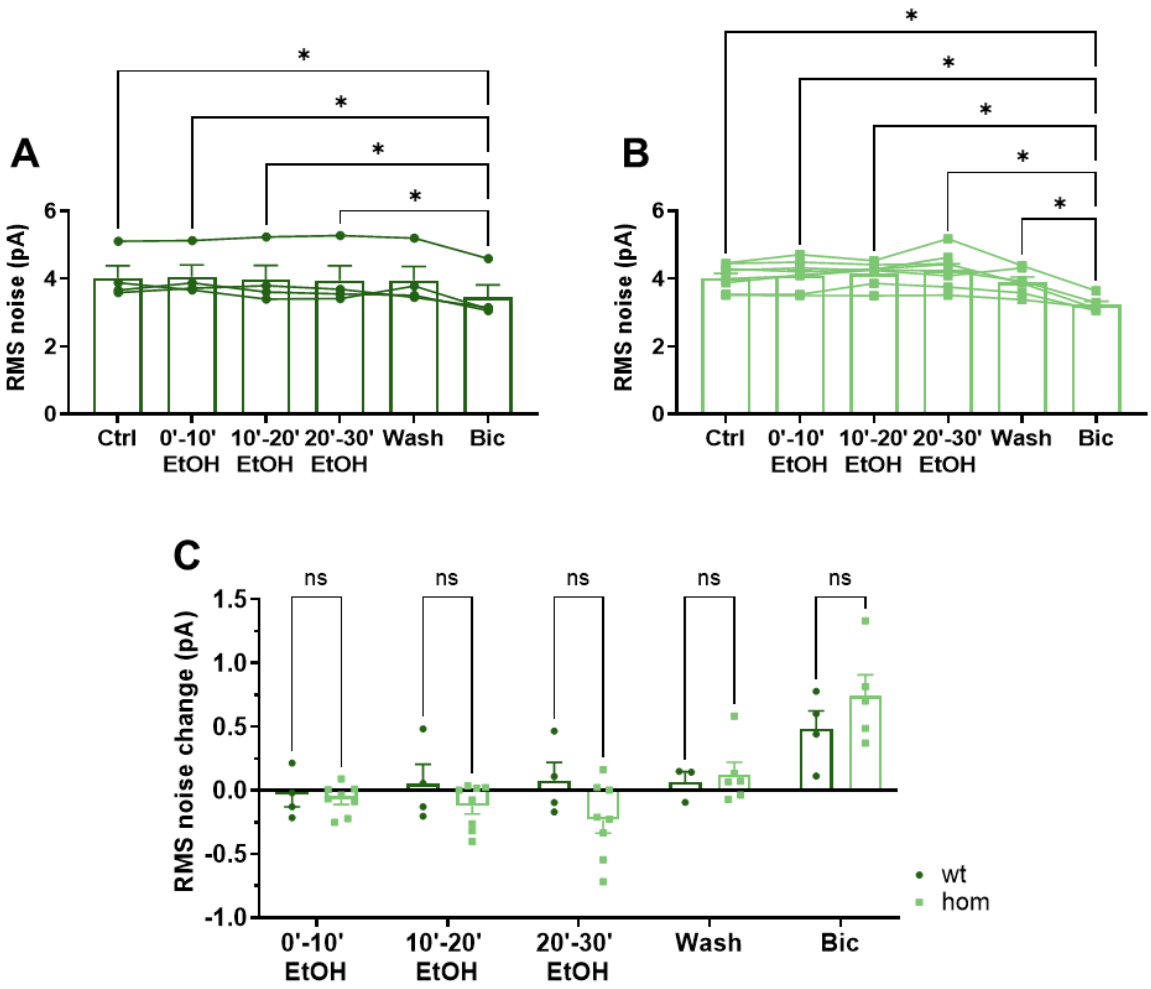


Figure 4.4 Modulation of tonic current (measured by RMS noise) by ethanol in ethanol naive wild type and homozygous α4^{Q246M} mutant animals

A and ***B*,** Mean RMS noise (pA) before (control; Ctrl), during 30 min bath application of 100 mM ethanol, during ethanol washout and during application of 50 µM bicuculline (Bic) in wild type (***A***) and homozygous mutant (***B***) mice. ***C,*** Mean RMS noise change (pA) induced in the presence of 100 mM ethanol and 50 µM bicuculline compared to control. Data are shown as mean ± SEM. Statistical tests used are one-way repeated measures ANOVA with comparisons using Tukey's test (to compare the effect of treatment: *A* and *B*) and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C*). Statistical significance indicated as * $p < 0.05$; where * is missing it indicates non-significance. Wild type: n = 4; hom: n = 5.

The paired recordings presented technical challenges and had a low success rate due to high cell mortality; therefore, we also conducted unpaired recordings. Additionally, unpaired recordings allowed us to study tonic currents in more detail. In these experiments, hippocampal slices were divided into two groups: control and ethanol-treated (*Figure 4.5 A, B, C* and *D*). Ethanol-treated slices were incubated for at least 30 min in 100 mM ethanol prior to recording. Experiments were completed by the bath application of 50 µM bicuculline to allow absolute GABA-mediated tonic currents to be measured.

The mean ± SEM values and statistical analysis results of these unpaired experiments are contained in *Table 4.17* (mIPSC parameters), *Table 4.18* (tonic current) and *Table 4.19* (statistics).

In contrast to paired recordings, we did not observe any differences in mIPSC amplitude between control and ethanol-treated cells in either genotype (*Figure 4.5 E*). Moreover, mIPSC amplitudes were unchanged between wild type and mutant cells.

Two-way ANOVA of mIPSC frequency revealed a significant treatment by genotype interaction. There was a significant increase in mIPSC frequency induced by ethanol in wild type animals (*Figure 4.5 F, Table 4.19,* page 202). However, we did not detect any differences between mutant cells. Ethanol-treated wild type cells had a significantly higher mIPSC frequency compared to EtOH-treated mutant cells. This result is in line with our findings from paired experiments.

We did not observe any differences in weighted decay times upon ethanol exposure in either $\alpha4^{Q/Q}$ or $\alpha4^{M/M}$ mice. (*Figure 4.6 A, B* and *C*). Additionally, decay times were unchanged between genotypes. We only observed a transient initial increase in τ_w for wild type animals during paired experiments; the increase reversed by the end of the 30-minute application of ethanol. Therefore, it is not surprising that we did not observe a difference between control and ethanol-treated wild type cells.

Similarly to paired recordings, bath application of ethanol did not impact upon mIPSC rise times (*Figure 4.6 D*). However, we detected a decrease in baseline mIPSC rise time of homozygous mutant cells compared to wild type.

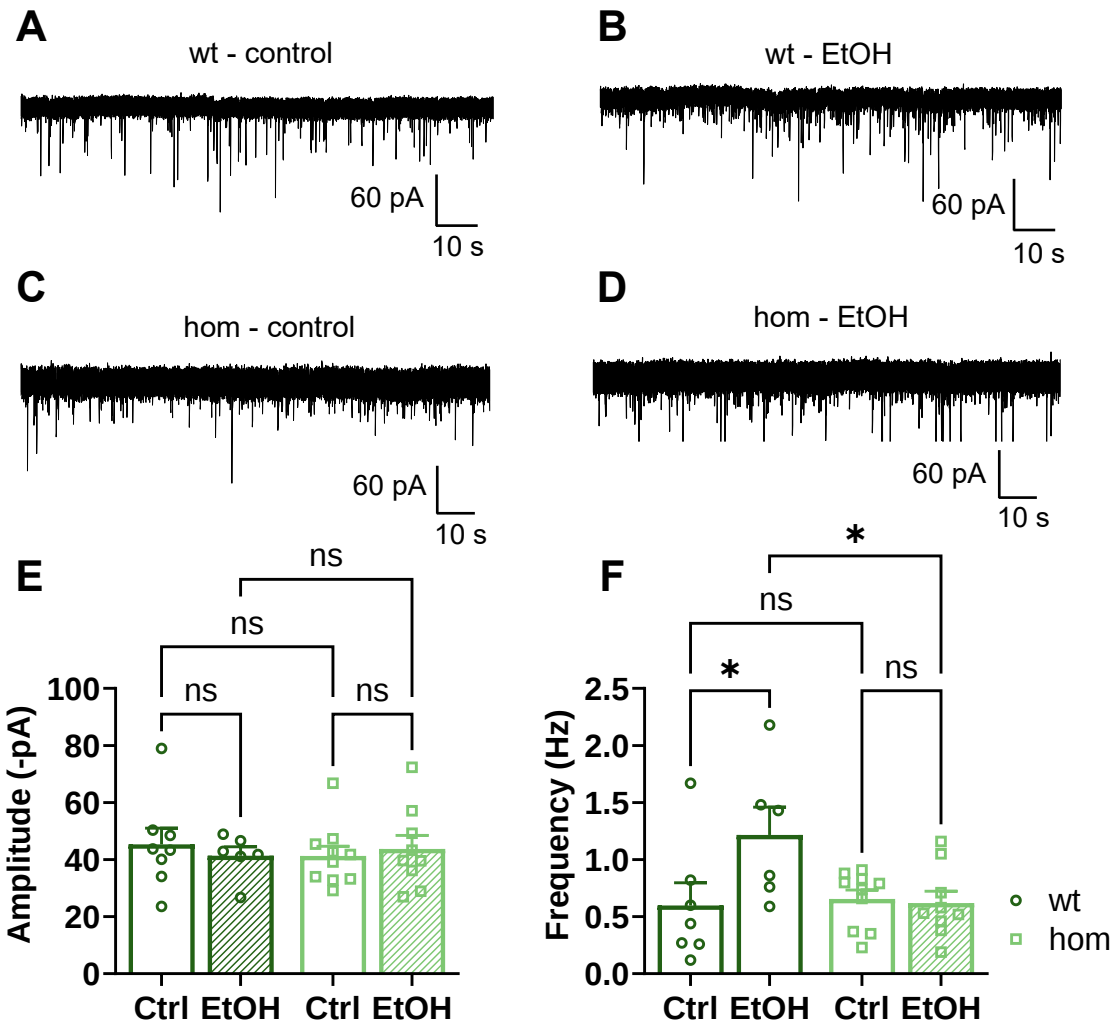


Figure 4.5 Modulation of $GABA_AR$-mediated mIPSC amplitude and frequency by ethanol in $\alpha4^{Q246M}$ animals – unpaired recordings

A and ***B***, Representative mIPSC recordings from wild type animals in control (**A**) and after > 30 min pre-incubation of 100 mM ethanol (**B**). ***C and D,*** Representative mIPSC recordings from homozygous $\alpha4^{Q246M}$ mutant animals in control (**C**) and after > 30 min bath application of 100 mM ethanol (**D**), respectively. ***E and F,*** Mean mIPSC amplitudes (**E**) and frequencies (**F**) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are two-way ANOVA with comparisons using uncorrected Fisher's LSD test (panels *E* and *F*). Statistical significance indicated as * $p < 0.05$. Wt: n = 8; wt + EtOH: n = 6; hom: n = 10; hom + EtOH: n = 9.

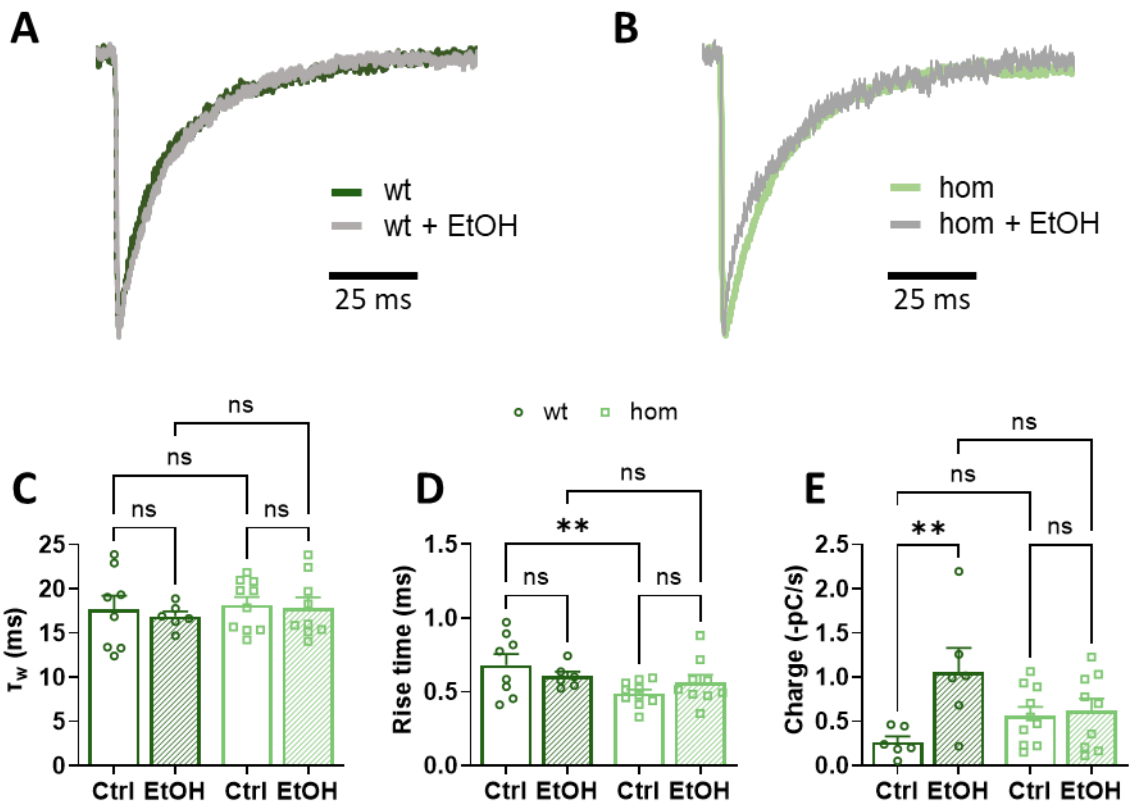


Figure 4.6 Modulation of $GABA_AR$-mediated mIPSC kinetics by ethanol in $\alpha4^{Q246M}$ animals – unpaired recordings

A and ***B***, Representative mean mIPSC waveform from wild type animals (**A**) and from homozygous $\alpha4^{Q246M}$ mutant (**B**) in control and after > 30 min pre-incubation of 100 mM ethanol. ***C, D***, and ***E***, Mean mIPSC weighted tau decay (ms; ***C***), mean mIPSC rise time (ms; ***D***) and mean mIPSC charge transfer (-pC/s; ***E***) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are two-way ANOVA with comparisons using uncorrected Fisher's LSD test (panels *C, D* and *E*). Statistical significance indicated as ** $p < 0.01$. Wt: n = 8; wt + EtOH: n = 6; hom: n = 10; hom + EtOH: n = 9.

Analysis of charge transfer revealed a significant effect of treatment and a significant interaction of treatment and genotype (*Figure 4.6 E, Table 4.19,* page 202). Ethanol significantly increased the charge transfer in wild type cells; this increase is mainly driven by the increase in frequency.

To study the effect of ethanol on tonic inhibitory currents (*Figure 4.7 A, B, C* and *D*), both the change in holding current (pA) and in baseline noise (RMS noise) were quantified. There were small but measurable tonic currents under both conditions in both genotypes, as revealed by the application of bicuculline (*Figure 4.7 E, F, G* and *H*). However, ethanol treatment did not influence the size of tonic GABA currents in either $\alpha4^{Q/Q}$ or $\alpha4^{M/M}$ animals. We did not find any changes in baseline tonic currents between the genotypes, which is in agreement with Minère (2019). Reflecting these observations, RMS noise analysis did not detect any change between genotypes and ethanol also had no impact (*Figure 4.8*).

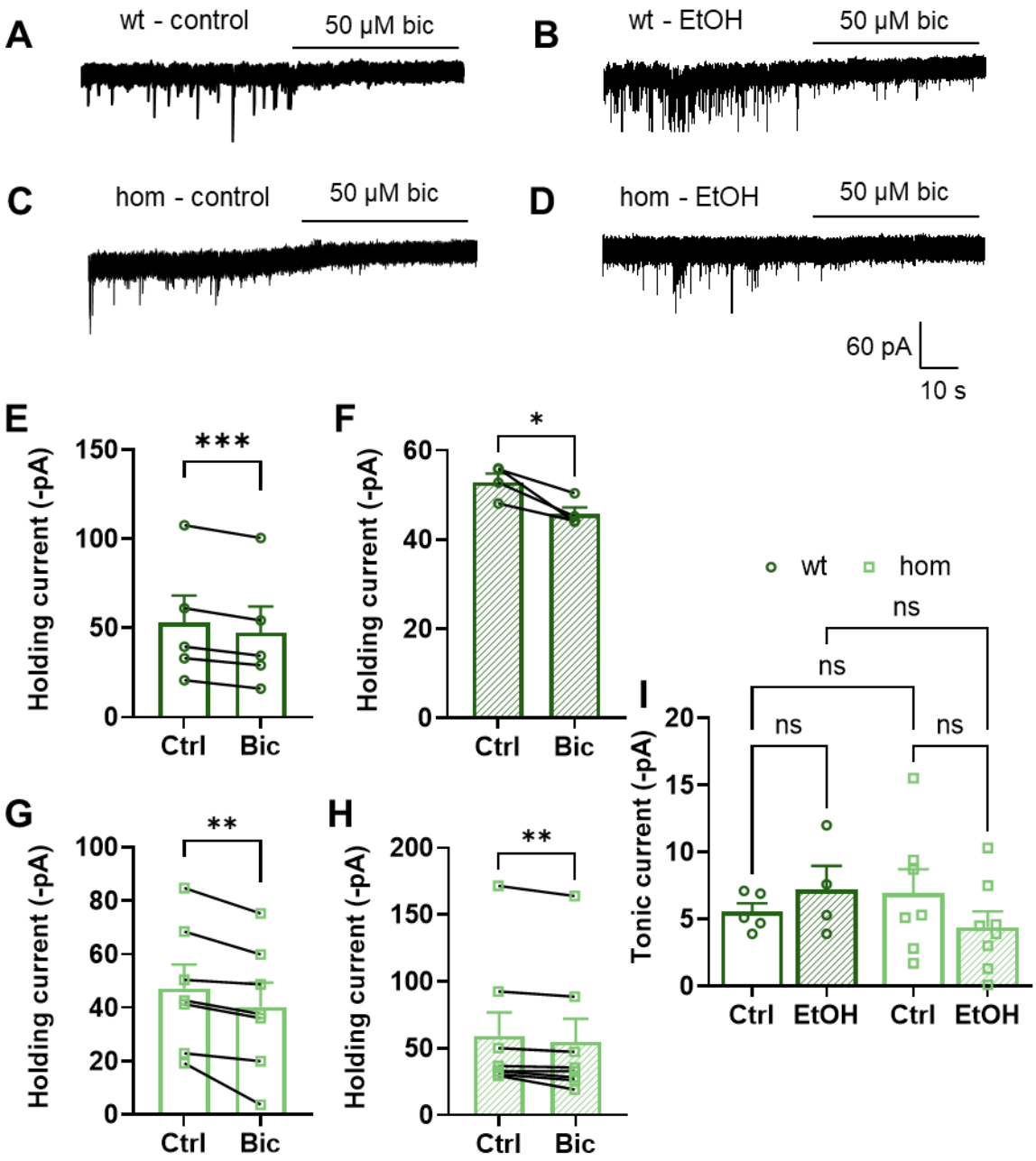


Figure 4.7 Modulation of $GABA_AR$-mediated tonic current by ethanol in $\alpha4^{Q246M}$ animals – unpaired recordings

A, B, C and ***D***, Representative traces showing mIPSC and tonic current block by 50 µM bicuculline (bic) from wild type animals under control conditions (**A**) and after 100mM ethanol application (**B**); and from homozygous $\alpha4^{Q246M}$ mutant mice in control (**C**) and after > 30 min pre-incubation of 100 mM ethanol (**D**). ***E, F***, ***G*** and ***H***, Mean holding current (-pA) before and application the application of 50 µM bic from wild type animals under control conditions (**E**) and after 100mM ethanol application (**F**); and from homozygous $\alpha4^{Q246M}$ mutant mice in control (**G**) and after > 30 min bath application of 100 mM ethanol (**H**). ***I,*** Mean tonic current (-pA) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are paired student's t test for panels *E, F, G* and *H*; two-way ANOVA with comparisons using uncorrected Fisher's LSD test for panel *I*. Statistical significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Wt: n = 5; wt + EtOH: n = 4; hom: n = 7; hom + EtOH: n = 8.

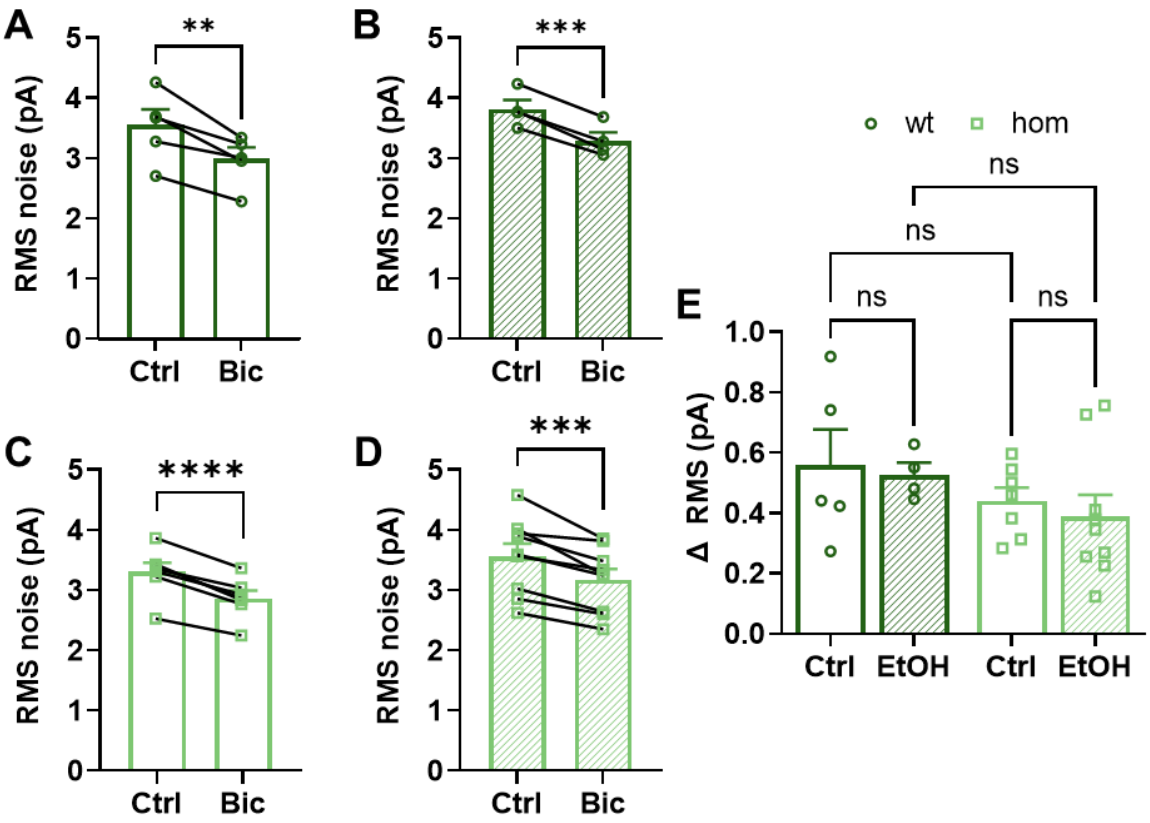


Figure 4.8 Modulation of $GABA_AR$-mediated tonic current (measured by RMS noise) by ethanol in $\alpha4^{Q246M}$ animals – unpaired recordings

A, B, ***C*** and ***D***, Mean RMS noise (pA) before and application the application of 50 µM bicuculline (bic) from wild type animals under control conditions (**A**) and after 100mM ethanol application (**B**); and from homozygous $\alpha4^{Q246M}$ mutant mice in control (**C**) and after > 30 min pre-incubation of 100 mM ethanol (**D**). ***E,*** Mean holding current change (-pA) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are paired student's t test for panels *A, B, C* and *D*; two-way ANOVA with comparisons using uncorrected Fisher's LSD test for panel *E*. Statistical significance indicated *** p < 0.01, *** p < 0.001, **** p < 0.0001.* Wt: n = 5; wt + EtOH: n = 4; hom: n = 7; hom + EtOH: n = 8.

4.2.2 Acute ethanol effects in α2^{Q241M} brain slices

We also investigated the impact of ethanol on $GABA_A$ receptor-mediated miniature IPSCs in dentate gyrus granule cells in ethanol-naïve α2$^{Q/Q}$ and α2$^{M/M}$ mice (*Figure 4.9 A* and *B*) using the same paired protocol in *Section 4.2.1*. The mean ± SEM values and statistical analysis results of paired experiments in the section are contained in *Table 4.3* (amplitude), *Table 4.4* (frequency), *Table 4.5* (weighted decay time), *Table 4.6* (rise time), *Table 4.7* (charge transfer), *Table 4.8* (% changes in mIPSC parameters), *Table 4.9* (RMS noise), *Table 4.10* (% RMS noise change), *Table 4.12* (RMS noise statistics) and *Table 4.14* (mIPSC parameter statistics) in *Section 4.5*.

Two-way RM ANOVA detected a significant main effect of treatment; ethanol increased mIPSC amplitude across both genotypes (*Figure 4.9 C and D, Table 4.14,* page 199). Percentage increases seen in mutant mice were larger compared to wild type, however, post-hoc comparisons revealed no statistical significance between genotypes. Ethanol had a significant effect on mIPSC amplitude in homozygous animals between 20 and 30 minutes compared to control. This effect was reversed during drug washout (EtOH 20-30 min: 21 ± 5 %; wash: 4 ± 4 %).

Analysis of mIPSC frequency revealed a significant effect of treatment; acute ethanol exposure resulted in an increase in frequency in both α2$^{Q/Q}$ and α2$^{M/M}$ mice (*Figure 4.9 E* and *F*). Two-way RM ANOVA also showed a significant interaction between treatment and genotype (*Table 4.14,* page 199); homozygous mutant cells exhibited a larger increase compared to wild type, however, post-hoc tests suggested that there were no significant differences between genotypes at any time point (*Table 4.4*, page 190). Wild type cells displayed the largest increase in the initial 10 min of ethanol application, whereas mutants had the most significant effect between 20 and 30 minutes. Drug washout partially reversed the effects induced by ethanol (wt: EtOH 20-30 min: 17 ± 16 % and wash: 6 ± 15 %; hom: EtOH 20-30 min: 101 ± 43 % and wash: 39 ± 15 %).

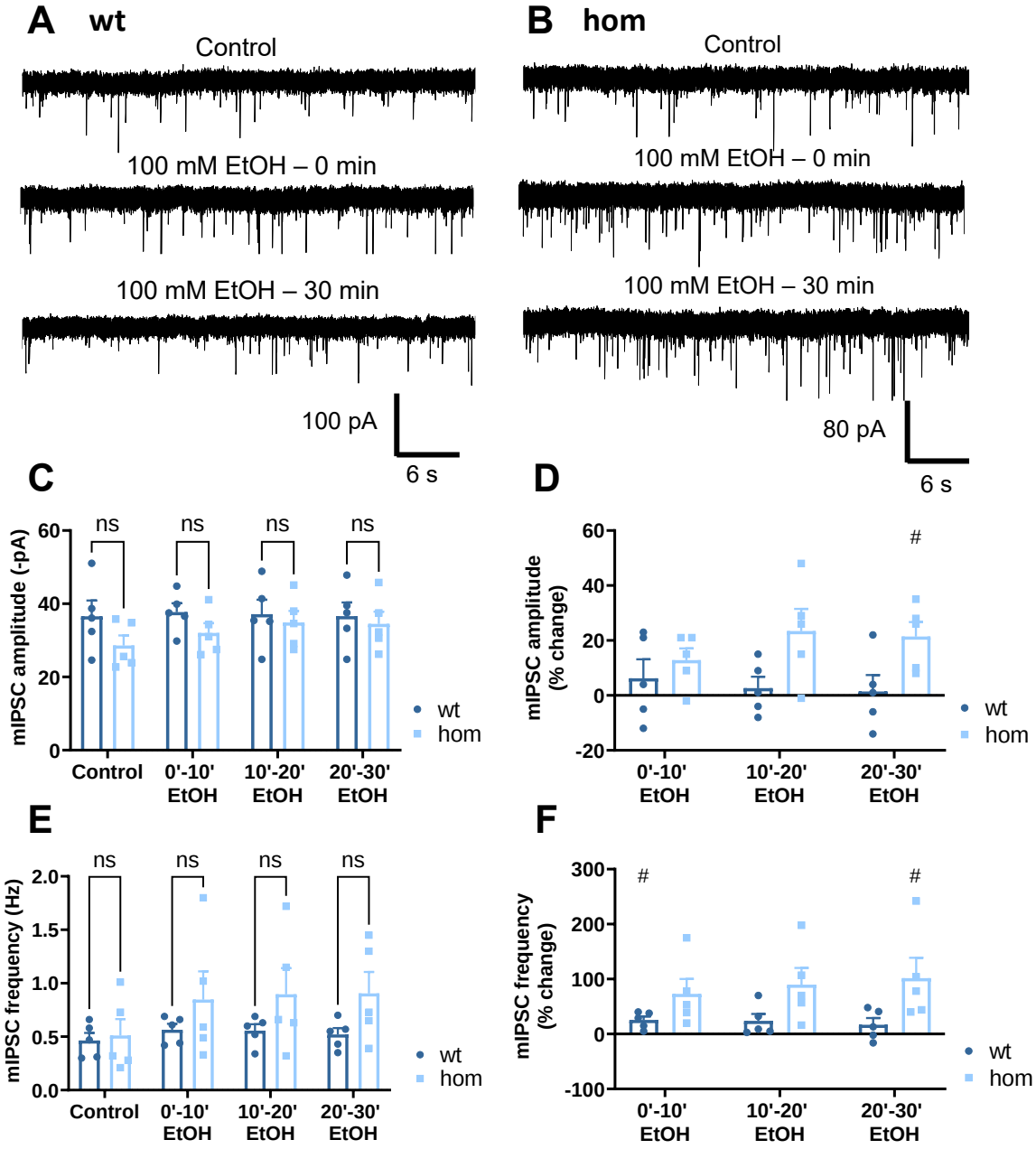


Figure 4.9 Modulation of $GABA_AR$-mediated mIPSC amplitude and frequency by ethanol in ethanol naïve wild type and homozygous $\alpha2^{Q241M}$ mutant animals

A and ***B***, Representative mIPSC recordings from wild type and homozygous mutant $\alpha2^{Q241M}$ mice before (control), during the initial 3 min (time 0) and at the end of a 30 min bath application of 100 mM ethanol (30 min), and 10 min after drug washout, respectively. ***C,*** Mean mIPSC amplitudes recorded during bath application of 100 mM ethanol. ***D***, Percentage change in mean mIPSC amplitude induced in the presence of 100 mM ethanol. ***E,*** Mean mIPSC frequencies recorded at various times during bath application of 100 mM ethanol. ***F,*** Percentage change in mean mIPSC frequency upon exposure to 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences (wt vs hom): panels *C* and *E*) and with comparisons using Dunnett's test (to compare the effect of ethanol treatment (control vs EtOH, symbol #): panels *D* and *F*; # $p < 0.05$; where # is missing it indicates non-significance). Wild type: n = 5; hom: n = 5.

Application of ethanol resulted in the prolongation of mIPSCs across both genotypes at every time point (*Figure 4.10 A, B, C* and *D, Table 4.14, page 199*), however, the extent of this increase in mIPSC τ_w was larger in wild type cells compared to mutants (*Table 4.8*, page 194); two-way RM ANOVA showed a significant effect of genotype, yet post-hoc multiple comparisons test indicated no differences between genotypes at any time point. Despite the effect being more prominent in $\alpha2^{Q/Q}$ animals, statistically only $\alpha2^{M/M}$ cells showed a significant increase during the initial 10 minutes of ethanol treatment. This prolongation of mIPSCs was reversed during the final 20 minutes of ethanol application in mutant cells.

Ethanol treatment had no effect on mIPSC rise times in both wild type and mutant cells (*Figure 4.10 E* and *F*). Furthermore, there were no differences in rise time between $\alpha2^{Q/Q}$ and $\alpha2^{M/M}$ animals.

Two-way RM ANOVA detected a significant effect of treatment on mIPSC charge transfer. (*Figure 4.10 G* and *H, Table 4.14*, page 199). Both wild type and mutant cells exhibited higher mIPSC charge transfers, however, statistically it was only significant for mutant cells in the last 20 minutes of recording. This increase in charge transfer is mainly driven by the increase in mIPSC amplitude and frequency. There were no detectable changes between genotypes.

Ethanol had no impact of RMS noise in either wild type or mutant animals (*Figure 4.11 A, B* and *C*). Bicuculline significantly reduced RMS noise in both genotypes, indicating the presence of GABAergic tonic currents. Wild type and mutant cells did not differ from each other.

These results show that ethanol treatment leads to an increase in mIPSC amplitude and charge transfer, however, the lack of difference between genotypes suggests that these effects are likely not mediated by neurosteroids acting via α2-$GABA_A$Rs in DGGCs. The effect on mIPSC frequency and weighted decay time potentially indicate the involvement of neurosteroid modulation of α2-type receptors. Frequency data suggests that removing neurosteroid sensitivity from α2-containing $GABA_A$ receptors enhances ethanol's effects, whereas the prolongation of mIPSCs is reduced in slices where α2-$GABA_A$Rs are rendered insensitive to the modulation by neurosteroids.

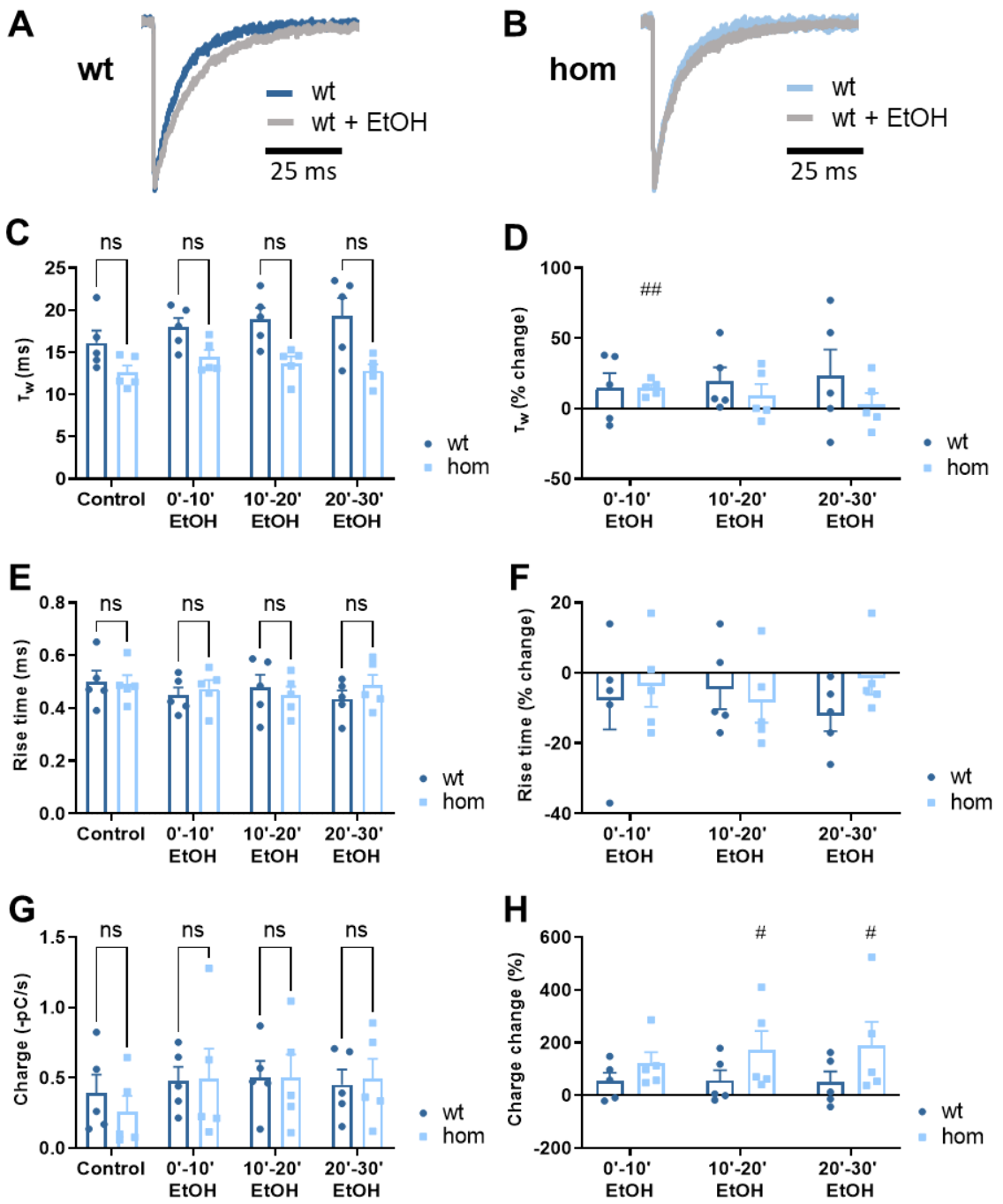


Figure 4.10 Modulation of $GABA_AR$-mediated mIPSC kinetics by ethanol in ethanol naive wild type and homozygous $\alpha2^{Q241M}$ mutant animals

A and ***B***, Representative mean mIPSC waveform from wild type and homozygous mutant $\alpha4^{Q246M}$ mice before (control) and at the end of a 30 min bath application of 100 mM ethanol (30 min), respectively. ***C, E***, and ***G***, Mean mIPSC weighted tau decay (ms; ***C***), mean mIPSC rise time (ms; ***E***) and mean mIPSC charge transfer (-pC/s; ***G***) recorded during bath application of 100 mM ethanol, respectively. ***D***, ***F*** and ***H***, Percentage change in mean mIPSC weighted tau (***D***), rise time (***F***) and charge transfer (***H***) induced in the presence of 100 mM ethanol, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences (wt vs hom): panels *C, E* and *G*) and with comparisons using Dunnett's test (to compare the effect of ethanol treatment (control vs EtOH): panels *D, F* and *H*; # $p < 0.05$ and ## $p < 0.01$; where # is missing it indicates non-significance). Wt: n = 4; hom: n = 8.

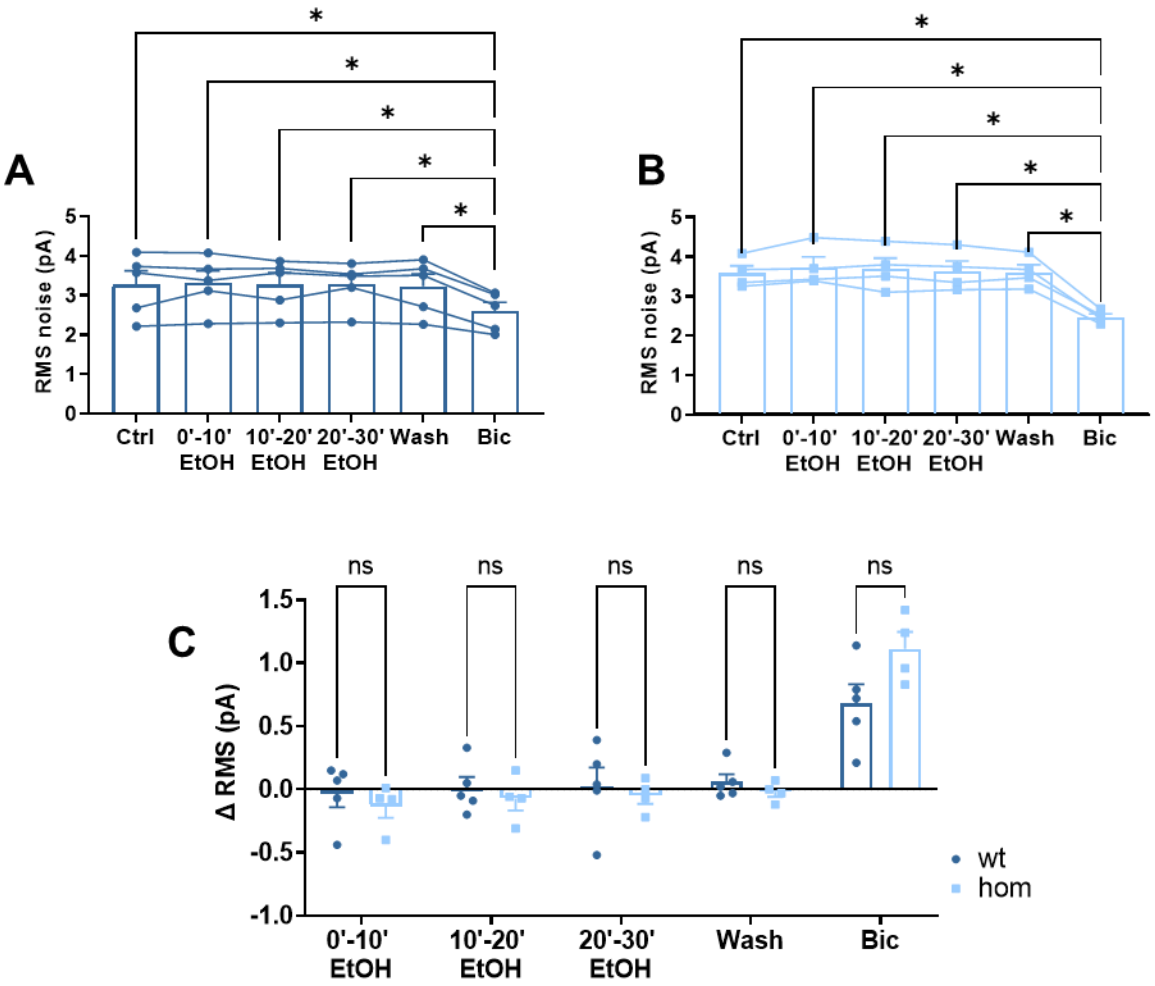


Figure 4.11 Modulation of tonic current (measured by RMS noise) by ethanol in naïve wild type and homozygous α2^{Q241M} mutant animals

A and ***B*,** Mean RMS noise (pA) before (control; Ctrl), during 30 min bath application of 100 mM ethanol, during ethanol washout and during application of 50 µM bicuculline (Bic) in wild type (***A***) and homozygous mutant (***B***) mice. ***C,*** Mean RMS noise change (pA) induced in the presence of 100 mM ethanol and 50 µM bicuculline compared to control. Data are shown as mean ± SEM. Statistical tests used are one-way repeated measures ANOVA with comparisons using Tukey's test (to compare the effect of treatment: *A* and *B*) and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C*). Statistical significance indicated as * $p < 0.05$; where * is missing it indicates non-significance. Wild type: n = 5; hom: n = 4.

Similar to the experiments conducted on the $\alpha4^{Q246M}$ mouse line, we also performed unpaired recordings on $\alpha2^{Q241M}$ mice to investigate the effects of ethanol on phasic and tonic currents (*Figure 4.12 A, B, C* and *D*). The mean ± SEM values and statistical analysis results of these unpaired experiments are contained in *Table 4.17* (mIPSC parameters), *Table 4.19* (tonic current) and *Table 4.20* (statistics).

Contrary to paired recordings, we did not observe any changes in mIPSC amplitude upon ethanol exposure in either wild type or mutant animals (*Figure 4.12 E*). Furthermore, mIPSC amplitudes were unchanged between genotypes.

Ethanol had no statistically significant effect on mIPSC frequency in either $\alpha2^{Q/Q}$ or $\alpha2^{M/M}$ mice (*Figure 4.12 F*). We observed a trend for increased frequency in ethanol-treated cells across both genotypes. These results in part mirror our findings from the paired recordings, with homozygous mutants (53 %) exhibiting a larger increase in mIPSC frequency compared to wild types (36 %).

Surprisingly, ethanol treatment had no impact on mIPSC decay in either genotype (*Figure 4.13 A, B* and *C*), in contrast to prolonging mIPSCs in wild type cells during paired recordings. The effect of ethanol was the same on mutant cells in both unpaired and paired experiments, i.e. no effect. We detected a baseline difference in mIPSC decay between $\alpha2^{Q/Q}$ and $\alpha2^{M/M}$ mice, with homozygous mutant animals showing faster decay kinetics (*Table 4.20*, page 203). This result is in agreement with the findings reported by Durkin et al. (2018).

Furthermore, ethanol exposure did not influence mIPSC rise time or charge transfer in either genotype, and there were no baseline differences between wild type and mutant cells (*Figure 4.13 D* and *E*).

Tonic currents were present in both wild type and mutant brain slices, evident by the holding current shifts produced by bicuculline (*Figure 4.14*). Ethanol did not potentiate these currents in $\alpha2^{Q/Q}$ or $\alpha2^{M/M}$ animals. RMS noise analysis also reflected these findings (*Figure 4.15*). However, we observed a reduction in the bicuculline-evoked shift in RMS control for mutant cells compared to wild type (*Figure 4.15 I*).

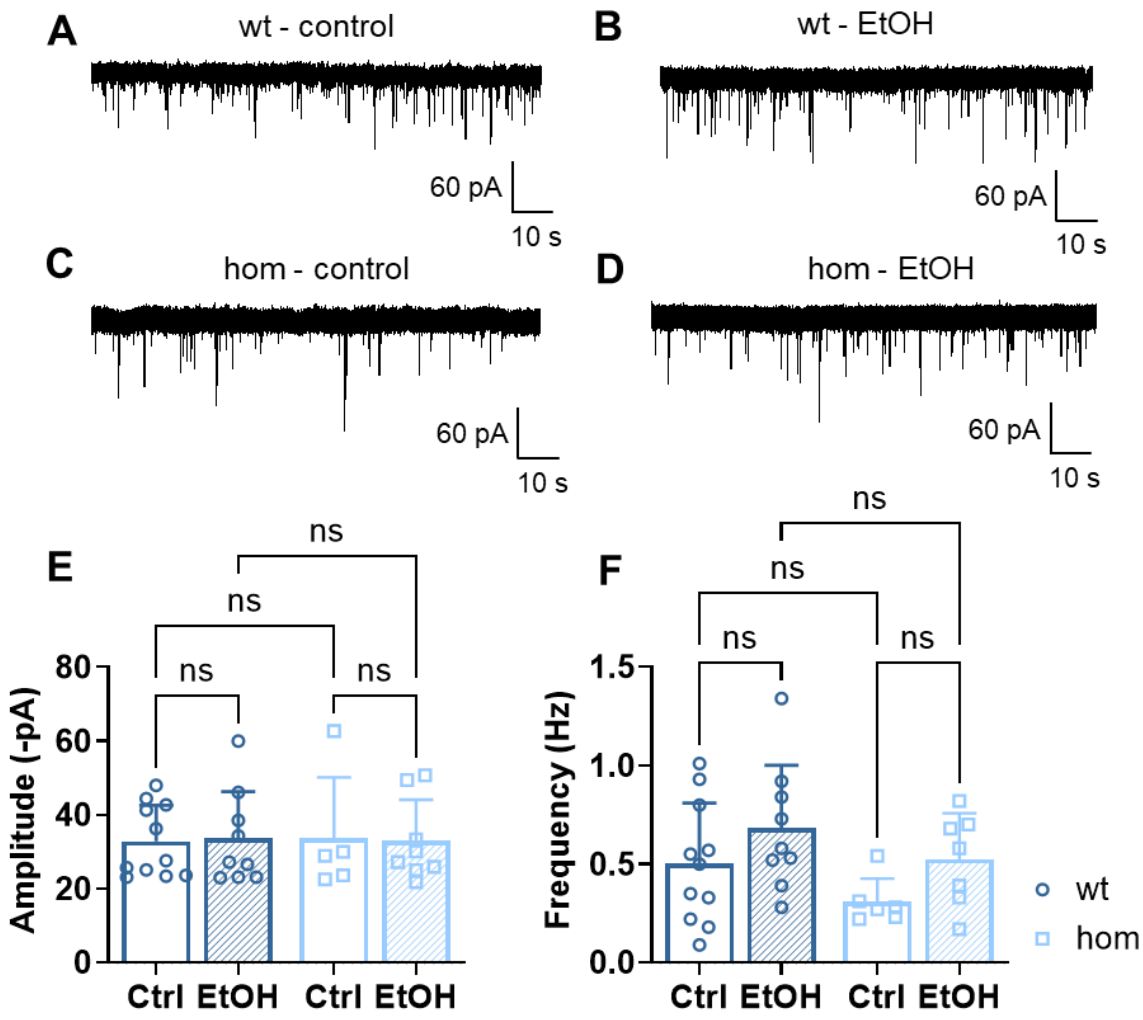


Figure 4.12 Modulation of $GABA_AR$-mediated mIPSC amplitude and frequency by ethanol in $\alpha2^{Q241M}$ animals – unpaired recordings

A and ***B***, Representative mIPSC recordings from wild type animals in control (**A**) and after > 30 min pre-incubation of 100 mM ethanol (**B**). ***C and D,*** Representative mIPSC recordings from homozygous $\alpha2^{Q241M}$ mutant animals in control (**C**) and after > 30 min bath application of 100 mM ethanol (**D**), respectively. ***E and F,*** Mean mIPSC amplitudes (**E**) and frequencies (**F**) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are two-way ANOVA with comparisons using uncorrected Fisher's LSD test (panels *E* and *F*). Statistical significance indicated as * $p < 0.05$. Wt: n = 8; wt + EtOH: n = 9; hom: n = 7; hom + EtOH: n = 7.

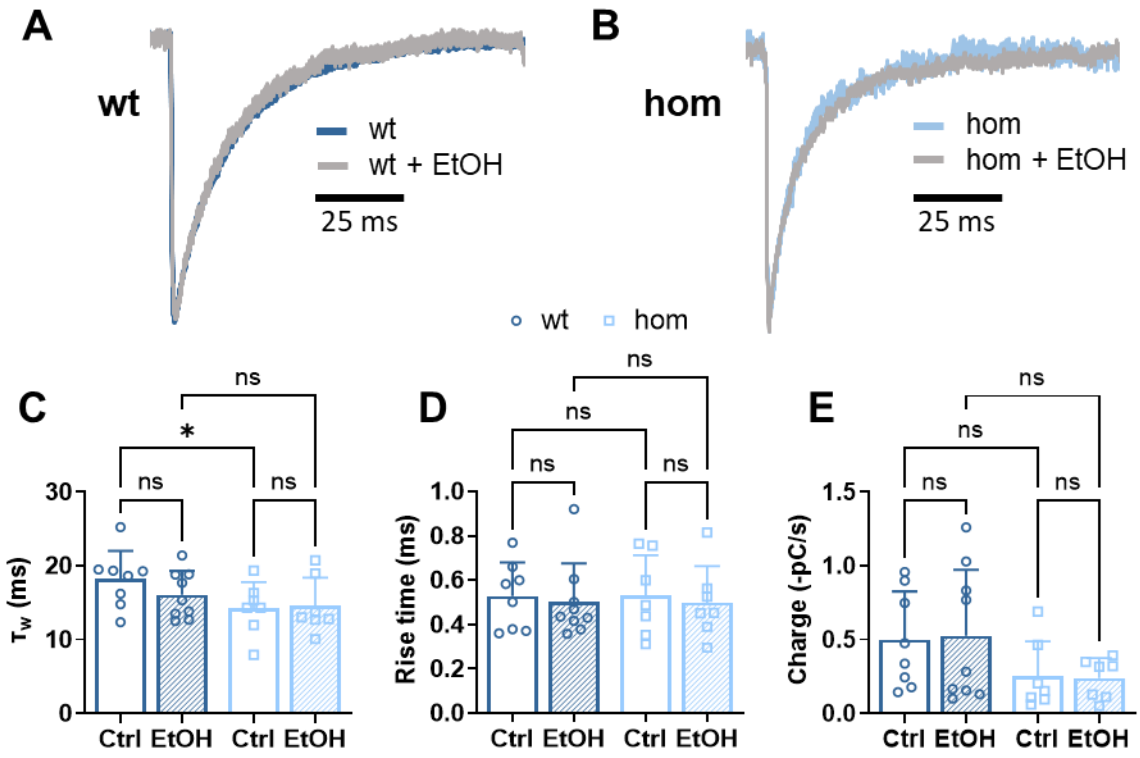


Figure 4.13 Modulation of $GABA_AR$-mediated mIPSC kinetics by ethanol in $\alpha2^{Q241M}$ animals – unpaired recordings

A and ***B***, Representative mean mIPSC waveform from wild type animals (**A**) and from homozygous $\alpha2^{Q241M}$ mutant (**B**) in control and after > 30 min bath application of 100 mM ethanol. ***C, D***, and ***E***, Mean mIPSC weighted tau decay (ms; ***C***), mean mIPSC rise time (ms; ***D***) and mean mIPSC charge transfer (-pC/s; ***E***) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are two-way ANOVA with comparisons using uncorrected Fisher's LSD test for panels *C, D* and *E*. Statistical significance indicated as * $p < 0.05$. Wt: n = 8; wt + EtOH: n = 9; hom: n = 7; hom + EtOH: n = 7.

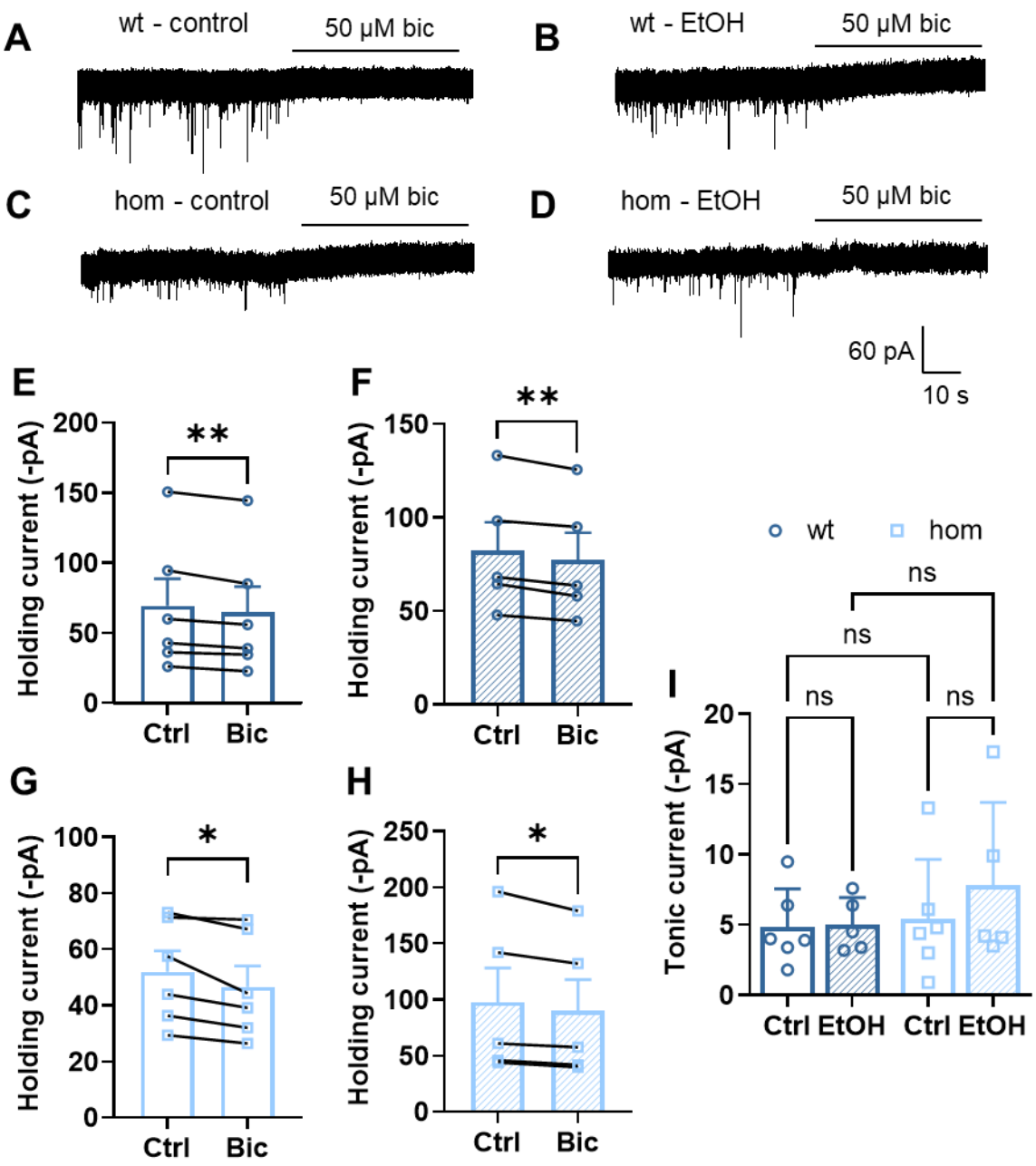


Figure 4.14 Modulation of $GABA_AR$-mediated tonic current by ethanol in $\alpha2^{Q241M}$ animals – unpaired recordings

A, B, C and ***D***, Representative traces showing mIPSC and tonic current block by 50 µM bicuculline (bic) from wild type animals under control conditions (**A**) and after 100mM ethanol application (**B**); and from homozygous $\alpha2^{Q241M}$ mutant mice in control (**C**) and after > 30 min bath application of 100 mM ethanol (**D**). ***E, F, G*** and ***H***, Mean holding current (-pA) before and application the application of 50 µM bic from wild type animals under control conditions (**E**) and after 100mM ethanol application (**F**); and from homozygous $\alpha2^{Q241M}$ mutant mice in control (**G**) and after > 30 min bath application of 100 mM ethanol (**H**). ***I,*** Mean tonic current (-pA) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are paired student's t test for panels *E, F, G* and *H*; two-way ANOVA with comparisons using uncorrected Fisher's LSD test for panel *I*. Statistical significance indicated as * $p < 0.05$ and ** $p < 0.01$. Wt: n = 6; wt + EtOH: n = 5; hom: n = 6; hom + EtOH: n = 5.

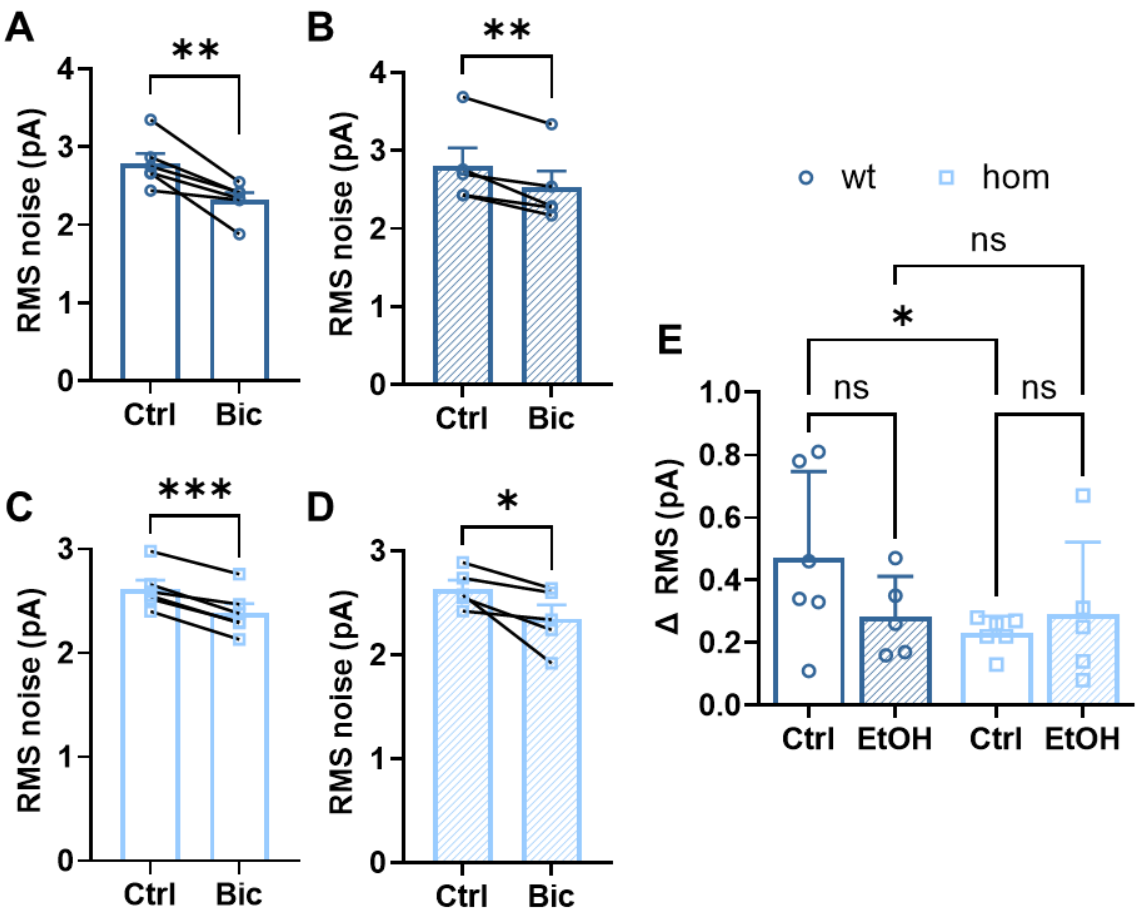


Figure 4.15 Modulation of $GABA_AR$-mediated tonic current (measured by RMS noise) by ethanol in $\alpha2^{Q241M}$ animals – unpaired recordings

A, B, ***C*** and ***D***, Mean RMS noise (pA) before and application the application of 50 µM bicuculline (bic) from wild type animals under control conditions (**A**) and after 100mM ethanol application (**B**); and from homozygous $\alpha2^{Q241M}$ mutant mice in control (**C**) and after > 30 min pre-incubation of 100 mM ethanol (**D**). ***E,*** Mean change (%) in RMS noise (-pA) recorded either under control conditions or during bath application of 100 mM ethanol. Data are shown as mean ± SEM. Statistical tests used are paired student's t test for panels *A, B, C* and *D*; two-way ANOVA with comparisons using uncorrected Fisher's LSD test for panel *E*. Statistical significance indicated as ** $p < 0.05$*, *** $p < 0.01$* and **** $p < 0.001$*. Wt: n = 6; wt + EtOH: n = 5; hom: n = 6; hom + EtOH: n = 5.

4.2.3 Chronic ethanol effects in $\alpha4^{Q246M}$ brain slices

To investigate the potential for inhibitory plasticity after voluntary consumption of alcohol, we recorded mIPSCs in DGGCs from mice that had participated in the two bottle choice experiment with ethanol. The mice were selected randomly. Slice recordings were made within 3 days after completion of the two bottle choice experiment and used the same experimental protocol as in the ethanol-naïve mice, with cells being exposed to 100 mM ethanol for 30 minutes after an initial period of control recording (*Figure 4.16 A* and *B*).

The mean ± SEM values and statistical analysis results of these experiments are contained in *Table 4.3* (amplitude), *Table 4.4* (frequency), *Table 4.5* (weighted decay time), *Table 4.6* (rise time), *Table 4.7* (charge transfer), *Table 4.8* (% changes in mIPSC parameters), *Table 4.9* (RMS noise), *Table 4.10* (% RMS noise change), *Table 4.11* (RMS noise statistics) and *Table 4.15* (mIPSC parameter statistics) in *Section 4.5*.

Analysis of mIPSC amplitudes recorded from $\alpha4^{Q/Q}$ and $\alpha4^{M/M}$ DGGCs showed a significant main effect of time, with amplitudes becoming larger during ethanol treatment (*Figure 4.16 C* and *D, Table 4.15,* page 200). The effects were evident from the start for the mutants and maximal at EtOH 10-20 min, and were slightly reduced in extent towards the end of ethanol perfusion. Wild type cells showed the largest increase between 20 and 30 minutes. There were no differences between the genotypes. Previous exposure to ethanol, during the two bottle choice experiment, did not affect mIPSC amplitudes in wild type cells, however, it led to a decrease in homozygous mutant cells (*Figure 4.2 C* and *Figure 4.16 C*).

Although two-way RM ANOVA detected a significant main effect of time on mIPSC frequency, no time point of ethanol perfusion differed appreciably from control in either wild type or mutant cells (*Figure 4.16 E* and *F*). We did not observe any differences in mIPSC frequency between the two genotypes. Participation in the two bottle choice experiment did not impact upon control mIPSC frequency of wild type mice. However, analysis of mutant data identified a main effect of previous ethanol exposure (F (1, 9) = 11.89, $p < 0.01$), leading to a decrease in control mIPSC frequency. However, mIPSC frequency change induced by ethanol was higher in two bottle choice mice compared to naïve animals (*Figure 4.2 F* and *Figure 4.16 F*).

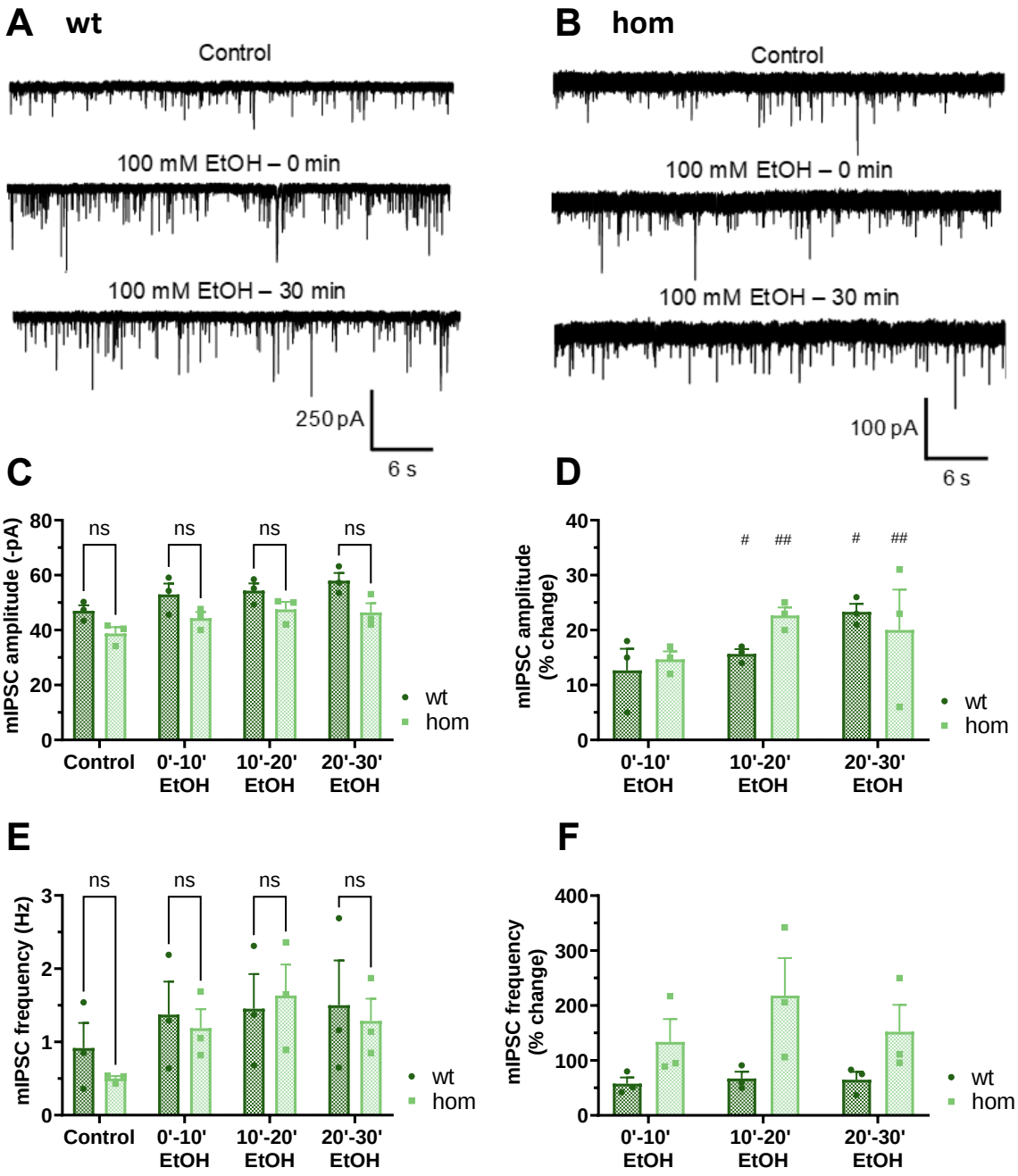


Figure 4.16 Modulation of $GABA_AR$-mediated mIPSCs by ethanol in $\alpha4^{Q246M}$ animals after two bottle choice experiment

A and ***B***, Representative mIPSC recordings from wild type and homozygous mutant $\alpha4^{Q246M}$ mice before (control), during the initial 3 min (time 0) and at the end of a 30 min bath application of 100 mM ethanol (30 min), and 10 min after drug washout, respectively. ***C,*** Mean mIPSC amplitudes recorded during bath application of 100 mM ethanol. ***D***, Percentage change in mean mIPSC amplitude induced in the presence of 100 mM ethanol compared to control. ***E,*** Mean mIPSC frequencies recorded at various times during bath application of 100 mM ethanol. ***F,*** Percentage change in mean mIPSC frequency upon exposure to 100 mM ethanol compared to control. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: panels C and E) and with comparisons using Dunnett's test (to compare the effect of treatment: panels D and F; # $p < 0.05$, ## $p < 0.01$, lack of # indicates non-significance). Wild type: n = 3; hom: n = 3.

Bath application of ethanol resulted in a significant increase in weighted decay times in both genotypes (*Figure 4.17 A, B, C* and *D*). However, the extent of mIPSC prolongation differed between wild type and mutant cells. Surprisingly, ethanol impacted upon homozygous mutant cells to a larger extent. In fact, between 20 and 30 minutes of 100 mM ethanol perfusion mutant cells had significantly prolonged mIPSCs compared to wild types (*Table 4.5*, page 191). Participation in the two bottle choice experiment had no effect on mIPSCs in wild type cells (i.e. naïve vs two bottle choice; *Figure 4.3 C*, *Figure 4.17 C*), however, two bottle choice homozygous animals showed slower kinetics upon ethanol exposure than naïve mutant animals (F (1, 9) = 10.30; P = 0.011; *Figure 4.3 C*, *Figure 4.17 C*).

Ethanol had no effect on mIPSC rise times in either genotype (Figure 4.17 E and F). Additionally, we observed no differences between $\alpha4^{Q/Q}$ and $\alpha4^{M/M}$ mice.

Analysis of charge transfer revealed a significant effect of treatment; both genotypes showed higher mIPSC charge transfer values upon bath application of ethanol (*Figure 4.17 G and H*). We found no significant differences between wild type and mutant cells. The changes in charge transfer likely reflect the increases in mIPSC amplitude, frequency and to some extent weighted decay times.

Two-way RM ANOVA detected a significant effect of ethanol on RMS noise in both $\alpha4^{Q/Q}$ and $\alpha4^{M/M}$ animals (*Figure 4.18 A, B and C, Table 4.15,* page 200). However, there were no genotypic differences. The increase in RMS noise indicates an increase in GABAergic tonic current. In fact, we saw a significant decrease in RMS noise upon bicuculline application, indicating the presence of such currents.

Taken together, these results suggest that participation in two bottle choice (i.e. previous exposure to ethanol through voluntary consumption) increases the sensitivity of homozygous mutant animals to prolongation of mIPSCs by ethanol. Additionally, the effect of ethanol on mIPSC frequency is more pronounced in two bottle choice animals compared to naïve counterparts; in both cases bath application of ethanol induced an increase, however, the extent of this increase was higher in two bottle choice animals relative to naïve mice. Furthermore, two bottle choice animals (both wild type and mutant) displayed larger changes in RMS noise upon ethanol exposure compared to naïve mice, indicating that previous voluntary intake of alcohol affects ethanol potentiation of tonic currents.

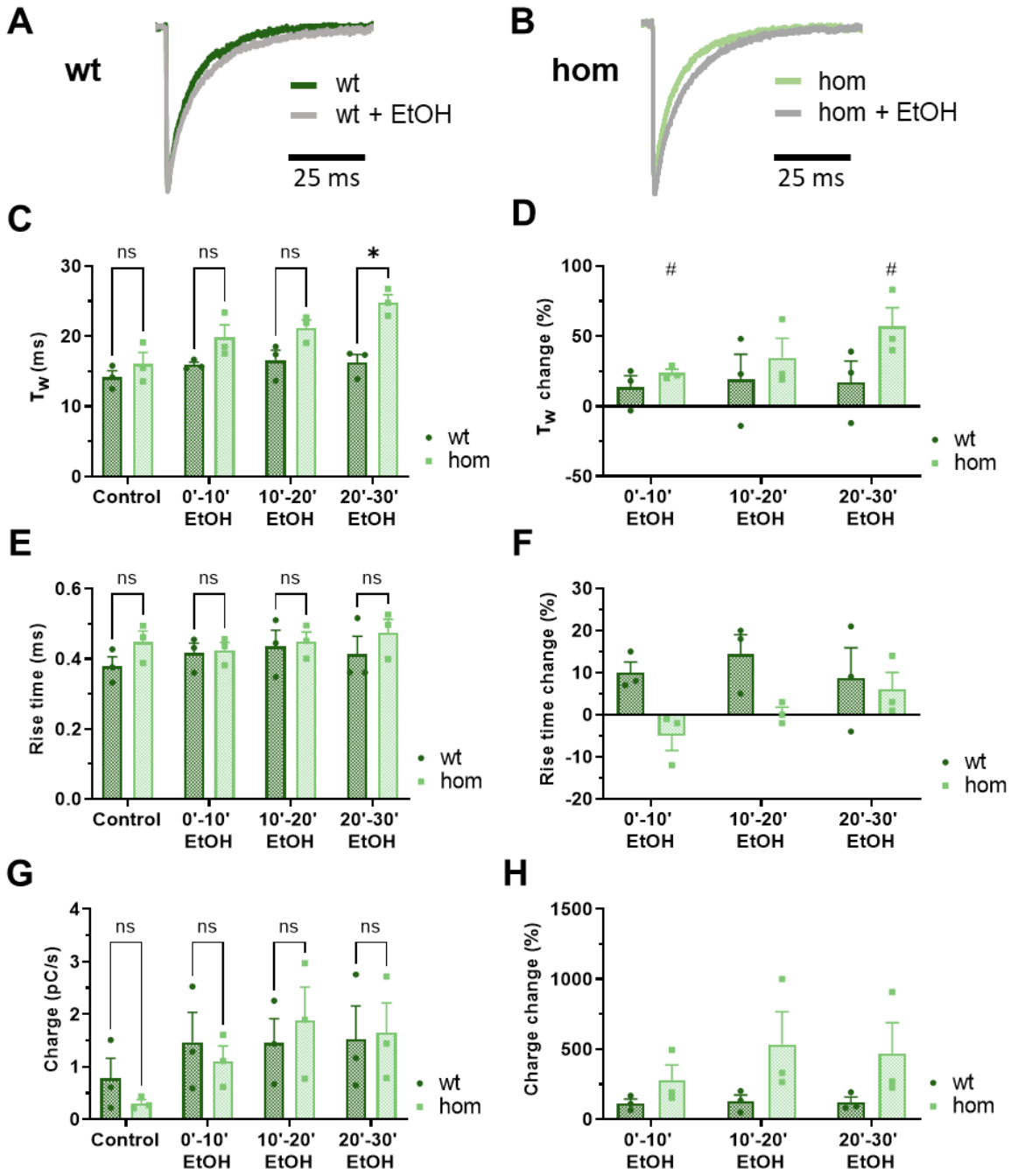


Figure 4.17 Modulation of $GABA_AR$-mediated mIPSC kinetics by ethanol in wild type and homozygous $\alpha4^{Q246M}$ mutant animals after two bottle choice

A and ***B***, Representative mean mIPSC waveform from wild type and homozygous mutant $\alpha4^{Q246M}$ mice before (control) and at the end of a 30 min bath application of 100 mM ethanol (30 min), respectively. ***C, E***, and ***G***, Mean mIPSC weighted tau decay (ms; ***C***), mean mIPSC rise time (ms; ***E***) and mean mIPSC charge transfer (-pC/s; ***G***) recorded during bath application of 100 mM ethanol, respectively. ***D***, ***F*** and ***H***, Percentage change in mean mIPSC weighted tau (***D***), mean mIPSC rise time (***F***) and mean mIPSC charge transfer (***H***) induced in the presence of 100 mM ethanol, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C, E* and *G; * $p < 0.05$*) and with comparisons using Dunnett's test (to compare the effect of ethanol treatment: *D, F* and *H*; # $p < 0.05$; where # is missing it indicates non-significance). Wild type: n = 3; hom: n = 3.

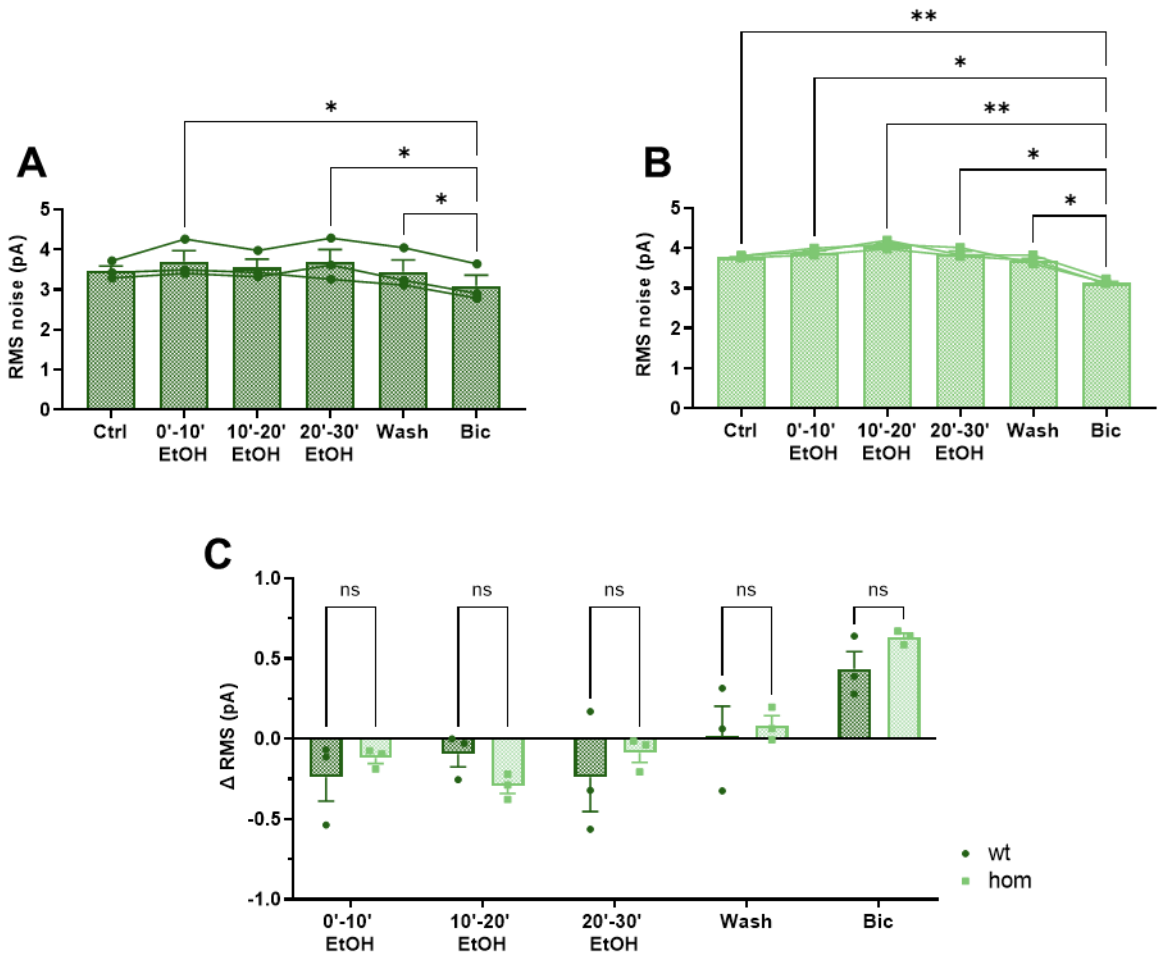


Figure 4.18 Modulation of tonic current (measured by RMS noise) by ethanol in wild type and homozygous α4^{Q246M} mutant animals after two bottle choice

A and ***B*,** Mean RMS noise (pA) before (control; Ctrl), during 30 min bath application of 100 mM ethanol, during ethanol washout and during application of 50 µM bicuculline (Bic) in wild type (***A***) and homozygous mutant (***B***) mice. ***C,*** Mean RMS noise change (pA) induced in the presence of 100 mM ethanol and 50 µM bicuculline compared to control. Data are shown as mean ± SEM. Statistical tests used are one-way repeated measures ANOVA with comparisons using Tukey's test (to compare the effect of treatment: *A* and *B*) and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C*). Statistical significance indicated as * $p < 0.05$, ** $p < 0.01$; where * is missing it indicates non-significance. Wild type: n = 3; hom: n = 3.

4.2.4 Chronic ethanol effects in α2^{Q241M} brain slices

We also examined the impact of bath application of ethanol on mIPSCs in α2$^{Q/Q}$ and α2$^{M/M}$ DGGCs after the two bottle choice paradigm (*Figure 4.19 A* and *B*). The mean ± SEM values and statistical analysis results of these experiments are contained in *Table 4.3* (amplitude), *Table 4.4* (frequency), *Table 4.5* (weighted decay time), *Table 4.6* (rise time), *Table 4.7* (charge transfer), *Table 4.8* (% changes in mIPSC parameters), *Table 4.9* (RMS noise), *Table 4.10* (% RMS noise change), *Table 4.12* (RMS noise statistics) and *Table 4.16* (mIPSC parameter statistics) in *Section 4.5*.

Analysis of mIPSC amplitude revealed no effect of ethanol in either wild type or mutant cells (*Figure 4.19 C* and *D*). Additionally, there was no statistical difference in mIPSC amplitude change between the genotypes, despite there being a clear trend, with homozygous mutants not showing any increase in mIPSC amplitude upon ethanol perfusion, compared with an approximately 20-30 % increase in amplitude for wild type cells (*Figure 4.19 D*). Furthermore, two bottle choice homozygous mutant animals displayed significantly smaller mIPSC amplitudes upon ethanol application compared to naïve animals (F (1, 6) = 22.00; P = 0.003; *Figure 4.9 D* and *Figure 4.19 D*). Participation in the two bottle choice experiment did not affect wild type mice.

Two-way RM ANOVA on mIPSC frequency values showed a main effect of treatment (*Figure 4.19 E, Table 4.16,* page 200). Both wild type and mutant cells had higher mIPSC frequencies after ethanol application. Again, there is a trend with α2$^{M/M}$ DGGCs showing less of a change in mIPSC frequency during ethanol application (~ 35-40 %) compared to α2$^{Q/Q}$ cells (~ 100-130 %) (*Figure 4.19 F*). However, post-hoc analyses revealed no significant differences between genotypes at any time point. mIPSC frequency of wild type mice was influenced by two bottle choice experiment participation; two bottle choice animals showed higher mIPSC frequencies compared to naïve animals following bath application of ethanol.

Decay kinetics of mIPSCs were not affected by acute ethanol application in either wild type or mutant cells (*Figure 4.20 A, B, C* and *D*). Furthermore, mIPSC weighted decay times were unchanged between genotypes.

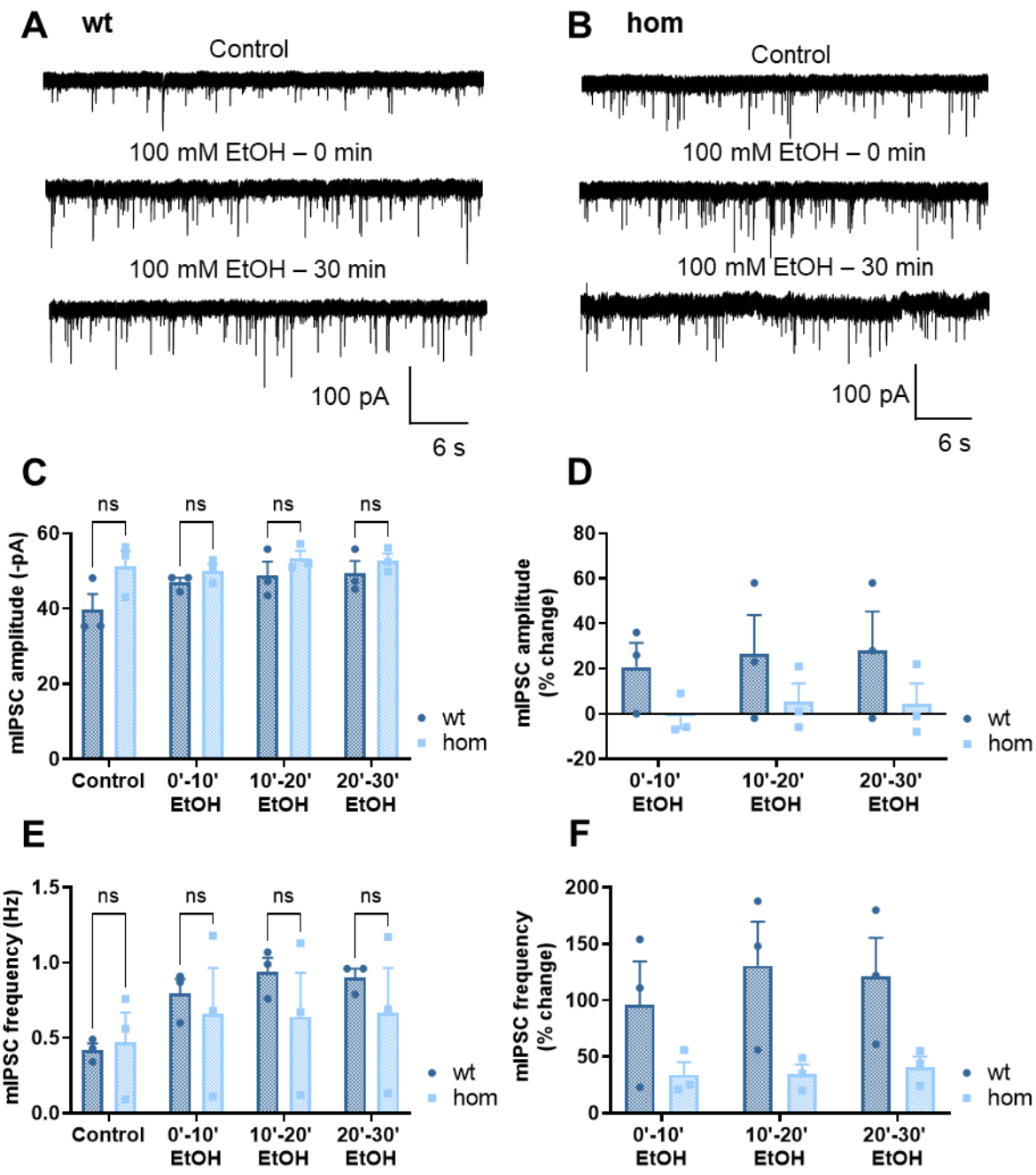


Figure 4.19 Modulation of $GABA_AR$-mediated mIPSCs by ethanol in $\alpha2^{Q246M}$ animals after two bottle choice experiment

A and ***B***, Representative mIPSC recordings from wild type and homozygous mutant $\alpha2^{Q241M}$ mice before (control), during the initial 3 min (time 0) and at the end of a 30 min bath application of 100 mM ethanol (30 min), and 10 min after drug washout, respectively. ***C,*** Mean mIPSC amplitudes recorded during application of 100 mM ethanol. ***D***, Percentage change in mean mIPSC amplitude induced in the presence of 100 mM ethanol. ***E,*** Mean mIPSC frequencies recorded at various times during application of 100 mM ethanol. ***F,*** Percentage change in mean mIPSC frequency upon exposure to 100 mM ethanol. Data are shown as mean ± SEM. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: panels *C* and *E*) and using Dunnett's test (to compare the effect of ethanol treatment: panels *D* and *F*, all comparisons are non-significant). Wild type: n = 3; hom: n = 3.

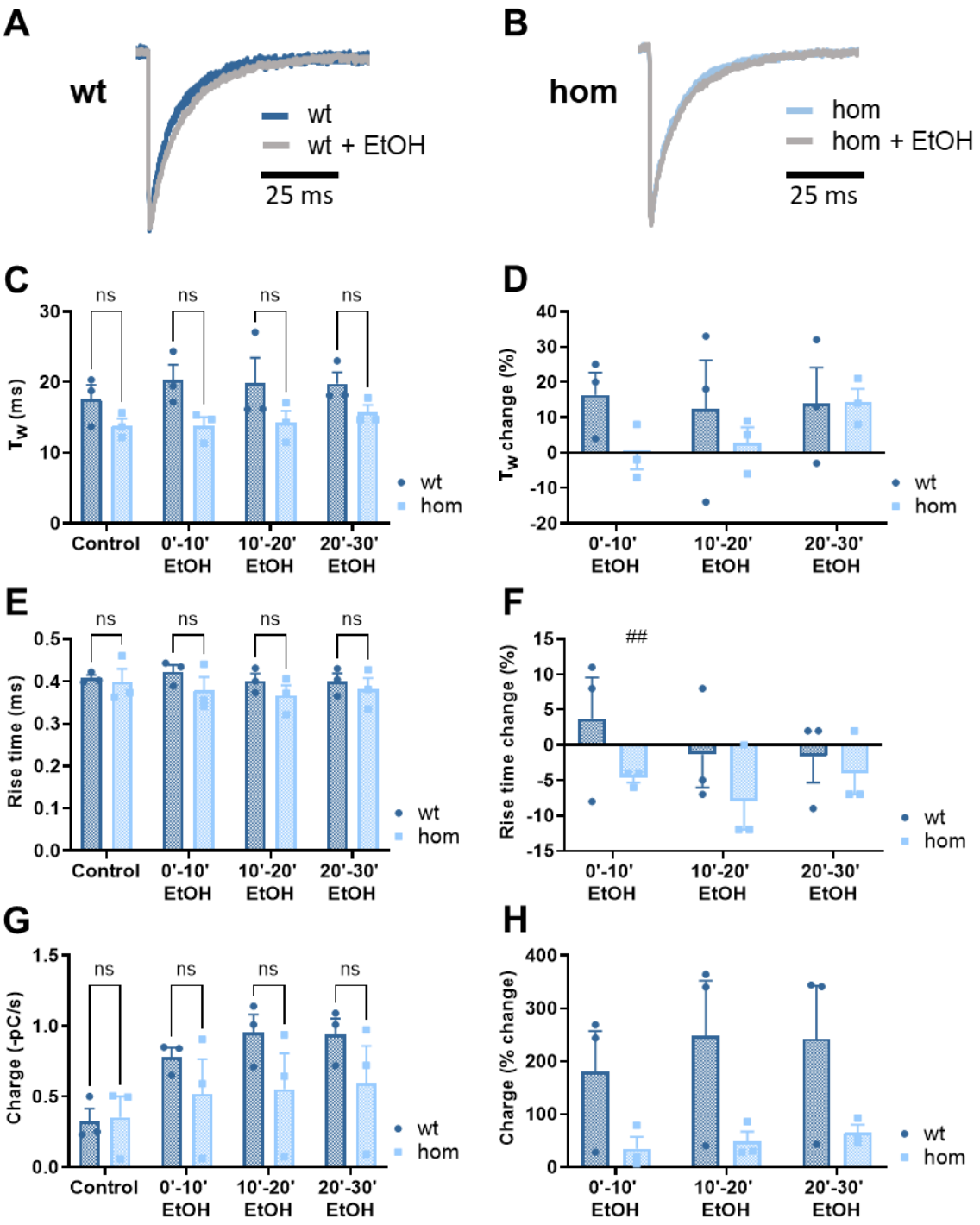


Figure 4.20 Modulation of $GABA_AR$-mediated mIPSC kinetics by ethanol in wild type and homozygous $\alpha2^{Q241M}$ mutant animals after two bottle choice

A and ***B***, Representative mean mIPSC waveform from wild type and homozygous mutant $\alpha2^{Q241M}$ mice before (control) and at the end of a 30 min bath application of 100 mM ethanol (30 min), respectively. ***C, E***, and ***G***, Mean mIPSC weighted tau decay (ms; ***C***), mean mIPSC rise time (ms; ***E***) and mean mIPSC charge transfer (-pC/s; ***G***) recorded during bath application of 100 mM ethanol, respectively. ***D***, ***F*** and ***H***, Percentage change in mean mIPSC weighted tau (***D***), mean mIPSC rise time (***F***) and mean mIPSC charge transfer (***H***) induced in the presence of 100 mM ethanol, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C, E* and *G*) and with comparisons using Dunnett's test (to compare the effect of ethanol treatment: *D, F* and *H*; *## p < 0.01;* where # is missing it indicates non-significance). Wild type: n = 3; hom: n = 3.

Ethanol had a transient effect on mIPSC rise time of homozygous mutant cells, showing a modest (5 %) but statistically significant reduction (*Figure 4.20 E* and *F*). Otherwise, had no overall impact and there were no observable differences between genotypes.

Analysis of mIPSC charge transfer detected a significant effect of treatment; with both genotypes showing an increase in charge transfer upon ethanol exposure (*Figure 4.20 G* and *H, Table 4.16*, page 200). This increase was more pronounced in wild type cells, although post-hoc analyses revealed no genotypic differences.

Ethanol treatment significantly increased RMS noise in both wild type and mutant animals (*Figure 4.21 A, B* and *C, Table 4.16,* page 200), indicating a potential increase in GABA-mediated tonic currents. Bicuculline application resulted in a significant decrease in RMS noise, suggesting the presence of such currents.

Overall, data from these experiments implies that chronic exposure to ethanol (i.e. voluntary consumption of alcohol during the two bottle choice experiment) enhances the effects of ethanol on mIPSC amplitude in wild type mice, whereas that effect is diminished in mutant animals. Similarly, bath application of ethanol resulted in a larger increase in mIPSC frequency for two bottle choice $\alpha2^{Q/Q}$ mice compared to naïve counterparts, while the extent of mIPSC frequency increase was smaller in two bottle choice $\alpha2^{M/M}$ compared to naïve neurons. Furthermore, RMS noise was increased upon acute ethanol exposure after two bottle choice in both genotypes.

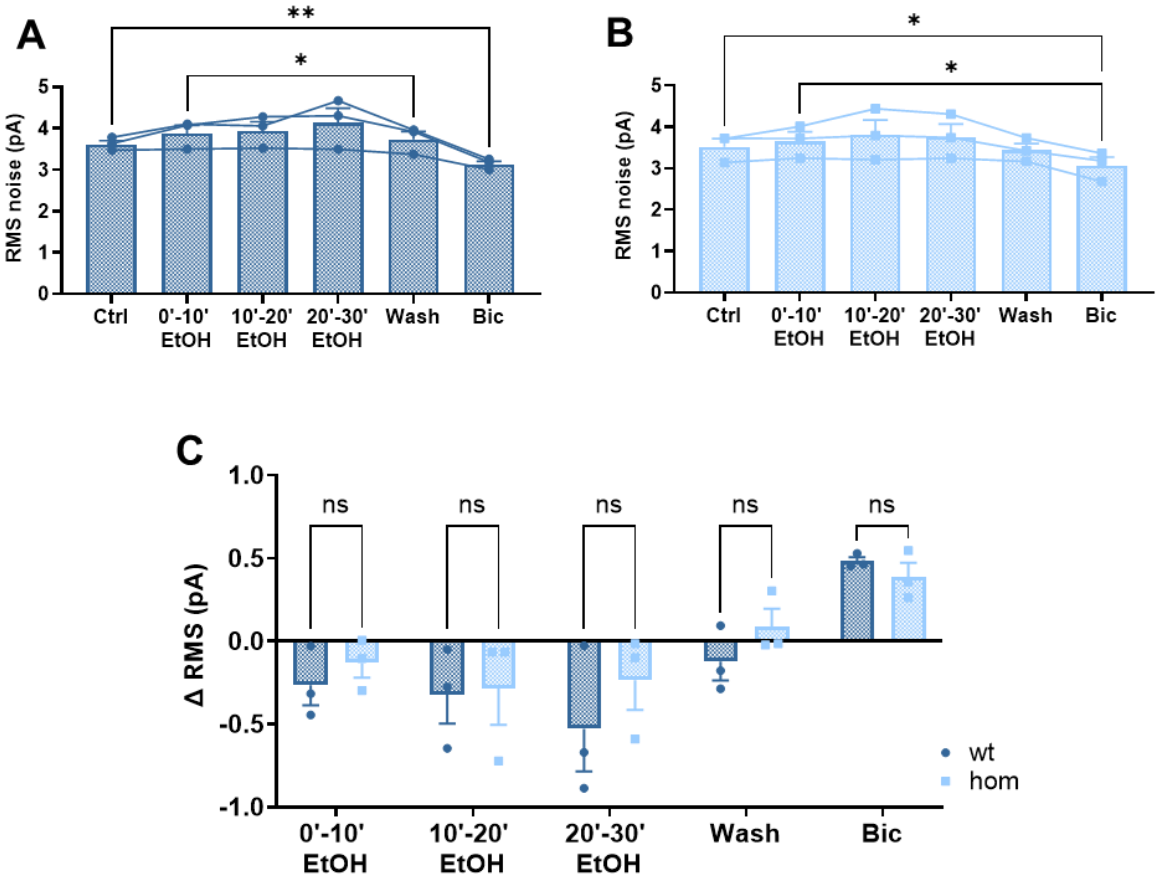


Figure 4.21 Modulation of tonic current (measured by RMS noise) by ethanol in wild type and homozygous α2^{Q241M} mutant animals after two bottle choice

A and ***B*,** Mean RMS noise (pA) before (control; Ctrl), during 30 min bath application of 100 mM ethanol, during ethanol washout and during application of 50 µM bicuculline (Bic) in wild type (***A***) and homozygous mutant (***B***) mice. ***C,*** Mean RMS noise change (pA) induced in the presence of 100 mM ethanol and 50 µM bicuculline compared to control. Data are shown as mean ± SEM. Statistical tests used are one-way repeated measures ANOVA with comparisons using Tukey's test (to compare the effect of treatment: *A* and *B*) and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare genotypic differences: *C*). Statistical significance indicated as * $p < 0.05$, ** $p < 0.01$; where * is missing it indicates non-significance. Wild type: n = 3; hom: n = 3.

4.2.5 Allopregnanolone concentration measurement in the hippocampus using confocal microscopy

Given the functional effects of ethanol in our electrophysiological recordings, we examined the effects of ethanol on allopregnanolone immunostaining in the hippocampus in C57BL/J6 mice using an anti-allopregnanolone antibody, which had not been characterised before. Our control experiments using only a secondary antibody (Alexa Fluor® anti-rabbit IgG) did not show any immunofluorescence in the absence of the primary allopregnanolone antibody (*Figure 4.22 A*).

We assessed allopregnanolone immunostaining under different conditions (control (i.e. ACSF); CTRL), ethanol (100 mM; EtOH), finasteride (1 µM; FIN) and allopregnanolone (100 nM; ALLO)) in three different regions of the hippocampus (dentate gyrus, CA1, CA3) using the protocol described in *Section 2.5.1*. The 150 µm thick slices were kept under constant oxygenation in Gibb's incubation chambers (Gibb and Edwards, 1999), and soaked for two hours in the different solutions prior to fixation with PFA.

Allopregnanolone staining was present and visible in all three hippocampal regions (*Figure 4.22 B, C* and *D*). Under control conditions, CA1 region exhibited the highest fluorescence intensity (161.8 ± 5.4 AU), CA3 region displayed similar values (154.2 ± 11.7 AU), while fluorescence intensity in the dentate gyrus was substantially lower (82.6 ± 6.6 AU).

We used the 'ALLO' condition as a form of control, as we hypothesised that supplementing the ACSF with allopregnanolone would lead to increased allopregnanolone staining. Indeed, ALLO treatment in DGGCs and CA1 neurons exhibited higher fluorescence intensities compared to CTRL (*Figure 4.22 E* and *F*), however, there was no increase in the CA3 region.

We also hypothesised that treatment with finasteride would result in decreased immunofluorescence, however, there was no difference between FIN and CTRL in any of the three areas studied (*Figure 4.22 E, F and G*).

Contrary to our functional experiments, which indicated changes in neurosteroid levels, we observed no increase in allopregnanolone staining under EtOH conditions in any of the brain regions examined (*Figure 4.22 E, F and G*).

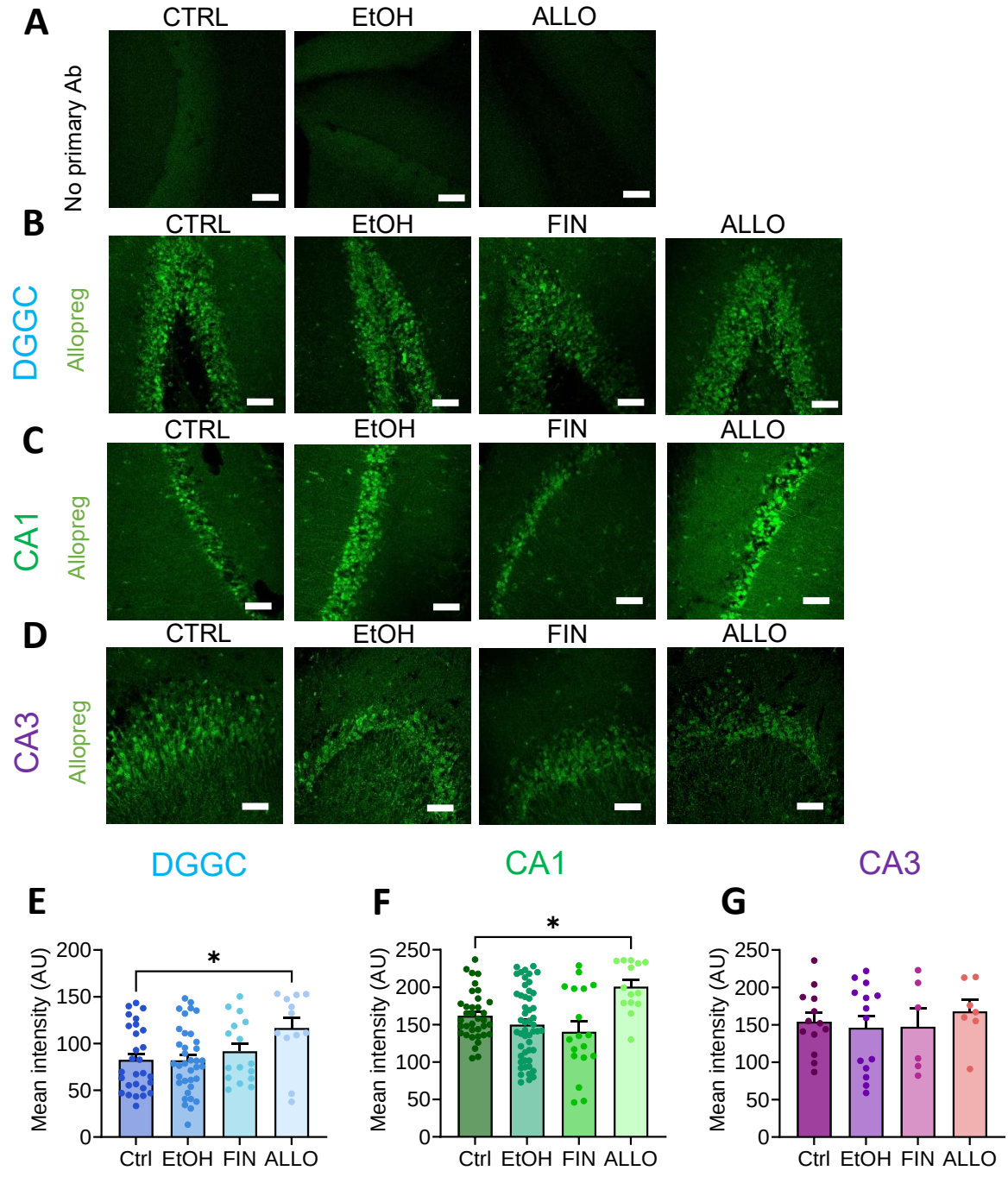


Figure 4.22 Modulation of allopregnanolone levels by ethanol in the hippocampus

A, Representative confocal microscopy images in the absence of the anti-allopregnanolone primary antibody (1° Ab) in different conditions – control (CTRL), in the presence of 100 mM ethanol (EtOH) and in the presence of 100 nM allopregnanolone (ALLO). ***B, C*** and ***D***, Example confocal images of allopregnanolone labelling (green) under different conditions (CTRL, EtOH, 1 µM finasteride (FIN) and ALLO) in different hippocampal regions: dentate gyrus granule cells (DGGC; ***B***), CA1 (***C***) and CA3 (***D***). Scale bar 50 µm. ***E*** (DGGC)***, F*** (CA1) and ***H*** (CA3), Bar charts representing mean fluorescence intensity in CTRL, EtOH, FIN and ALLO conditions of allopregnanolone determined by anti-allopregnanolone 1° Ab (1:500) and secondary antibody (1:750) conjugated to AlexaFluor®-488. Each dot represents the mean fluorescence intensity for the cell body layer per hemisphere. At least 3 animals (C57BL/J6) were used for each condition. Data are shown as mean ± SEM. **E:** CTRL: 82.6. ± 6.6, n = 28; EtOH: 82.1 ± 8.9, n = 36; FIN: 91.6 ± 8.4, n = 16; ALLO: 117.0 ± 11.4, n = 12. **F**: CTRL: 161.8 ± 5.4, n = 36; EtOH: 150 ± 6.6, n = 51; FIN:

140.6 ± 13.6, n = 18; ALLO: 220.8 ± 8.9, n = 14. **F**: CTRL: 154.2 ± 11.7, n = 13; EtOH: 146.1 ± 15.7, n = 14; FIN: 147.3 ± 24.9, n = 6; ALLO: 168.0 ± 15.7, n = 7. Statistical test used is an ordinary one-way ANOVA with comparisons using Dunnett's test for panels *E, F* and *G*. Statistical significance indicated as ** $p < 0.05$*; all other comparisons are non-significant.

4.3 Discussion

The impact of ethanol on inhibitory neurotransmission has been extensively studied, including its effects on dentate gyrus granule cells. However, to our knowledge, no research to date has examined the role of neurosteroids in the ethanol modulation of $GABA_A$ receptor-mediated transmission in these cells. Sanna et al. (2004) investigated ethanol's effects on rat CA1 pyramidal neurons and found that pre-incubation with finasteride attenuated some of these effects, providing a link between ethanol, GABAergic transmission and neurosteroids.

The $\alpha4^{Q246M}$ and $\alpha2^{Q241M}$ knock-in mouse lines used here offer a unique opportunity to elucidate the roles of α4- and α2-containing receptor isoforms in the neurosteroid-mediated modulation of ethanol effects.

4.3.1 Functional role of neurosteroid modulation of α4-$GABA_A$ receptors in mediating the effects of ethanol in DGGCs

The main findings from electrophysiological recordings using the $\alpha4^{Q246M}$ mouse line are summarised in *Table 4.1*.

We recorded mIPSCs from hippocampal DGGCs in ethanol-naïve animals. We found no statistical difference in mIPSC amplitude, frequency or decay kinetics between wild type and mutant cells under control conditions or during ethanol application. Ethanol treatment resulted in an increase in mIPSC amplitude in both $\alpha4^{Q/Q}$ and $\alpha4^{M/M}$ cells, which was evident after 10 and 20 minutes, respectively. Sanna et al. (2004) showed that incubation of hippocampal tissue with ethanol leads to an increase in neurosteroid levels after 20 minutes. Therefore, our results suggest that the increase in mIPSC amplitude is not mediated via neurosteroids acting on α4-$GABA_A$ receptors, since it is also present in slices containing the mutant receptors. Ethanol did have an impact on mIPSC frequency in wild type but not in mutant cells in our study, indicating that neurosteroid modulation of α4-type $GABA_A$Rs may be responsible for this effect. Sanna et al. (2004) reported an increase in both mIPSC amplitude and frequency in rat hippocampal CA1 neurons after bath application of ethanol. Finasteride was able to inhibit the effect of ethanol on mIPSC amplitude but not on frequency, which led them to conclude that the change in

amplitude is likely mediated via neurosteroids, whereas the change in frequency is probably independent of neurosteroid action. Later studies suggested that neurosteroid levels are increased after ethanol administration in CA1 pyramidal neurons and in the polymorphic layer of dentate gyrus but not in DGGCs (our study region), and that this increase is still present in adrenalectomised animals suggesting a local effect (Cook et al., 2014a, Cook et al., 2014b). However, the enzymes required for neurosteroid biosynthesis are expressed in DGGCs (Agís-Balboa et al., 2006), and our functional data implies that there is an increase in neurosteroid levels. Thus, it is possible that immunohistochemical techniques are not sensitive enough to detect these changes.

Table 4.1 Summary of functional effects of ethanol in the dentate gyrus in $\alpha4^{Q246M}$ mice
Symbols: ↑ increase, ↓ decrease, = no change, n/a not applicable

$\alpha4^{Q246M}$	NAÏVE PAIRED		2BC PAIRED	
	Treatment Ctrl vs EtOH	Genotype Wt vs Hom	Treatment Ctrl vs EtOH	Genotype Wt vs Hom
Amplitude	↑	=	↑	=
Frequency	trend ↑	trend wt > hom	↑	=
Weighted decay	↑	=	↑	wt < hom
Rise time	=	=	=	=
Charge transfer	↑	=	↑	=
Holding current	n/a	n/a	n/a	n/a
RMS noise	=	=	↑	=
$\alpha4^{Q246M}$	UNPAIRED			
	EtOH Wt	EtOH Hom	Untreated Wt vs Hom	Treated Wt vs Hom
Amplitude	=	=	=	=
Frequency	↑	=	=	wt > hom
Weighted decay	=	=	=	=
Rise time	=	=	wt > hom	=
Charge transfer	↑	=	=	=
Holding current	trend ↑	trend ↓	=	=
RMS noise	=	=	=	=

Moreover, our knock-in mouse model only removes neurosteroid sensitivity from α4-$GABA_A$Rs, but the α1 subunit, which is heavily involved in generating synaptic inhibition, is also abundantly expressed in DGGCs (Pirker et al., 2000). It was previously reported that global δ subunit knockout does not impact upon mIPSC amplitude in DGGCs, suggesting that α4βδ receptors contribute minimally, if at all, to peak synaptic currents (Mihalek et al., 1999). Therefore, a possible explanation for the increase in mIPSC

amplitude in mutant cells could be that $GABA_A$ receptors other than the α4-type are subject to neurosteroid modulation induced by ethanol.

Surprisingly, we did not observe any potentiation of mIPSC amplitude by ethanol in either genotype in our unpaired recordings. There is one main difference between our paired and unpaired protocols is the timing of the recordings. In the paired experiments, we looked at effects of ethanol between 0 and 30 minutes, whereas unpaired recordings were made after 30 minutes (30-50 min) of ethanol exposure. Therefore, it could be that the increase in mIPSC amplitude is transient.

The increase in mIPSC frequency in wild type controls in paired recordings, but not in the mutants, indicates that neurosteroid modulation of α4-$GABA_A$Rs potentially plays a role in synaptic GABA release. Our unpaired data corroborates this finding, we saw a potentiation of mIPSC frequency in wild type but not in mutant cells. There is evidence for α4βδ receptors acting presynaptically to regulate transmitter release from mossy fibre afferents to CA3 pyramidal cells (Ruiz et al., 2010). Low concentrations of THDOC facilitate glutamate release, whereas the $GABA_A$ receptor antagonist, gabazine, reduces glutamate release. Therefore, we speculate that interneurons in the dentate gyrus may express α4-$GABA_A$Rs in their axons or synaptic terminals, which upon ethanol-evoked neurosteroid modulation increase GABA release, thus leading to a higher mIPSC frequency in wild type DGGCs.

Bath application of ethanol resulted in the prolongation of mIPSCs in both genotypes during paired recordings. Sanna et al. (2004) observed an increase in mIPSC decay times after 30 min by ethanol, and suggested that this effect is mediated via neurosteroids. Our data suggests that the prolongation of mIPSC induced by ethanol is likely not via neurosteroid modulation of α4-containing receptors. Surprisingly, our unpaired experiments showed no increase in mIPSC decay times in either wild type or mutant cells. We speculate, similarly to ethanol effects on amplitude, that ethanol's impact on decay kinetics is also transient.

Ethanol has no effect on mIPSC rise time in either wild type or mutant cells in any of the experimental protocols used. Unexpectedly, we detected a decrease in baseline (i.e. untreated) rise time in homozygous mutant cells compared to wild type in our unpaired recordings. We did not see this effect in the other two paradigms used. Furthermore, neurosteroids are believed to not play a role in GABA binding (Akk et al., 2007).

Therefore, we speculate that this effect might be a 'false positive' and unlikely to be physiologically significant.

Acute ethanol exposure leads to an increase in charge transfer, as a result of increasing amplitude, frequency and decay. The extent of this increase was trending to be lower in mutants compared to wild types in paired recordings, and it was significantly lower in our unpaired experiments. This suggests that some of ethanol's effects are mediated via neurosteroid modulation of α4-containing $GABA_A$ receptor in dentate gyrus granule cells.

We also probed ethanol's effect on tonic currents. We used RMS noise as a measure in our paired recordings, whereas in our unpaired experiments we quantified both holding current shifts and changes in RMS noise. Both experiments suggested no significant potentiation of tonic currents by ethanol in either wild type or homozygous mutant animals. However, unpaired recordings displayed an interesting trend; a ~30 % increase in holding current shifts was observed between control and ethanol-treatment in wild type cells; whereas mutant cells showed an approximately 35 % *decrease*. Potentiation of tonic currents by ethanol has been surrounded by controversy – some labs observe a change (Wei et al., 2004, Liang et al., 2006, Fleming et al., 2007), while others do not (Borghese et al., 2006). A single dose of ethanol leads to acute changes in $GABA_A$ receptor subunit expression (Liang et al., 2007). Both α4 and δ subunit expression is decreased 1 hour after ethanol exposure, which is also reflected in a reduction in GABAergic tonic currents and a reduction in ethanol-mediated potentiation of these currents. Both of these subunits, α4 and δ, might be reduced already at 40 minutes (time of our recordings), likely resulting in smaller potentiation of tonic currents by ethanol in our experiments. Nonetheless, our data implies a potential effect of ethanol on GABAergic tonic currents, and suggests that neurosteroid modulation of α4-$GABA_A$ receptors possibly plays a role.

We also recorded GABA-mediated mIPSCs from mice that had participated in the two bottle choice assay with ethanol to investigate whether chronic consumption of ethanol leads to any alteration in DGGC function in mice with ablated neurosteroid binding sites. In our study, participation in the two-bottle choice experiment is defined as 'chronic consumption' or 'chronic ethanol exposure', as mice underwent seven 24-hour periods of voluntary alcohol (20 % w/v) intake over 14 days. It is worth noting that other studies

may refer to chronic consumption following the CIE protocol, which involves 60 days of ethanol exposure (Olsen and Spigelman, 2010).

Similar to experiments with ethanol-naïve animals, we found that ethanol application leads to an increase in mIPSC amplitude across both $\alpha4^{Q/Q}$ and $\alpha4^{M/M}$ cells. mIPSC amplitudes under control conditions were smaller in two bottle choice animals compared to naïve animals (both wild type and hom), indicating an ethanol-induced plasticity change. In fact, it has been shown that chronic exposure to ethanol leads to the downregulation of the α1 subunit, which is consistent with smaller mIPSC amplitudes (Cagetti et al., 2003). Despite suggestions that α4 subunits localise to synapses after chronic exposure (Liang et al., 2006), we did not observe a significant differences in mIPSC amplitude between wild type and mutant mice. Thus, our findings suggest that the increased mIPSC amplitude is not dependent on neurosteroid modulation of α4-$GABA_A$Rs in the dentate gyrus. There is evidence for the upregulation of other α subunits, such as α2, which potentially plays a more important role in phasic inhibition (Lindemeyer et al., 2017).

Ethanol administration resulted in an increase in mIPSC frequency in both wild type and mutant animals, as observed in ethanol-naïve mice. However, there was no difference between the two genotypes. Previous ethanol exposure (during the two bottle choice experiment) did not seem to affect mIPSC frequency of wild type cells, but did cause an increase in mutant cells. There is much evidence for neurosteroids regulating $GABA_AR$ subunit expression (Herd et al., 2007). Therefore, it could be that ethanol exposure leads to elevated neurosteroid concentrations which in turn results in alterations in $GABA_A$ receptor subunit expression, thus leading to a change in mIPSC frequency in mutant animals.

Consistent with our naïve paired recordings, we found that bath application of ethanol leads to the prolongation of mIPSC in both genotypes. Participation in the two bottle choice experiment did not affect wild type animals, however, it resulted in an increase in the extent of ethanol's effects in mutant cells. The reason for this is unclear. One possible explanation could be differential localisation of mutant receptors; e.g. switch to perisynaptic sites.

Ethanol significantly increased RMS noise in animals after two bottle choice compared to naïve. This finding is in line with studies showing increased α4 subunit expression after chronic ethanol exposure (Liang et al., 2007). There was a trend (P = 0.075) for wild type cells showing a higher increase relative to mutants, implying a possible involvement of neurosteroid modulation of α4-containing $GABA_A$ receptors in ethanol's potentiating effect on tonic inhibition.

Our electrophysiological data utilising the knock-in $\alpha4^{Q246M}$ mouse line suggest that certain effects of ethanol are mediated through neurosteroid modulation of α4-type receptors. Acutely, the neurosteroid sensitivity of these receptors appears crucial for the presynaptic changes induced by ethanol, illustrated by the alterations in mIPSC frequency. Chronically, this sensitivity may contribute to ethanol-induced modifications in tonic currents.

4.3.2 Functional role of neurosteroid modulation of α2-GABA$_A$ receptors in mediating the effects of ethanol in DGGCs

The main findings from electrophysiological recordings using the α2^{Q241M} mouse line are summarised in *Table 4.2*.

Table 4.2 Summary of functional effects of ethanol in the dentate gyrus in α2^{Q241M} mice

Symbols: ↑ increase, = no change, n/a not applicable

α2^{Q241M}	NAÏVE PAIRED		2BC PAIRED	
	Treatment Ctrl vs EtOH	Genotype Wt vs Hom	Treatment Ctrl vs EtOH	Genotype Wt vs Hom
Amplitude	↑	=	↑ wt only	strong trend wt > hom
Frequency	↑	=	↑	strong trend wt > hom
Weighted decay	wt trend ↑	wt > hom	trend ↑	=
Rise time	=	=	=	=
Charge transfer	↑	=	↑	strong trend wt > hom
Holding current	n/a	n/a	n/a	n/a
RMS noise	=	=	↑	=
α2^{Q241M}	UNPAIRED			
	EtOH Wt	EtOH Hom	Untreated Wt vs Hom	Treated Wt vs Hom
Amplitude	=	=	=	=
Frequency	trend ↑	trend ↑	=	=
Weighted decay	=	=	wt > hom	=
Rise time	=	=	=	=
Charge transfer	=	=	trend wt > hom	trend wt > hom
Holding current	=	=	=	=
RMS noise	=	=	wt > hom	=

We recorded mIPSCs from hippocampal DGGCs in ethanol-naïve animals for the α2^{Q241M} mouse line. We found that ethanol increased mIPSC amplitude and frequency in both genotypes during paired recordings, suggesting that neurosteroid modulation of α2-type receptors is likely not responsible for these changes. Again, it is possible that ethanol mediates its effects via a different receptor isoform, with α1 being a likely candidate.

We observed no ethanol effect on mIPSC amplitude in either wild type or mutant cells during unpaired recordings. Our unpaired experiments, with both α4^{Q246M} and α2^{Q241M} mouse lines, hint at the possibility that the ethanol-induced increase in mIPSC amplitude effect, observed in our paired recordings, is transient, that it diminishes after 30 minutes of continuous ethanol perfusion.

Ethanol did not significantly affect mIPSC frequency in either $\alpha2^{Q/Q}$ or $\alpha2^{M/M}$ mice during unpaired recordings, although there was a trend toward increased frequency in ethanol-treated cells across both genotypes.

Bath application of ethanol during paired recordings resulted in the prolongation of mIPSCs in wild type cells, showing a strong trend (P = 0.055) though not statistically significant. Between 20 and 30 minutes, ethanol increased mIPSC decay by ~25 % in wild type, while only by ~3 % in mutant cells, indicating the involvement of neurosteroid modulation of α2-type receptor in mediating some of ethanol's effects. Additionally, $\alpha2^{M/M}$ cells displayed faster kinetics compared to $\alpha2^{Q/Q}$ cells under control conditions. Our unpaired recordings also demonstrated this baseline difference. These findings are consistent with Durkin et al. (2018), suggesting that neurosteroid modulation of α2-$GABA_A$ receptors is important for phasic inhibition in the hippocampus. However, we found no evidence for ethanol modulation of mIPSC kinetics in our unpaired recordings. The discrepancy between the paired and unpaired protocols may be attributed to the timing of the experiments. Alternatively, it could be due to the different conditions: perfusion in the bath with a slice harp might lead to greater neurosteroid accumulation compared to slices maintained on a mesh in a Gibb's incubation chamber.

Ethanol had no overall effect on mIPSC rise time in either wild type or mutant cells in any of the experimental protocols used.

RMS noise was unchanged in both $\alpha2^{Q/Q}$ or $\alpha2^{M/M}$ cells upon ethanol application during paired recordings. Furthermore, we did not detect any change in holding currents induced by ethanol in our unpaired recordings. Consistent with the $\alpha4^{Q246M}$ mouse line data, we did not observe potentiation of tonic currents with ethanol. Again, this could be due to the timing of tonic current measurements. α2-type $GABA_A$ receptors are thought to play a minor role in tonic currents in the dentate gyrus. However, their involvement cannot be dismissed, as they have been implicated in tonic inhibition in other hippocampal regions (Durkin et al., 2018). Furthermore, it has been shown that α2-containing $GABA_A$ receptors play a role in the potentiation of tonic currents in the dentate gyrus by THDOC (Durkin, 2012). In fact, we observe a baseline (i.e. between untreated cells) decrease in RMS noise in mutant cells compared to wild type. The reduction in RMS noise was not accompanied by a change in holding current. Therefore, while α2-type receptors may contribute, their role is unlikely to be substantial. Tonic

inhibition is largely mediated by α4βδ receptors in DGGCs (Herd et al., 2007, Stell et al., 2003), therefore the lack of potentiation by ethanol is likely due to the ethanol-induced changes in subunit expression described in the previous section.

We have also assessed the effects of ethanol on $GABA_A$ receptor function in DGGCs in $\alpha2^{Q/Q}$ and $\alpha2^{M/M}$ animals after the two bottle choice experiment. Interestingly, an earlier study showed that ethanol administration leads to an upregulation of α2-$GABA_A$Rs at the cell surface in the CA1 region and the dentate gyrus (Lindemeyer et al., 2017). However, we found no statistical difference in mIPSC amplitude or frequency between wild type and mutant cells under control conditions or following acute ethanol administration.

We did observe a clear trend in mIPSC amplitude between the two genotypes, in that further ethanol treatment failed to elicit an increase in mutant cells (*Table 4.8*, page 194), suggesting that α2-$GABA_A$Rs might play a role in mediating the effects of ethanol via neurosteroid modulation.

Ethanol administration impacted similarly upon mIPSC frequency as upon amplitude, indicating that the observed increase in mIPSC frequency is potentially dependent on neurosteroid action via α2-type $GABA_A$Rs. While α2-type receptors are traditionally thought to be postsynaptic, evidence suggests α2-containing $GABA_A$ receptors are also present presynaptically in primary sensory neurons (Witschi et al., 2011).

Bath application of ethanol at each time point during paired recordings prolonged mIPSCs in wild type cells, showing a strong trend but not reaching statistical significance. This effect was absent in mutant cells throughout most of the experiment, suggesting the involvement of neurosteroid modulation of α2-type receptors in mediating some of ethanol's effects.

The differential ethanol modulation of wild type and mutant cells was evident in the differences in charge transfer ($P = 0.092$). Ethanol markedly increased mIPSC charge transfer in wild type cells (~250 %), while the effect was much lower in mutants (~60 %). The increase in charge transfer is mainly due to changes in amplitude, frequency, and decay kinetics, which are more pronounced in $\alpha2^{Q/Q}$ relative to $\alpha2^{M/M}$ cells. This highlights the role of neurosteroid modulation of α2-containing $GABA_A$ receptors in influencing certain effects of ethanol.

Ethanol substantially increased RMS noise in animals after two bottle choice compared to naïve conditions. Although we observed no statistical differences between genotypes, there was a trend for more pronounced increases in wild type cells. Considering our data from unpaired recordings, which suggest a potential role for α2-type receptors in tonic inhibition in DGGCs, it is plausible that these receptors contribute to the potentiation of tonic inhibition by ethanol.

We identified a more prominent role of neurosteroid modulation of α2-$GABA_A$ receptors after chronic ethanol exposure compared to acute treatment. This is consistent with studies indicating an elevation in α2 subunit expression following ethanol administration (Lindemeyer et al., 2017).

Overall, our electrophysiological data suggest that ethanol's effects in the dentate gyrus are partially dependent on the neurosteroid sensitivity of α2-containing $GABA_A$ receptors. These receptors appear to be targets for ethanol potentiation of both phasic and tonic currents, primarily after chronic rather than acute exposure.

4.3.3 The effect of ethanol on allopregnanolone levels in the hippocampus

We probed the effect of acute ethanol treatment on neurosteroid levels in hippocampal brain slices using immunohistochemistry. We demonstrated the presence of allopregnanolone immunoreactivity in all three areas of the hippocampus: the dentate gyrus, CA1 and CA3 regions. However, allopregnanolone levels were higher in the *cornu ammonis* regions relative to the dentate gyrus. This finding is in line with previous immunohistochemical studies (Agís-Balboa et al., 2006, Saalmann et al., 2007).

We used an anti-allopregnanolone antibody which had not been characterised before. Our control experiments without the primary antibody showed no immunofluorescence. We hypothesised that adding exogenous allopregnanolone to the brain slices would increase fluorescence. We conducted this experiment as a form of control, to validate the effectiveness of the antibody. Dentate gyrus granule cells and CA1 pyramidal neurons displayed elevated mean fluorescence intensity upon supplementation with allopregnanolone compared to control, however, there were no significant differences in CA3.

We also hypothesised that pre-treatment of slices with finasteride would lead to a reduction in immunostaining. However, finasteride did not alter allopregnanolone levels in any hippocampal regions. There was a slight indication of a trend in CA1 pyramidal neurons. Interestingly, some studies claim that finasteride eliminates staining for allopregnanolone within the hippocampus (Tokuda et al., 2011, Tokuda et al., 2010). However, examination of their quantification of mean fluorescence intensity reveals no effect of finasteride compared to control.

We detected no increase in allopregnanolone staining upon ethanol exposure in any of the regions studied, contrary to the finding of Tokuda et al. (2011), where they observe significant increases in allopregnanolone levels in CA1 pyramidal neurons.

The primary difference between our methodology and theirs is that they pre-screened the slices using electrophysiology to minimise slice-to-slice variability, whereas we did not. Additionally, they used a different primary antibody. Therefore, it is possible that the lack of ethanol potentiation in our study is due to these differences.

Moreover, the absence of ethanol-induced enhancement directly contrasts with our functional data. This, along with the lack of an increase in immunostaining following the exogenous application of allopregnanolone in CA3 neurons, might indicate that the antibody is not suitable for detecting minor differences in neurosteroid levels.

4.3.4 Limitations

The properties of synaptic and tonic currents recorded from cells in brain slices can be influenced by various parameters, including recording temperature (Bright and Smart, 2013a, Thompson et al., 1985, Kim and Connors, 2012).

If we assume that ethanol increases neurosteroid production in the brain through enzymatic reactions, we might observe larger differences if electrophysiological recordings were made at temperatures closer to physiological levels (37 °C) rather than at room temperature. Recording at room temperature may not fully reveal the role of neurosteroids in ethanol's central actions.

Furthermore, tonic currents are elevated in dentate gyrus granule cells closer to physiological temperatures (Bright and Smart, 2013b). Therefore, raising recording temperatures could help discerning the role of neurosteroid modulation of $GABA_A$ receptors in mediating ethanol’s effect on tonic inhibition.

Another limitation of the functional experiments is the sample size, which may affect the generalisability of the findings. Small sample sizes can limit the statistical power of the analyses, making it challenging to draw robust conclusions. Future studies with larger sample sizes are necessary to confirm these findings and enhance the validity of the conclusions drawn from these experiments.

4.4 Conclusions

1. Upon acute exposure, neurosteroid sensitivity of α4-type $GABA_A$ receptors appears crucial for ethanol-induced presynaptic changes, such as modifications in mIPSC frequency, in dentate gyrus granule cells.

2. Following chronic ethanol treatment, intact neurosteroid sensitivity of α4-$GABA_A$ receptors is key for ethanol-induced modifications of tonic currents in dentate gyrus.

3. Ethanol's effects in the dentate gyrus are partially mediated by neurosteroid modulation of α2-containing $GABA_A$ receptors. These receptors seem to be targets for ethanol potentiation of both phasic and tonic currents, mainly after chronic rather than acute exposure.

4. The primary anti-allopregnanolone antibody used in this study might not be suitable for determining small changes in neurosteroid levels.

4.5 Appendix

Table 4.3 Mean mIPSC amplitude (-pA) upon exposure to ethanol in dentate gyrus granule cells during paired recordings

	mIPSC amplitude (-pA)				
	Mean ± SEM		P value		
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**	**Wt vs Hom**	**Wt: Control vs EtOH**	**Hom: Control vs EtOH**
Control	49.6 ± 5.8	52.7 ± 2.8	0.985	-	-
EtOH 0-10 min	53.4 ± 4.9	56.1 ± 2.2	0.983	0.270	0.181
EtOH 10-20 min	55.4 ± 4.9	60.6 ± 2.6	0.862	**0.045**	**0.012**
EtOH 20-30 min	55.3 ± 6.0	58.1 ± 2.8	0.992	**0.003**	**0.042**
$\alpha4^{Q246M}$ two bottle choice					
Control	47.0 ± 2.0	38.8 ± 2.3	0.202	-	-
EtOH 0-10 min	53.0 ± 4.0	44.3 ± 2.3	0.473	0.174	**0.009**
EtOH 10-20 min	54.3 ± 2.7	47.5 ± 2.7	0.479	**0.018**	**0.010**
EtOH 20-30 min	58.0 ± 2.8	46.4 ± 3.4	0.221	**0.015**	0.223
$\alpha2^{Q241M}$ naïve					
Control	36.6 ± 4.3	28.6 ± 2.8	0.514	-	-
EtOH 0-10 min	37.7 ± 2.4	32.0 ± 2.7	0.488	0.935	0.087
EtOH 10-20 min	37.1 ± 4.0	34.9 ± 3.2	0.988	0.967	0.098
EtOH 20-30 min	36.6 ± 3.8	34.5 ± 3.4	0.990	0.999	**0.037**
$\alpha2^{Q241M}$ two bottle choice					
Control	39.6 ± 4.3	51.2 ± 4.1	0.408	-	-
EtOH 0-10 min	47.0 ± 1.2	50.0 ± 1.8	0.660	0.344	0.934
EtOH 10-20 min	48.9 ± 3.6	53.4 ± 1.9	0.822	0.472	0.866
EtOH 20-30 min	49.4 ± 3.2	52.8 ± 1.8	0.897	0.440	0.955

Table 4.4 Mean mIPSC frequency (Hz) upon exposure to ethanol in dentate gyrus granule cells during paired recordings

	mIPSC frequency (Hz)				
	Mean ± SEM		P value		
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**	**Wt vs Hom**	**Wt: Control vs EtOH**	**Hom: Control vs EtOH**
Control	0.4 ± 0.1	0.9 ± 0.2	0.300	-	-
EtOH 0-10 min	0.6 ± 0.2	1.0 ± 0.3	0.778	0.358	0.998
EtOH 10-20 min	0.5 ± 0.2	1.1 ± 0.3	0.313	0.120	0.102
EtOH 20-30 min	0.6 ± 0.1	1.1 ± 0.3	0.400	**0.023**	0.451
$\alpha4^{Q246M}$ two bottle choice					
Control	0.9 ± 0.3	0.5 ± 0.1	0.819	-	-
EtOH 0-10 min	1.4 ± 0.4	1.2 ± 0.3	0.996	0.097	0.192
EtOH 10-20 min	1.5 ± 0.5	1.6 ± 0.4	0.998	0.103	0.193
EtOH 20-30 min	1.5 ± 0.6	1.3 ± 0.3	0.998	0.319	0.201
$\alpha2^{Q241M}$ naïve					
Control	0.5 ± 0.1	0.5 ± 0.2	0.998	-	-
EtOH 0-10 min	0.6 ± 0.1	0.8 ± 0.3	0.823	**0.018**	0.122
EtOH 10-20 min	0.6 ± 0.1	0.9 ± 0.2	0.670	0.188	0.051
EtOH 20-30 min	0.5 ± 0.1	0.9 ± 0.2	0.431	0.682	**0.019**
$\alpha2^{Q241M}$ two bottle choice					
Control	0.4 ± 0.1	0.5 ± 0.2	0.999	-	-
EtOH 0-10 min	0.8 ± 0.1	0.7 ± 0.3	0.993	0.198	0.457
EtOH 10-20 min	0.9 ± 0.1	0.6 ± 0.3	0.883	0.101	0.424
EtOH 20-30 min	0.9 ± 0.1	0.7 ± 0.3	0.942	0.070	0.400

Table 4.5 Mean mIPSC weighted decay times (ms) upon ethanol exposure during paired recordings

	mIPSC τ_W (ms)				
	Mean ± SEM		P value		
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**	**Wt vs Hom**	**Wt: Control vs EtOH**	**Hom: Control vs EtOH**
Control	14.3 ± 1.2	15.6 ± 0.7	0.886	-	-
EtOH 0-10 min	15.7 ± 1.2	17.6 ± 0.6	0.623	**0.021**	0.073
EtOH 10-20 min	16.1 ± 1.8	17.5 ± 0.5	0.943	0.328	0.071
EtOH 20-30 min	14.9 ± 1.2	17.8 ± 0.6	0.325	0.593	0.055
$\alpha4^{Q246M}$ two bottle choice					
Control	14.2 ± 0.9	16.1 ± 1.6	0.855	-	-
EtOH 0-10 min	15.9 ± 0.4	19.8 ± 1.8	0.500	0.440	**0.019**
EtOH 10-20 min	16.5 ± 1.5	21.2 ± 1.1	0.249	0.691	0.159
EtOH 20-30 min	16.2 ± 1.2	24.8 ± 1.1	**0.024**	0.673	**0.037**
$\alpha2^{Q241M}$ naïve					
Control	16.1 ± 1.5	12.6 ± 0.8	0.289	-	-
EtOH 0-10 min	18.0 ± 1.1	14.4 ± 0.9	0.138	0.570	**0.008**
EtOH 10-20 min	18.9 ± 1.3	13.7 ± 0.8	0.055	0.200	0.592
EtOH 20-30 min	19.3 ± 2.1	12.8 ± 0.8	0.137	0.544	0.991
$\alpha2^{Q241M}$ two bottle choice					
Control	17.6 ± 2.0	13.8 ± 1.0	0.576	-	-
EtOH 0-10 min	20.3 ± 2.1	13.8 ± 1.2	0.256	0.222	0.999
EtOH 10-20 min	19.8 ± 3.6	14.3 ± 1.6	0.712	0.776	0.825
EtOH 20-30 min	19.8 ± 1.6	15.7 ± 1.0	0.390	0.488	0.095

Table 4.6 Mean mIPSC rise times (ms) upon ethanol exposure during paired recordings

	mIPSC rise time (ms)				
	Mean ± SEM		P value		
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**	**Wt vs Hom**	**Wt: Control vs EtOH**	**Hom: Control vs EtOH**
Control	0.48 ± 0.12	0.48 ± 0.09	0.999	-	-
EtOH 0-10 min	0.44 ± 0.04	0.50 ± 0.09	0.952	0.974	0.440
EtOH 10-20 min	0.42 ± 0.03	0.47 ± 0.08	0.972	0.949	0.966
EtOH 20-30 min	0.40 ± 0.01	0.49 ± 0.07	0.708	0.891	0.985
$\alpha4^{Q246M}$ two bottle choice					
Control	0.38 ± 0.03	0.45 ± 0.03	0.531	-	-
EtOH 0-10 min	0.42 ± 0.03	0.42 ± 0.02	0.999	0.103	0.533
EtOH 10-20 min	0.43 ± 0.05	0.45 ± 0.03	0.999	0.207	0.996
EtOH 20-30 min	0.41 ± 0.05	0.47 ± 0.04	0.869	0.621	0.530
$\alpha2^{Q241M}$ naïve					
Control	0.50 ± 0.04	0.49 ± 0.03	0.999	-	-
EtOH 0-10 min	0.45 ± 0.03	0.47 ± 0.03	0.978	0.670	0.865
EtOH 10-20 min	0.48 ± 0.05	0.45 ± 0.03	0.984	0.796	0.427
EtOH 20-30 min	0.43 ± 0.03	0.49 ± 0.04	0.822	0.177	0.985
$\alpha2^{Q241M}$ two bottle choice					
Control	0.41 ± 0.01	0.40 ± 0.03	0.998	-	-
EtOH 0-10 min	0.42 ± 0.02	0.38 ± 0.03	0.771	0.874	**0.007**
EtOH 10-20 min	0.40 ± 0.02	0.37 ± 0.02	0.773	0.977	0.332
EtOH 20-30 min	0.40 ± 0.02	0.38 ± 0.03	0.975	0.910	0.530

Table 4.7 Mean mIPSC charge transfer (-pC/s) upon ethanol exposure during paired recordings

	mIPSC charge transfer (-pC/s)				
	Mean ± SEM		P value		
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**	**Wt vs Hom**	**Wt: Control vs EtOH**	**Hom: Control vs EtOH**
Control	0.31 ± 0.08	0.87 ± 0.26	0.649	-	-
EtOH 0-10 min	0.52 ± 016	1.14 ± 0.31	0.569	0.170	**0.043**
EtOH 10-20 min	0.55 ± 0.14	1.41 ± 0.37	0.253	0.052	0.053
EtOH 20-30 min	0.55 ± 0.11	1.27 ± 0.31	0.420	**0.011**	0.168
$\alpha4^{Q246M}$ two bottle choice					
Control	0.78 ± 0.38	0.31 ± 0.07	0.810	-	-
EtOH 0-10 min	1.47 ± 0.57	1.11 ± 0.29	0.977	0.125	0.188
EtOH 10-20 min	1.45 ± 0.46	1.88 ± 0.63	0.979	0.053	0.233
EtOH 20-30 min	1.52 ± 0.63	1.65 ± 0.57	0.999	0.186	0.261
$\alpha2^{Q241M}$ naïve					
Control	0.39 ± 0.13	0.26 ± 0.12	0.941	-	-
EtOH 0-10 min	0.48 ± 0.10	0.49 ± 0.22	0.999	0.675	0.184
EtOH 10-20 min	0.50 ± 0.12	0.50 ± 0.16	0.999	0.475	**0.034**
EtOH 20-30 min	0.45 ± 0.11	0.49 ± 0.14	0.999	0.918	**0.025**
$\alpha2^{Q241M}$ two bottle choice					
Control	0.33 ± 0.09	0.35 ± 0.15	0.999	-	-
EtOH 0-10 min	0.78 ± 0.07	0.52 ± 0.25	0.872	0.180	0.518
EtOH 10-20 min	0.95 ± 0.13	0.55 ± 0.25	0.690	0.181	0.436
EtOH 20-30 min	0.94 ± 0.11	0.60 ± 0.26	0.789	0.168	0.348

Table 4.8 Mean mIPSC amplitude, frequency, τ_W, rise time and charge transfer % changes upon ethanol exposure during paired recordings

	Amplitude change (%)		Frequency change (%)	
	Mean ± SEM		Mean ± SEM	
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**	**Wt**	**Hom**
Control vs EtOH 0-10 min	9 ± 5	8 ± 4	36 ± 12	8 ± 11
Control vs EtOH 10-20 min	13 ± 4	16 ± 5	30 ± 9	25 ± 8
Control vs EtOH 20-30 min	12 ± 1	11 ± 4	52 ± 22	26 ± 16
$\alpha4^{Q246M}$ two bottle choice				
Control vs EtOH 0-10 min	13 ± 4	15 ± 1	58 ± 11	134 ± 42
Control vs EtOH 10-20 min	16 ± 1	23 ± 1	67 ± 12	218 ± 68
Control vs EtOH 20-30 min	23 ± 1	20 ± 7	65 ± 14	152 ± 49
$\alpha2^{Q241M}$ naïve				
Control vs EtOH 0-10 min	6 ± 7	13 ± 4	25 ± 7	73 ± 27
Control vs EtOH 10-20 min	3 ± 4	23 ± 8	24 ± 13	90 ± 31
Control vs EtOH 20-30 min	1 ± 6	21 ± 5	17 ± 12	102 ± 37
$\alpha2^{Q241M}$ two bottle choice				
Control vs EtOH 0-10 min	21 ± 11	-1 ± 5	96 ± 39	34 ± 11
Control vs EtOH 10-20 min	26 ± 17	5 ± 8	131 ± 39	35 ± 8
Control vs EtOH 20-30 min	28 ± 17	4 ± 9	121 ± 34	41 ± 9
	τ_W change (%)		**Rise time change (%)**	
	Mean ± SEM		Mean ± SEM	
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**	**Wt**	**Hom**
Control vs EtOH 0-10 min	10 ± 2	14 ± 5	10 ± 7	6 ± 4
Control vs EtOH 10-20 min	13 ± 7	14 ± 5	7 ± 9	1 ± 5
Control vs EtOH 20-30 min	4 ± 4	16 ± 5	1 ± 8	7 ± 7
$\alpha4^{Q246M}$ two bottle choice				
Control vs EtOH 0-10 min	13 ± 8	24 ± 3	10 ± 3	-5 ± 4
Control vs EtOH 10-20 min	19 ± 18	35 ± 14	14 ± 5	1 ± 1
Control vs EtOH 20-30 min	17 ± 15	57 ± 13	9 ± 7	6 ± 4
$\alpha2^{Q241M}$ naïve				
Control vs EtOH 0-10 min	15 ± 11	15 ± 2	-8 ± 8	-4 ± 6
Control vs EtOH 10-20 min	19 ± 10	9 ± 8	-5 ± 6	-8 ± 6
Control vs EtOH 20-30 min	24 ± 18	3 ± 8	-12 ± 4	-1 ± 5
$\alpha2^{Q241M}$ two bottle choice				
Control vs EtOH 0-10 min	16 ± 6	1 ± 4	4 ± 6	-5 ± 1
Control vs EtOH 10-20 min	12 ± 14	3 ± 4	-1 ± 5	-8 ± 4
Control vs EtOH 20-30 min	14 ± 10	14 ± 4	-2 ± 4	-4 ± 3
	Charge transfer change (%)			
	Mean ± SEM			
$\alpha4^{Q246M}$ naïve	**Wt**	**Hom**		
Control vs EtOH 0-10 min	66 ± 10	39 ± 12		
Control vs EtOH 10-20 min	82 ± 5	77 ± 18		
Control vs EtOH 20-30 min	93 ± 15	65 ± 19		
$\alpha4^{Q246M}$ two bottle choice				
Control vs EtOH 0-10 min	116 ± 30	280 ± 108		
Control vs EtOH 10-20 min	129 ± 44	534 ± 235		
Control vs EtOH 20-30 min	122 ± 36	469 ± 220		
$\alpha2^{Q241M}$ naïve				
Control vs EtOH 0-10 min	55 ± 32	120 ± 43		
Control vs EtOH 10-20 min	56 ± 39	171 ± 73		
Control vs EtOH 20-30 min	50 ± 41	187 ± 91		
$\alpha2^{Q241M}$ two bottle choice				
Control vs EtOH 0-10 min	180 ± 77	35 ± 22		
Control vs EtOH 10-20 min	248 ± 104	48 ± 19		
Control vs EtOH 20-30 min	243 ± 100	66 ± 14		

Table 4.9 Mean RMS noise (pA) upon ethanol exposure during paired recordings

	RMS noise (pA)	
	Wt	**Hom**
$\alpha4^{Q246M}$ naïve	**Mean ± SEM**	
Control	4.0 ± 0.4	4.0 ± 0.1
EtOH 0-10 min	4.1 ± 0.4	4.1 ± 0.2
EtOH 10-20 min	4.0 ± 0.4	4.1 ± 0.1
EtOH 20-30 min	4.0 ± 0.4	4.3 ± 0.2
Wash	4.0 ± 0.6	3.9 ± 0.2
Bicuculline	3.4 ± 0.4	3.2 ± 0.1
$\alpha4^{Q246M}$ two bottle choice		
Control	3.5 ± 0.1	3.8 ± 0.1
EtOH 0-10 min	3.7 ± 0.3	3.9 ± 0.1
EtOH 10-20 min	3.6 ± 0.2	4.1 ± 0.1
EtOH 20-30 min	3.7 ± 0.3	3.9 ± 0.1
Wash	3.4 ± 0.3	3.7 ± 0.1
Bicuculline	3.1 ± 0.3	3.1 ± 0.1
$\alpha2^{Q241M}$ naïve		
Control	3.3 ± 0.4	3.6 ± 0.4
EtOH 0-10 min	3.3 ± 0.3	3.7 ± 0.3
EtOH 10-20 min	3.3 ± 0.3	3.7 ± 0.3
EtOH 20-30 min	3.3 ± 0.3	3.6 ± 0.3
Wash	3.2 ± 0.3	3.6 ± 0.2
Bicuculline	2.6 ± 0.2	2.5 ± 0.1
$\alpha2^{Q241M}$ two bottle choice		
Control	3.6 ± 0.1	3.5 ± 0.2
EtOH 0-10 min	3.9 ± 0.2	3.7 ± 0.2
EtOH 10-20 min	3.9 ± 0.2	3.8 ± 0.4
EtOH 20-30 min	4.1 ± 0.4	3.8 ± 0.3
Wash	3.7 ± 0.2	3.4 ± 0.2
Bicuculline	3.1 ± 0.1	3.1 ± 0.2

Table 4.10 Mean RMS noise change (pA) upon ethanol exposure during paired recordings

	RMS noise change (pA)		
	Wt	**Hom**	**Wt vs Hom**
$\alpha4^{Q246M}$ naïve	**Mean ± SEM**		**P value**
Control vs EtOH 0-10 min	-0.04 ± 0.09	-0.07 ± 0.04	0.999
Control vs EtOH 10-20 min	0.05 ± 0.15	-0.12 ± 0.06	0.887
Control vs EtOH 20-30 min	0.08 ± 0.14	-0.23 ± 0.11	0.498
Control vs Wash	0.07 ± 0.08	0.12 ± 0.10	0.995
Control vs Bicuculline	0.48 ± 0.14	0.74 ± 0.17	0.802
$\alpha4^{Q246M}$ two bottle choice			
Control vs EtOH 0-10 min	-0.24 ± 0.15	-0.12 ± 0.04	0.971
Control vs EtOH 10-20 min	-0.10 ± 0.08	-0.30 ± 0.05	0.452
Control vs EtOH 20-30 min	-0.24 ± 0.22	-0.09 ± 0.06	0.984
Control vs Wash	0.02 ± 0.19	0.08 ± 0.06	0.999
Control vs Bicuculline	0.43 ± 0.11	0.63 ± 0.03	0.674
$\alpha2^{Q241M}$ naïve			
Control vs EtOH 0-10 min	0.03 ± 0.11	0.14 ± 0.09	0.968
Control vs EtOH 10-20 min	-0.01 ± 0.09	0.07 ± 0.09	0.983
Control vs EtOH 20-30 min	-0.02 ± 0.15	0.05 ± 0.07	0.997
Control vs Wash	-0.06 ± 0.06	0.02 ± 0.04	0.856
Control vs Bicuculline	-0.68 ± 0.15	-1.11 ± 0.13	0.306
$\alpha2^{Q241M}$ two bottle choice			
Control vs EtOH 0-10 min	-0.26 ± 0.12	-0.13 ± 0.09	0.943
Control vs EtOH 10-20 min	-0.32 ± 0.17	-0.29 ± 0.22	0.999
Control vs EtOH 20-30 min	-0.53 ± 0.26	-0.24 ± 0.18	0.929
Control vs Wash	-0.12 ± 0.11	0.09 ± 0.11	0.755
Control vs Bicuculline	0.48 ± 0.02	0.39 ± 0.08	0.909

Table 4.11 $\alpha4^{Q246M}$ - RMS noise statistics during paired recordings

$\alpha4^{Q246M}$ naïve wt	**P values**				
	RMS noise				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.997	0.999	0.987	0.999	**0.019**
0'-10' EtOH	-	0.938	0.868	0.999	**0.012**
10'-20' EtOH	-	-	0.953	0.999	**0.035**
20'-30' EtOH	-	-	-	0.989	**0.032**
Wash	-	-	-	-	0.132
$\alpha4^{Q246M}$ naïve hom	**RMS noise**				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.577	0.444	0.341	0.673	**0.036**
0'-10' EtOH	-	0.934	0.480	0.366	**0.038**
10'-20' EtOH	-	-	0.851	0.143	**0.016**
20'-30' EtOH	-	-	-	0.242	**0.032**
Wash	-	-	-	-	**0.015**
$\alpha4^{Q246M}$ two bottle choice wt	**RMS noise**				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.662	0.821	0.850	0.999	0.455
0'-10' EtOH	-	0.503	0.999	0.191	**0.034**
10'-20' EtOH	-	-	0.913	0.886	**0.127**
20'-30' EtOH	-	-	-	0.217	**0.047**
Wash	-	-	-	-	**0.019**
$\alpha4^{Q246M}$ two bottle choice hom	**RMS noise**				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.254	0.081	0.726	0.739	**0.006**
0'-10' EtOH	-	0.282	0.847	0.472	**0.019**
10'-20' EtOH	-	-	0.397	0.079	**0.004**
20'-30' EtOH	-	-	-	0.728	**0.046**
Wash	-	-	-	-	**0.017**

Table 4.12 $\alpha2^{Q241M}$ - RMS noise statistics during paired recordings

$\alpha2^{Q241M}$ naïve wt	P values				
	RMS noise				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.997	0.999	0.999	0.851	**0.049**
0'-10' EtOH	-	0.993	0.995	0.900	**0.034**
10'-20' EtOH	-	-	0.999	0.719	**0.014**
20'-30' EtOH	-	-	-	0.993	**0.031**
Wash	-	-	-	-	**0.021**
$\alpha2^{Q241M}$ naïve hom	**RMS noise**				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.522	0.839	0.957	0.992	**0.017**
0'-10' EtOH	-	0.988	0.573	0.690	**0.039**
10'-20' EtOH	-	-	0.775	0.821	**0.044**
20'-30' EtOH	-	-	-	0.995	**0.043**
Wash	-	-	-	-	**0.015**
$\alpha2^{Q241M}$ two bottle choice wt	**RMS noise**				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.484	0.571	0.515	0.856	**0.009**
0'-10' EtOH	-	0.937	0.670	0.017	0.096
10'-20' EtOH	-	-	0.887	0.402	0.140
20'-30' EtOH	-	-	-	0.426	0.218
Wash	-	-	-	-	0.124
$\alpha2^{Q241M}$ two bottle choice hom	**RMS noise**				
	0'-10' EtOH	10'-20' EtOH	20'-30' EtOH	Wash	Bicuculline
Control	0.702	0.777	0.772	0.938	**0.049**
0'-10' EtOH	-	0.855	0.857	0.280	**0.013**
10'-20' EtOH	-	-	0.877	0.546	0.169
20'-30' EtOH	-	-	-	0.458	0.114
Wash	-	-	-	-	0.119

Table 4.13 $\alpha4^{Q246M}$ – naïve paired recordings - two-way ANOVA statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Figure 4.2 C Amplitude (pA)	Treatment	0.000	***	F (2.554, 25.54) = 11.58
	Genotype	0.508	ns	F (1, 10) = 0.4706
	Treatment x Genotype	0.712	ns	F (3, 30) = 0.4611
Figure 4.2 E Frequency (Hz)	Treatment	0.151	ns	F (2.254, 22.54) = 2.024
	Genotype	0.226	ns	F (1, 10) = 1.663
	Treatment x Genotype	0.329	ns	F (3, 30) = 1.194
Figure 4.3 C τw (ms)	Treatment	0.008	**	F (2.296, 22.96) = 5.713
	Genotype	0.128	ns	F (1, 10) = 2.755
	Treatment x Genotype	0.360	ns	F (3, 30) = 1.112
Figure 4.3 E Rise time (ms)	Treatment	0.493	ns	F (1.448, 14.48) = 0.6406
	Genotype	0.694	ns	F (1, 10) = 0.1643
	Treatment x Genotype	0.530	ns	F (3, 30) = 0.7513
Figure 4.3 G Charge transfer (pC/s)	Treatment	0.024	*	F (1.573, 15.73) = 5.218
	Genotype	0.151	ns	F (1, 10) = 2.421
	Treatment x Genotype	0.531	ns	F (3, 30) = 0.7495
Figure 4.4 C Δ RMS (pA)	Treatment	0.081	ns	F (2.398, 21.59) = 2.708
	Genotype	0.234	ns	F (1, 10) = 1.600
	Treatment x Genotype	0.225	ns	F (3, 27) = 1.547

Table 4.14 $\alpha2^{Q241M}$ – naïve paired recordings - two-way ANOVA statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Figure 4.9 C Amplitude (pA)	Treatment	0.049	*	F (2.463, 19.71) = 3.316
	Genotype	0.349	ns	F (1, 8) = 0.9918
	Treatment x Genotype	0.055	ns	F (3, 24) = 2.914
Figure 4.9 E Frequency (Hz)	Treatment	0.002	**	F (2.012, 16.10) = 9.888
	Genotype	0.267	ns	F (1, 8) = 1.423
	Treatment x Genotype	0.014	*	F (3, 24) = 4.312
Figure 4.10 C τw (ms)	Treatment	0.179	ns	F (1.525, 12.20) = 2.021
	Genotype	0.009	**	F (1, 8) = 11.80
	Treatment x Genotype	0.322	ns	F (3, 24) = 1.227
Figure 4.10 E Rise time (ms)	Treatment	0.326	ns	F (2.040, 16.32) = 1.206
	Genotype	0.834	ns	F (1, 8) = 0.04677
	Treatment x Genotype	0.308	ns	F (3, 24) = 1.266
Figure 4.10 G Charge transfer (pC/s)	Treatment	0.022	*	F (2.120, 16.96) = 4.749
	Genotype	0.919	ns	F (1, 8) = 0.01106
	Treatment x Genotype	0.382	ns	F (3, 24) = 1.067
Figure 4.11 C Δ RMS (pA)	Treatment	0.3824	ns	F (1.953, 13.67) = 1.027
	Genotype	0.5764	ns	F (1, 7) = 0.3432
	Treatment x Genotype	0.953	ns	F (2, 14) = 0.04873

Table 4.15 $\alpha4^{Q246M}$ – paired recordings after 2 bottle choice - two-way ANOVA statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Figure 4.16 C Amplitude (pA)	Treatment	0.002	**	F (1.551, 6.205) = 22.06
	Genotype	0.076	ns	F (1, 4) = 5.667
	Treatment x Genotype	0.315	ns	F (3, 12) = 1.316
Figure 4.16 E Frequency (Hz)	Treatment	0.009	**	F (1.571, 6.283) = 12.05
	Genotype	0.779	ns	F (1, 4) = 0.08996
	Treatment x Genotype	0.292	ns	F (3, 12) = 1.395
Figure 4.17 C τw (ms)	Treatment	0.023	*	F (1.114, 4.454) = 11.26
	Genotype	0.026	*	F (1, 4) = 12.05
	Treatment x Genotype	0.029	*	F (3, 12) = 4.241
Figure 4.17 E Rise time (ms)	Treatment	0.223	ns	F (1.959, 7.838) = 1.827
	Genotype	0.454	ns	F (1, 4) = 0.6870
	Treatment x Genotype	0.182	ns	F (3, 12) = 1.907
Figure 4.17 G Charge transfer (pC/s)	Treatment	0.018	*	F (1.332, 5.326) = 10.51
	Genotype	0.916	ns	F (1, 4) = 0.01251
	Treatment x Genotype	0.205	ns	F (3, 12) = 1.775
Figure 4.18 C Δ RMS (pA)	Treatment	0.022	*	F (2.099, 8.398) = 6.149
	Genotype	0.832	ns	F (1, 4) = 0.05126
	Treatment x Genotype	0.075	ns	F (3, 12) = 2.966

Table 4.16 $\alpha2^{Q241M}$ – paired recordings after 2 bottle choice - two-way ANOVA statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Figure 4.19 C Amplitude (pA)	Treatment	0.189	ns	F (1.084, 4.336) = 2.450
	Genotype	0.127	ns	F (1, 4) = 3.701
	Treatment x Genotype	0.311	ns	F (3, 12) = 1.327
Figure 4.19 E Frequency (Hz)	Treatment	0.015	*	F (1.075, 4.302) = 15.28
	Genotype	0.605	ns	F (1, 4) = 0.3138
	Treatment x Genotype	0.055	ns	F (3, 12) = 3.375
Figure 4.20 C τw (ms)	Treatment	0.265	ns	F (1.625, 6.500) = 1.615
	Genotype	0.120	ns	F (1, 4) = 3.892
	Treatment x Genotype	0.452	ns	F (3, 12) = 0.9392
Figure 4.20 E Rise time (ms)	Treatment	0.402	ns	F (1.803, 7.214) = 1.008
	Genotype	0.406	ns	F (1, 4) = 0.8594
	Treatment x Genotype	0.543	ns	F (3, 12) = 0.7499
Figure 4.20 G Charge transfer (pC/s)	Treatment	0.023	*	F (1.067, 4.266) = 11.85
	Genotype	0.351	ns	F (1, 4) = 1.112
	Treatment x Genotype	0.092	ns	F (3, 12) = 2.701
Figure 4.21 C Δ RMS (pA)	Treatment	0.026	*	F (2.032, 8.130) = 5.881
	Genotype	0.462	ns	F (1, 4) = 0.6616
	Treatment x Genotype	0.572	ns	F (3, 12) = 0.6955

Table 4.17 Mean mIPSC parameters in dentate gyrus granule cells during unpaired recordings

	Amplitude (-pA)	Frequency (Hz)	τw (ms)	Rise time (ms)	Charge transfer (pC/s)
α4^{Q246M}	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
Wt	45.3 ± 5.7	0.6 ± 0.2	17.6 ± 1.6	0.68 ± 0.08	0.27 ± 0.07
Wt + EtOH	41.3 ± 3.2	1.2 ± 0.2	16.9 ± 0.6	0.60 ± 0.03	1.06 ± 0.27
Hom	41.2 ± 3.4	0.7 ± 0.1	18.2 ± 0.9	0.49 ± 0.03	0.56 ± 0.10
Hom + EtOH	43.7 ± 4.7	0.6 ± 0.1	17.9 ± 1.2	0.56 ± 0.05	0.62 ± 0.14
α2^{Q241M}					
Wt	32.8 ± 2.9	0.5 ± 0.3	18.2 ± 1.4	0.53 ± 0.05	0.50 ± 0.12
Wt + EtOH	33.5 ± 4.3	0.7 ± 0.3	16.1 ± 1.1	0.50 ± 0.06	0.53 ± 0.15
Hom	33.5 ± 7.4	0.3 ± 0.1	14.2 ± 1.3	0.53 ± 0.07	0.25 ± 0.09
Hom + EtOH	32.9 ± 3.9	0.5 ± 0.2	14.6 ± 1.4	0.50 ± 0.06	0.24 ± 0.05

Table 4.18 Mean tonic current parameters in dentate gyrus granule cells during unpaired recordings

	Holding current (-pA)			RMS noise (pA)		
	Control	Bic	Control vs Bic	Control	Bic	Control vs Bic
α4^{Q246M}	**Mean ± SEM**		**P value**	**Mean ± SEM**		**P value**
Wt	52.8 ± 15.3	47.3 ± 14.8	**<0.001**	3.6 ± 0.3	3.0 ± 0.2	**0.009**
Wt + EtOH	53.0 ± 1.8	45.8 ± 1.5	**0.026**	3.8 ± 0.2	3.3 ± 0.1	**<0.001**
Hom	47.2 ± 8.9	40.3 ± 9.1	**0.008**	3.3 ± 0.2	2.9 ± 0.1	**<0.001**
Hom + EtOH	59.1 ± 17.6	54.7 ± 17.3	**0.007**	3.6 ± 0.2	3.2 ± 0.2	**<0.001**
α2^{Q241M}						
Wt	69.4 ± 19.2	64.6 ± 18.4	**0.007**	2.8 ± 0.1	2.3 ± 0.1	**0.008**
Wt + EtOH	82.4 ± 15.1	77.3 ± 14.6	**0.005**	2.8 ± 0.2	2.5 ± 0.2	**0.009**
Hom	51.9 ± 7.5	46.5 ± 7.5	**0.026**	2.6 ± 0.1	2.4 ± 0.1	**<0.001**
Hom + EtOH	97.7 ± 30.5	89.9 ± 28.0	**0.041**	2.6 ± 0.1	2.3 ± 0.1	**0.047**

	Tonic current (-pA)	Δ RMS (pA)
α4^{Q246M}	**Mean ± SEM**	**Mean ± SEM**
Wt	5.5 ± 0.6	0.6 ± 0.1
Wt + EtOH	7.2 ± 1.8	0.5 ± 0.1
Hom	6.9 ± 1.8	0.4 ± 0.1
Hom + EtOH	4.4 ± 1.2	0.4 ± 0.1
α2^{Q241M}		
Wt	4.8 ± 1.1	0.5 ± 0.1
Wt + EtOH	5.0 ± 0.9	0.3 ± 0.1
Hom	5.4 ± 1.7	0.2 ± 0.1
Hom + EtOH	7.8 ± 2.6	0.3 ± 0.1

Table 4.19 $\alpha4^{Q246M}$ – unpaired recording statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Figure 4.5 E Amplitude (pA)	Treatment	0.868	ns	F (1, 29) = 0.02816
	Genotype	0.852	ns	F (1, 29) = 0.03548
	Treatment x Genotype	0.483	ns	F (1, 29) = 0.5042
Figure 4.5 F Frequency (Hz)	Treatment	0.060	ns	F (1, 28) = 3.843
	Genotype	0.082	ns	F (1, 28) = 3.264
	Treatment x Genotype	0.036	*	F (1, 28) = 4.850
Figure 4.6 C τw (ms)	Treatment	0.646	ns	F (1, 29) = 0.2157
	Genotype	0.522	ns	F (1, 29) = 0.4193
	Treatment x Genotype	0.846	ns	F (1, 29) = 0.03850
Figure 4.6 D Rise time (ms)	Treatment	0.984	ns	F (1, 29) = 0.0003925
	Genotype	0.031	*	F (1, 29) = 5.175
	Treatment x Genotype	0.148	ns	F (1, 29) = 2.205
Figure 4.6 E Charge transfer (pC/s)	Treatment	0.010	**	F (1, 27) = 7.693
	Genotype	0.636	ns	F (1, 27) = 0.2291
	Treatment x Genotype	0.023	*	F (1, 27) = 5.837
Figure 4.7 I Tonic current (pA)	Treatment	0.776	ns	F (1, 20) = 0.08293
	Genotype	0.645	ns	F (1, 20) = 0.2190
	Treatment x Genotype	0.180	ns	F (1, 20) = 1.929
Figure 4.8 E Δ RMS (pA)	Treatment	0.601	ns	F (1, 21) = 0.2821
	Genotype	0.120	ns	F (1, 21) = 2.621
	Treatment x Genotype	0.905	ns	F (1, 21) = 0.01470

	Uncorrected Fisher's LSD multiple comparison P values			
	CTRL Wt vs Hom	**EtOH** Wt vs Hom	**WT** Ctrl vs EtOH	**HOM** Ctrl vs EtOH
Amplitude (pA)	0.507	0.728	0.569	0.679
Frequency (Hz)	0.774	**0.011**	**0.011**	0.851
τw (ms)	0.738	0.575	0.668	0.838
Rise time (ms)	**0.009**	0.599	0.331	0.268
Charge transfer (pC/s)	0.178	0.053	**0.003**	0.776
Tonic current (pA)	0.513	0.214	0.495	0.185
Δ RMS (pA)	0.295	0.238	0.800	0.593

Table 4.20 $\alpha2^{Q241M}$ – unpaired recording statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Figure 4.12 E Amplitude (pA)	Treatment	0.991	ns	F (1, 29) = 0.0001351
	Genotype	0.992	ns	F (1, 29) = 0.0001126
	Treatment x Genotype	0.882	ns	F (1, 29) = 0.02251
Figure 4.12 F Frequency (Hz)	Treatment	0.053	ns	F (1, 29) = 4.076
	Genotype	0.083	ns	F (1, 29) = 3.233
	Treatment x Genotype	0.849	ns	F (1, 29) = 0.03699
Figure 4.13 C τw (ms)	Treatment	0.508	ns	F (1, 27) = 0.4511
	Genotype	0.045	*	F (1, 27) = 4.433
	Treatment x Genotype	0.351	ns	F (1, 27) = 0.9022
Figure 4.13 D Rise time (ms)	Treatment	0.640	ns	F (1, 27) = 0.2234
	Genotype	0.991	ns	F (1, 27) = 0.0001292
	Treatment x Genotype	0.971	ns	F (1, 27) = 0.001320
Figure 4.13 E Charge transfer (pC/s)	Treatment	0.957	ns	F (1, 27) = 0.002904
	Genotype	0.030	*	F (1, 27) = 5.222
	Treatment x Genotype	0.860	ns	F (1, 27) = 0.03158
Figure 4.14 I Tonic current (pA)	Treatment	0.458	ns	F (1, 18) = 0.5751
	Genotype	0.334	ns	F (1, 18) = 0.9850
	Treatment x Genotype	0.525	ns	F (1, 18) = 0.4202
Figure 4.15 E Δ RMS (pA)	Treatment	0.444	ns	F (1, 18) = 0.6123
	Genotype	0.176	ns	F (1, 18) = 1.989
	Treatment x Genotype	0.149	ns	F (1, 18) = 2.270

	Uncorrected Fisher's LSD multiple comparison P values			
	CTRL Wt vs Hom	**EtOH** Wt vs Hom	**WT** Ctrl vs EtOH	**HOM** Ctrl vs EtOH
Amplitude (pA)	0.915	0.918	0.897	0.930
Frequency (Hz)	0.174	0.264	0.157	0.166
τw (ms)	**0.042**	0.415	0.239	0.852
Rise time (ms)	0.986	0.973	0.748	0.734
Charge transfer (pC/s)	0.153	0.089	0.865	0.934
Tonic current (pA)	0.801	0.281	0.939	0.333
Δ RMS (pA)	**0.044**	0.947	0.123	0.615

Chapter 5 Voluntary alcohol consumption after finasteride treatment

5.1 Introduction

Several lines of evidence suggest a significant interaction between neurosteroids and ethanol. This conclusion is supported by findings from various experimental approaches and methodologies.

Firstly, acute ethanol administration increases the concentration of THDOC and allopregnanolone in the plasma, cerebral cortex and hippocampus (Barbaccia et al., 1999). Furthermore, oral consumption of ethanol leads to an increase of allopregnanolone levels in the brain of male mice (Finn et al., 2004b, Gabriel et al., 2004). In humans, alcohol intoxication increases allopregnanolone levels in adolescent male and female subjects (Torres and Ortega, 2003, Torres and Ortega, 2004).

Secondly, a number of studies demonstrate that exogenous administration of allopregnanolone or its synthetic analogue, ganaxolone, increases ethanol consumption in mice (Ford et al., 2005, Morrow et al., 2001a, Ramaker et al., 2014) and in rats (Janak and Michael Gill, 2003, Nie and Janak, 2003). Conversely, other studies provide evidence for decreased ethanol drinking following allopregnanolone or ganaxolone treatment (Besheer et al., 2010, Cook et al., 2014c, Ramaker et al., 2015).

Thirdly, manipulations of endogenous neurosteroid biosynthesis alter ethanol intake and ethanol-induced functional effects. Pre-treatment of animals with finasteride, which inhibits the biosynthesis of allopregnanolone, reduces the extent of the ethanol-induced increase in neurosteroid levels (Khisti et al., 2002, VanDoren et al., 2000). Furthermore, finasteride impedes acquisition of ethanol drinking measured using lickometer chambers (Ford et al., 2008a, Ford et al., 2005, Ford et al., 2008b). In contrast, finasteride increased alcohol preference in late adolescent rats, whereas it had no significant effect in adult rats (Milivojevic and Covault, 2012). An electrophysiological study in hippocampal tissue determined that the action of ethanol on GABAergic inhibition was biphasic, and consisted of a rapid direct effect on $GABA_A$ receptor activity and an indirect effect that appeared to be mediated by neurosteroid biosynthesis, documenting that

ethanol-induced allopregnanolone synthesis can occur in brain slices (Sanna et al., 2004). That is, pre-treatment with finasteride did not affect the rapid increase in IPSC amplitude and decay time induced by ethanol, while it abolished the secondary increase of both parameters that was apparent between 20- and 40 minutes during bath application of ethanol.

Lastly, a strong interaction between ethanol and pregnane neurosteroids (notably allopregnanolone and pregnanolone) has been consistently shown in drug discrimination studies, where allopregnanolone and pregnanolone often fully substitute for the ethanol training dose (Bowen et al., 1999, Engel et al., 2001, Hodge et al., 2001).

To investigate the role of neurosteroid modulation of specific populations of $GABA_A$ receptors in alcohol-driven reward behaviour, we assessed ethanol consumption in two novel knock-in mouse models, $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$, which remove neurosteroid potentiation from α2-$GABA_A$ and α4-$GABA_A$ receptors, respectively. Our data suggest that both the α2 and α4 neurosteroid insensitive mice show decreased ethanol intake in the intermittent access two bottle choice protocol. Whereas saccharin and quinine intake were unchanged for the same animal models.

Taking into account the aforementioned observations and their indication of an interaction between allopregnanolone and multiple ethanol-related behaviours, we hypothesise that endogenous neurosteroids modulate voluntary consumption of ethanol under physiologically relevant conditions. Thus, the aim of this chapter is to determine whether pharmacological suppression of endogenous allopregnanolone levels with finasteride would lead to alterations in ethanol intake or preference. We aim to characterise the effects of finasteride in C57BL/J6 mice and our two knock-in mouse lines, $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ mice.

5.2 Results

5.2.1 Protocol optimisation

Results from the intermittent access paradigm used in *Chapter 3* indicate that the most significant difference in ethanol consumption between wild type and homozygous mutant mice, in both $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ mouse lines, occurs within the first 24 hours of the experiment. Furthermore, it was suggested that rodents consume half of their daily intake within the first hour (Quadir et al., 2021). Consequently, our experiments with finasteride focused on the initial few hours during the first day of ethanol access.

The finasteride (FIN) dose was derived from studies that demonstrated that a 50 mg/kg dose reduced plasma and brain allopregnanolone levels by 66 % and 80 %, respectively, 24 hours post-injection (Finn et al., 2004a). Numerous other studies have also found this dose to be effective in other non-ethanol related behavioural paradigms when administered intraperitoneally (Kudryashov et al., 2020, Mosher et al., 2017, Mukai et al., 2008, Kokate et al., 1999, Mendell et al., 2020). Additionally, this FIN dose has been shown to effectively alter ethanol-related behaviours (Hirani et al., 2005; Gorin et al., 2005).

The timing of the finasteride injection (50 mg/kg) was based on studies reporting that the peak effect of finasteride on endogenous allopregnanolone levels occurs 24 hours post-injection in C57BL/J6 mice (Gorin-Meyer et al., 2007, Finn et al., 2004a). Therefore, we administered treatments (vehicle and finasteride) 20 hours prior to the drinking session. The mice were given 6-hour access to ethanol, which we postulated would cover the peak effect range of finasteride (20-26 hours post-injection) reported by Gorin-Meyer et al. (2007). Mice were habituated to the drinking bottles, inverted light cycle and single housing prior to the experiment.

The timeline of the experiment is illustrated in *Figure 5.1 A* and described in more detail in *Section 2.3.4*. The mean ± SEM values and statistical analysis of this experiment are contained in *Table 5.1* (control parameters), *Table 5.2* (ethanol intake and preference) and *Table 5.5* (statistics).

Vehicle- and finasteride-treated mice had no difference in their body weight (*Figure 5.1 B*) and their fluid intake during habituation was unchanged (*Figure 5.1 C*).

Two-way RM ANOVA of ethanol intake over the 6-hour period revealed no significant effect of treatment (*Figure 5.2 A*). Furthermore, there was no significant difference at any time point between vehicle and finasteride treatment groups (*Figure 5.2 C, D and E*). Animals consumed the highest quantity of alcohol in the first two-hour period, and their consumption decreased thereafter over the course of the experiment.

Similarly, analysis of ethanol preference over 6 hours detected no significant effect of treatment (*Figure 5.2 B*). Furthermore, at no time point was there a significant difference between vehicle and finasteride treatment groups (*Figure 5.2 F, G and H*).

Overall, these results suggest that finasteride does not alter ethanol intake or preference of C57BL/J6 mice at 20-26 hours post-injection.

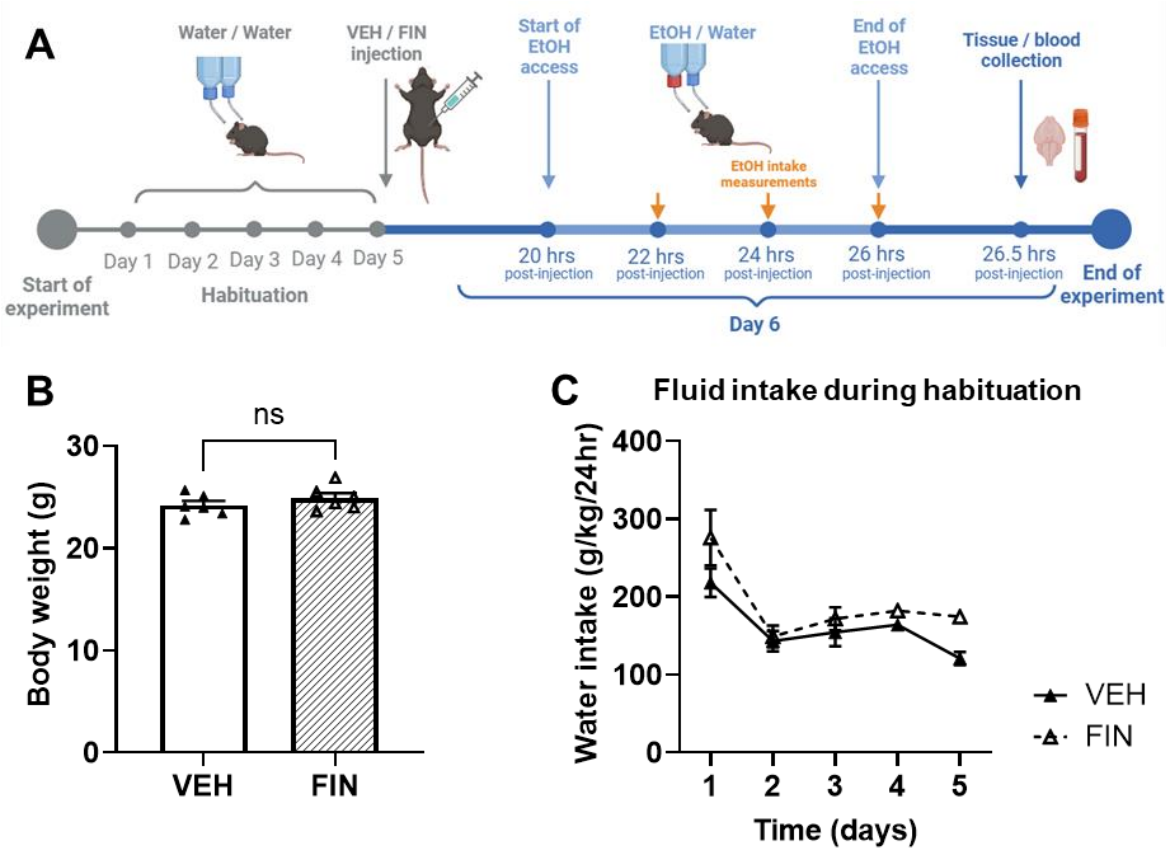


Figure 5.1 Protocol (A) and control parameters of the alcohol two bottle choice limited access paradigm for C57BL/J mice 20 hours post-injection

A, Schematic diagram of the limited access protocol used in the experiment (Protocol A). ***B,*** Body weight (in grams) of animals at the start of the experiment. ***C,*** Water intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are unpaired student's t test for panel *B*, and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panel *C*. VEH: n = 6; FIN: n = 6.

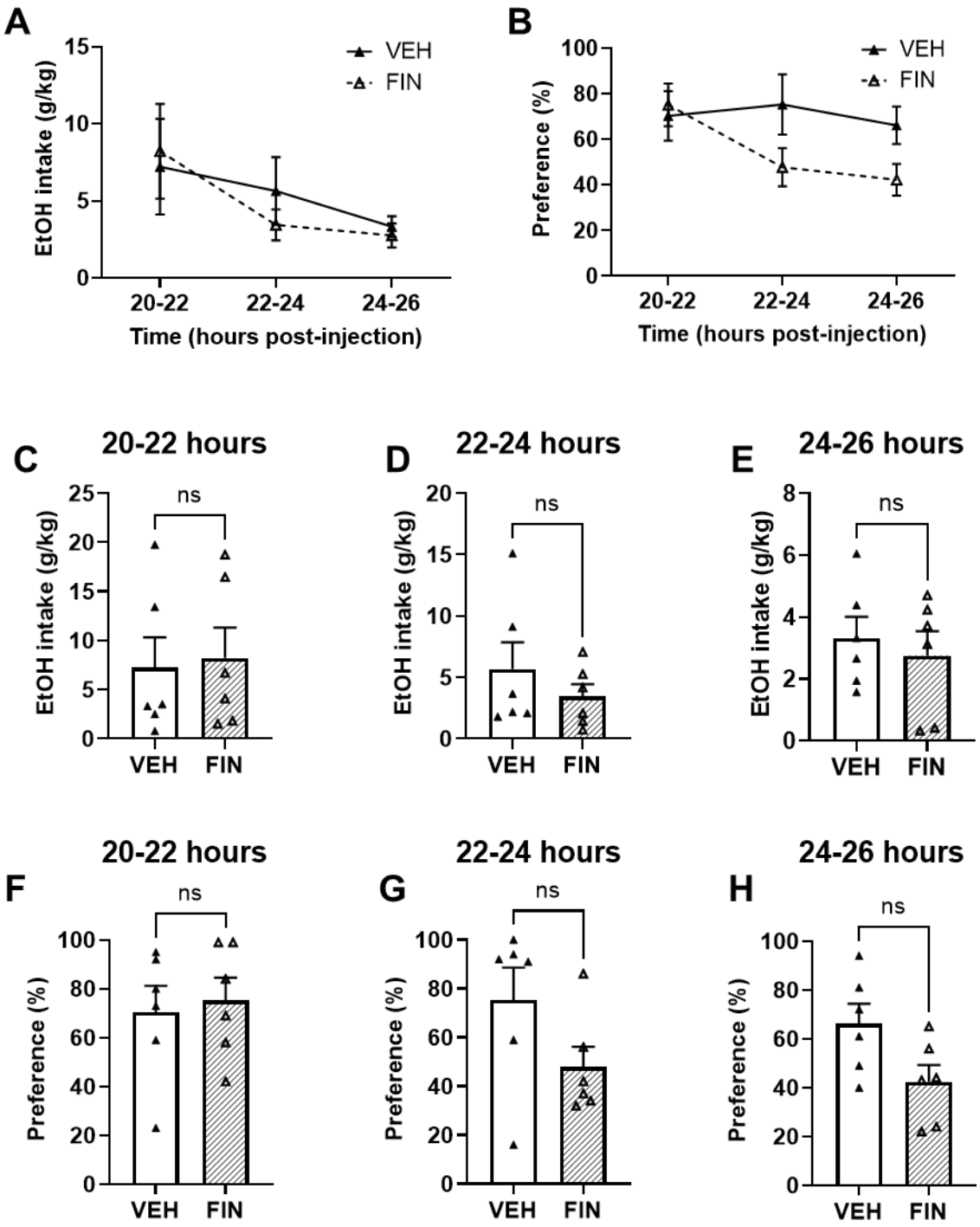


Figure 5.2 Protocol A - Finasteride does not affect ethanol intake or preference of C57BL/J6 at 20-26 hours post-injection

A and ***B,*** Ethanol intake (g/kg) and preference (%) over the course of the whole experiment, respectively. ***C, D*** *and* ***E,*** Ethanol intake (g/kg) at 22, 24 and 26 hours post-injection, respectively. ***F, G*** *and* ***H,*** Ethanol preference (%) at 22, 24 and 26 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, unpaired student's t test for panels *C, E, F* and *H*, and Mann-Whitney test for panels *D* and *G*. VEH: n = 6; FIN: n = 6.

Upon reviewing the results of these experiments, we considered the possibility that we may have missed the optimal time window for finasteride's effectiveness. A review of the literature revealed that several other research groups employ much shorter timing protocols for finasteride administration, starting from 15 minutes (Kudryashov et al., 2020, Mosher et al., 2017, Bortolato et al., 2008, Frau et al., 2013, Pallarès et al., 2015). Consequently, we decided to conduct an exploratory study in which we assessed ethanol intake of mice over a 24-hour period, starting 2 hours post-injection.

The timeline of the experiment is illustrated in *Figure 5.3 A* and described in more detail in *Section 2.3.4*. The mean ± SEM values and statistical analysis of this experiment are contained in *Table 5.1* (control parameters), *Table 5.3* (ethanol intake and preference) and *Table 5.5* (statistics).

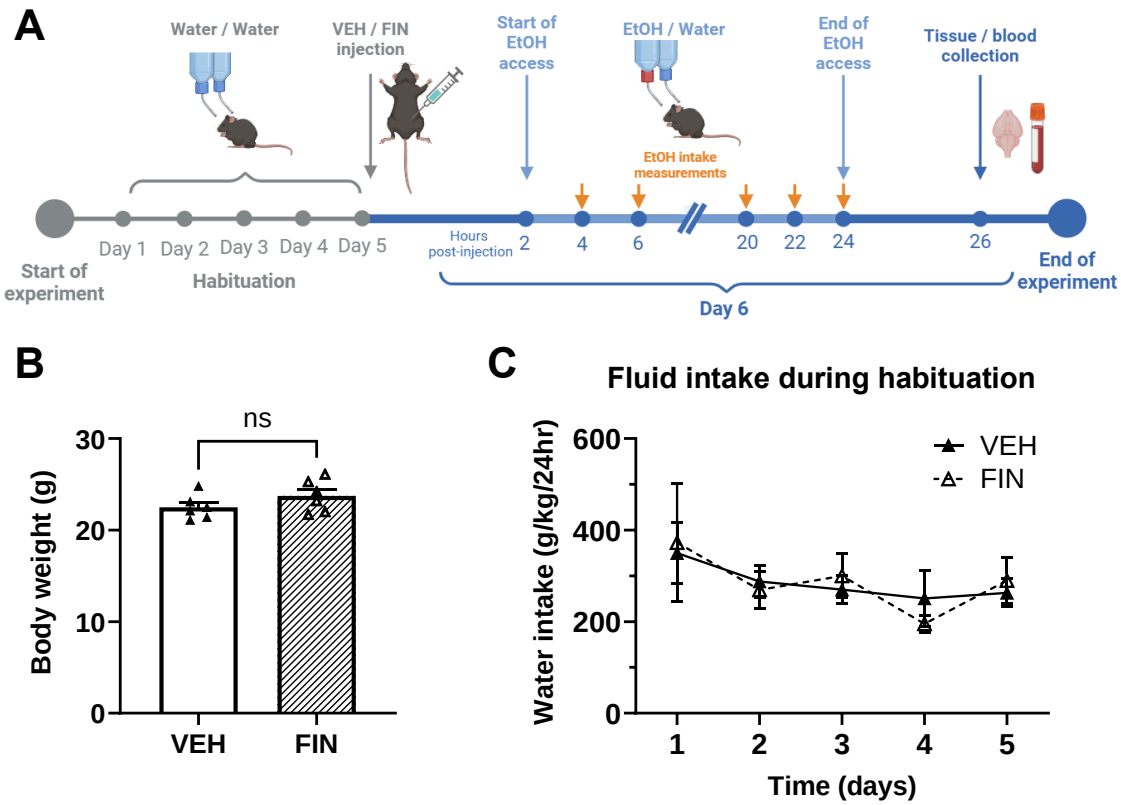


Figure 5.3 Protocol (B) and control parameters of the 24-hour alcohol two bottle choice limited access paradigm for C57BL/J6 mice 2 hours post-injection

A, Schematic diagram of the limited access protocol used in the experiment (Protocol B). ***B,*** Body weight (in grams) of animals at the start of the experiment. ***C,*** Water intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are unpaired student's t test for panel *B*, and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences*;* all comparisons are non-significant) for panel *C*. VEH: n = 6; FIN: n = 6.

C57BL6/J6 mice treated with either the vehicle or finasteride showed no differences in body weight (Figure 5.3 B), and their fluid intake during habituation remained consistent (*Figure 5.3 C*).

Analysis of ethanol intake over 24 hours revealed a significant effect of treatment; with finasteride-treated mice consuming less ethanol (*Figure 5.4 A*). Surprisingly, the difference was the largest in the initial few hours (2-8 hours) post-injection. Moreover, this experiment effectively highlighted the necessity of conducting limited access ethanol drinking paradigms during the dark phase, as mice exhibited minimal ethanol consumption during the light phase.

Finasteride had a significant effect on ethanol preference over the course of the whole experiment (*Figure 5.4 B*). FIN mice had lower preference relative to VEH treated mice. Similar to ethanol intake, the largest difference between the treatment groups was observed in the initial hours. Finasteride-treated mice displayed significantly lower preference for ethanol between 2 to 4 hours post-injection. Preference measurements during the light phase show high variability due to the minimal fluid consumption of the mice but there are no significant differences between vehicle and finasteride groups during this period.

The findings from the exploratory experiment indicated that finasteride influences ethanol drinking behaviour more rapidly than previously anticipated. The most pronounced differences were observed between 2 and 4 hours post-injection, with the effect diminishing over time. Consequently, we hypothesised that the effect might be even more pronounced at earlier time points. We conducted an experiment with a small cohort of animals (VEH: n = 4, FIN: n = 4, data not shown) where mice were given access to ethanol immediately after injection. However, we observed that the mice exhibited minimal movement and did not interact with the drinking bottles for at least an hour, resulting in skewed data.

Therefore, we decided to adhere to the protocol of providing access to ethanol bottles 2 hours post-injection, when the mice had resumed normal behaviour. The final protocol (Protocol C) is illustrated in *Figure 5.5 A*.

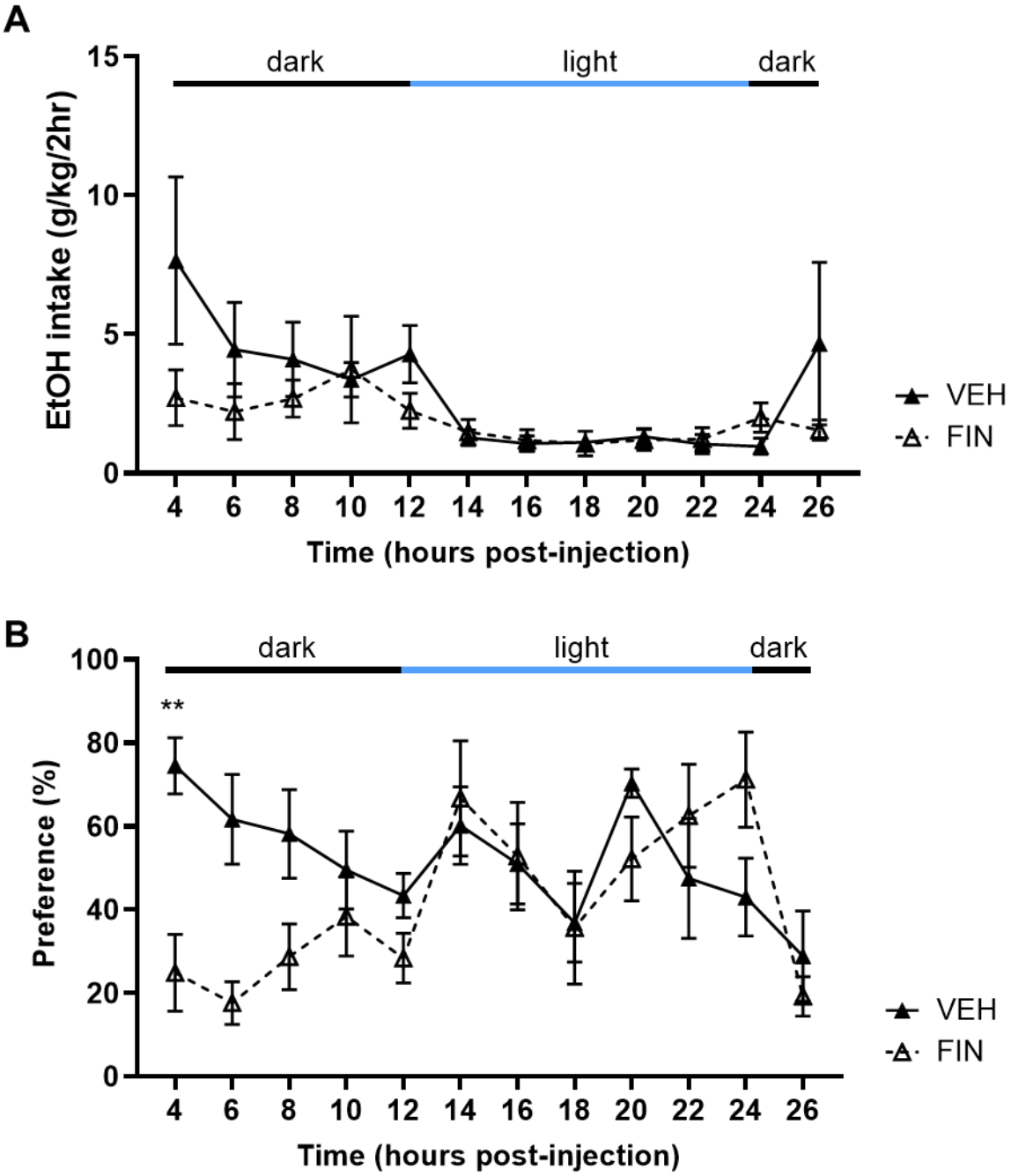


Figure 5.4 Protocol B - Finasteride impacts upon ethanol intake and preference of C57BL/J6 mice at 4-12 hours post-injection

A, Ethanol intake (g/kg) over the course of whole experiment. ***B,*** Ethanol intake preference (%) over the course of the whole experiment. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences). Statistical significance indicated as *** p < 0.01* compared to VEH. VEH: n = 6; FIN: n = 6.

5.2.2 Alcohol consumption of C57BL/J6 mice after finasteride treatment

We assessed ethanol intake of C57BL/J6 mice after finasteride pre-treatment using the protocol (Protocol C) outlined in *Figure 5.5 A.* The mean ± SEM values and statistical analysis of this experiment are contained in *Table 5.1* (control parameters)*, Table 5.4* (results) and *Table 5.5* (statistics) in *Section 5.5*.

The animals were age-matched (6-8 week old), had no difference in body weight (*Figure 5.5 B*) and their fluid intake during habituation was unchanged between the treatment groups (*Figure 5.5 C*).

Analysis of ethanol intake data over the 6-hour period revealed no significant difference between vehicle- and finasteride-treated mice (*Figure 5.6 A*). However, mice receiving vehicle injections consistently displayed higher intakes at every time point. Overall, the finasteride group showed a 37 % decrease in ethanol consumption compared to the vehicle group. Both treatment groups reduced their intake over the course of the experiment. Despite these patterns, ethanol consumption did not differ significantly between the treatment groups at any specific time point (*Figure 5.6 C, D and E*).

Two-way RM ANOVA detected a significant main effect of treatment on ethanol preference, with finasteride-treated mice showing lower preference for ethanol (*Figure 5.6 B*). Ethanol preference was significantly decreased in the finasteride group relative to the vehicle group at every time point (*Figure 5.6 F, G and H*).

Overall fluid intake was not influenced by finasteride treatment (*Figure 5.7 A*), both VEH and FIN mice consumed the same amount of fluid (water + ethanol) and their consumption decreased over time. There was no significant difference between treatment groups at any time point (*Figure 5.7 C, D and E*).

However, finasteride-treated mice consumed significantly more water than vehicle-treated mice over the 6-hour experimental period (*Figure 5.7 B*). The largest difference between the two groups was observed during the initial two hours of the experiment (*Figure 5.7 F*). This difference gradually decreased over time. A significant difference was still evident during the middle two hours of the experiment (*Figure 5.7 G*). However, while the final two hours did not show a statistically significant difference, a trend was still present (*Figure 5.7 H*).

The substantial difference in ethanol preference between the treatment groups is attributed to the trend towards reduced ethanol consumption and the significant increase in water intake by finasteride-treated mice upon ethanol exposure. The lack of change in overall fluid intake is due to the reduction in ethanol consumption and the corresponding increase in water intake by finasteride-treated mice, resulting in no net change in total fluid intake.

Nevertheless, pre-treatment of mice with finasteride alters voluntary consumption patterns of ethanol in C57BL/J6 mice studied in a 6-hour limited access paradigm.

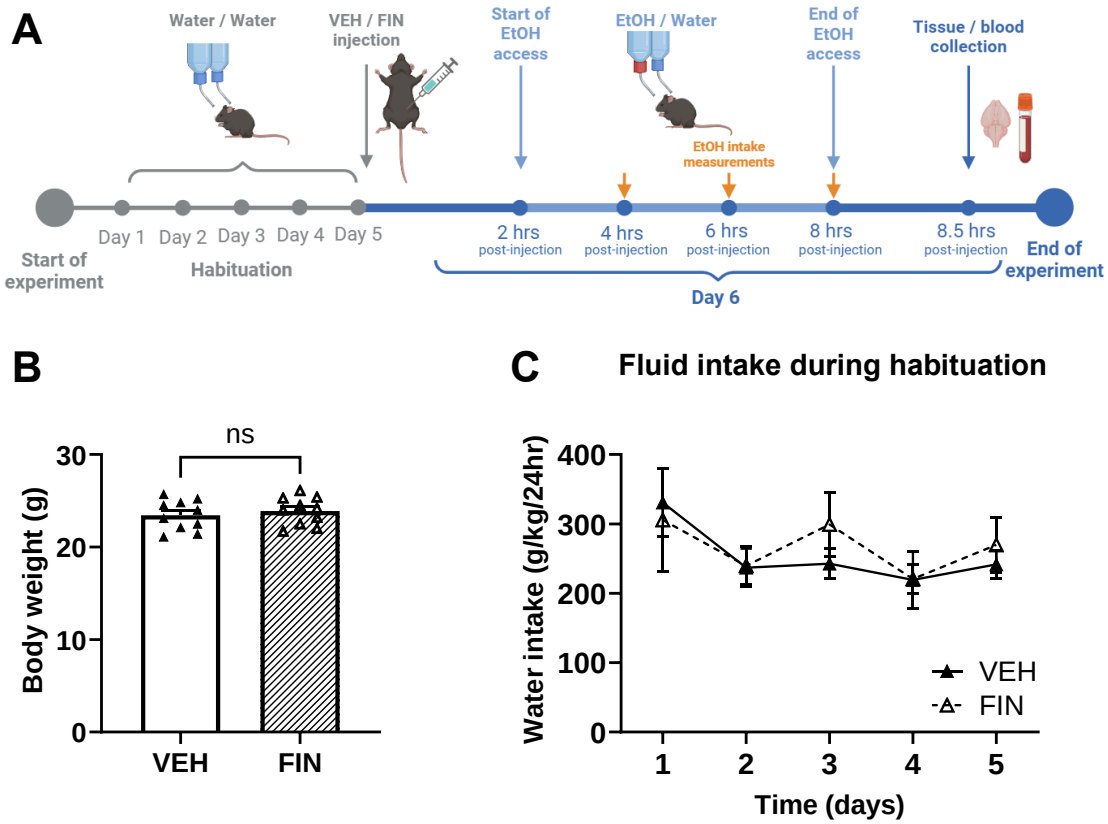


Figure 5.5 Protocol (C) and control parameters of the alcohol two bottle choice limited access paradigm for C57BL/J6 mice 2 hours post-injection for 6 hours

A, Schematic diagram of the limited access protocol used in the experiment (Protocol C). ***B,*** Body weight (in grams) of animals at the start of the experiment. ***C,*** Water intake (g/kg/24 hrs) of animals during the habituation period. Data are shown as mean ± SEM. Statistical tests used are unpaired student's t test for panel *B*, and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences; all comparisons are non-significant) for panel *C*. VEH: n = 10; FIN: n = 10.

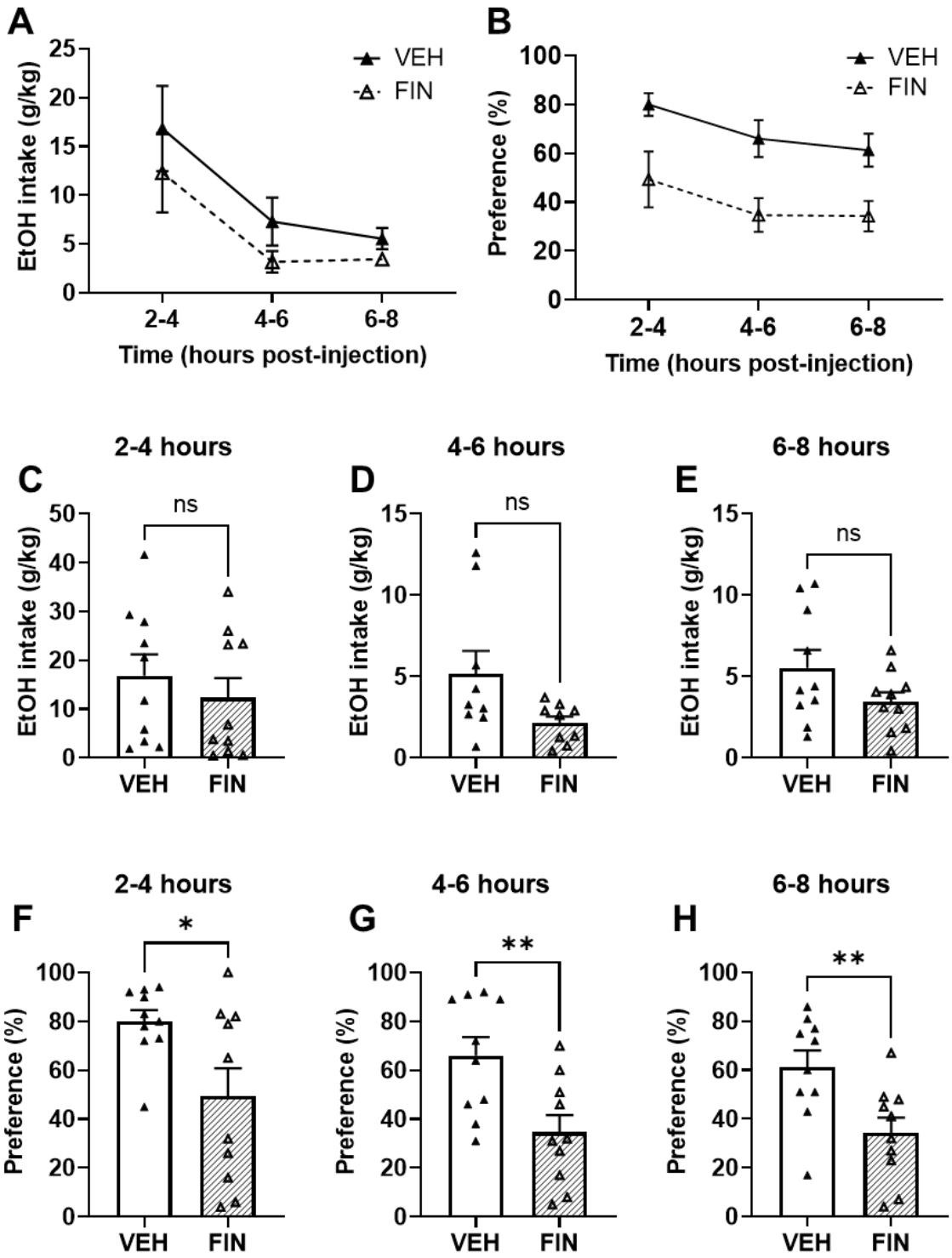


Figure 5.6 Protocol C - Finasteride influences ethanol preference of C57BL/J6 mice at 2-8 hours post-injection

A and ***B,*** Ethanol intake (g/kg) and preference (%) over the course of whole experiment, respectively. ***C, D*** *and* ***E,*** Ethanol intake (g/kg) at 4, 6 and 8 hours post-injection, respectively. ***F, G*** *and* ***H,*** Ethanol preference (%) at 4, 6 and 8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, Mann-Whitney test for panel *C,* unpaired student's t test for panels *E, G* and *H*, and unpaired student's t test with Welch's correction for panels *D* and *F.* Statistical significance indicated as * $p < 0.05$, ** $p < 0.01$ compared to VEH. VEH: n = 10; FIN: n = 10.

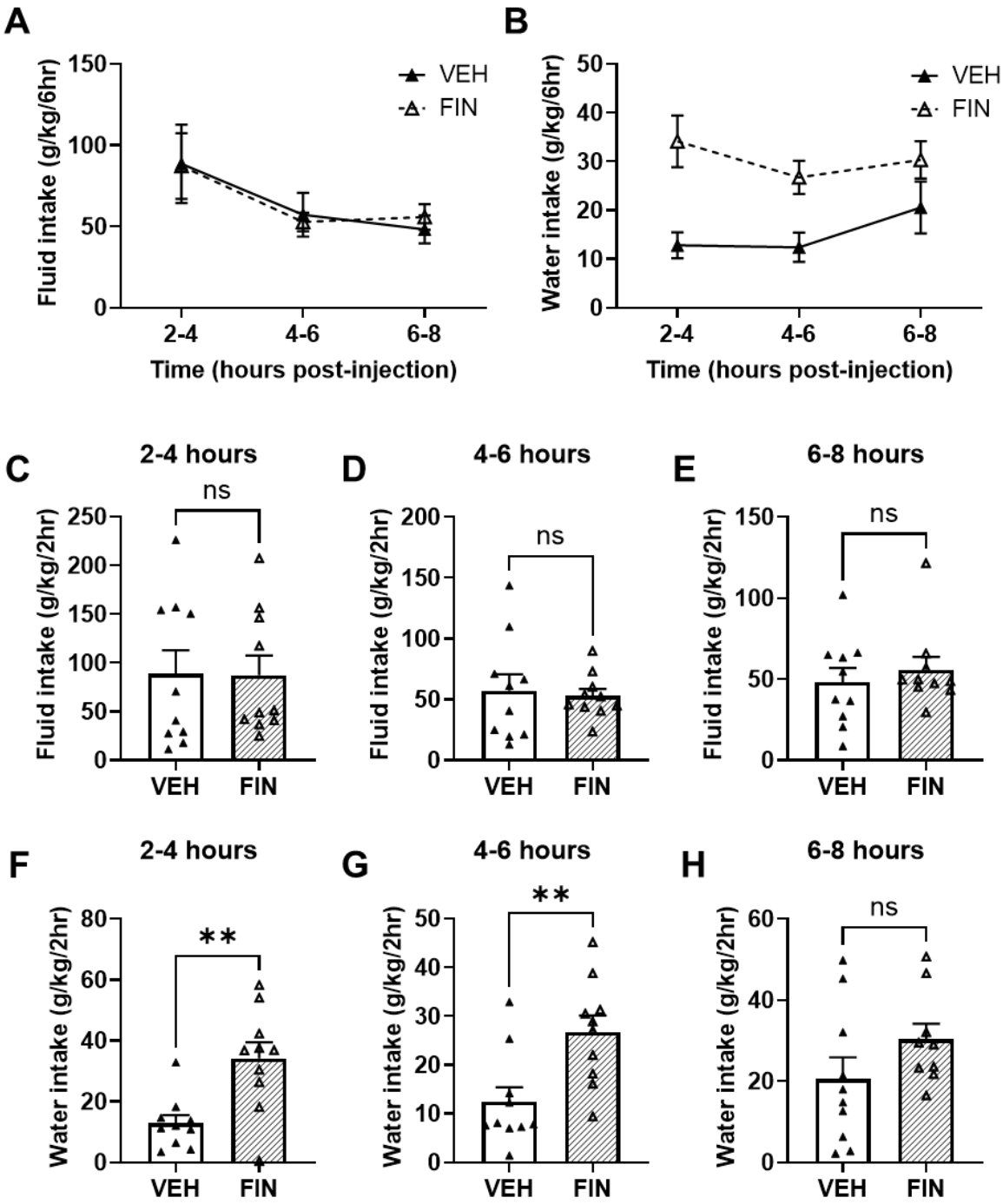


Figure 5.7 Protocol C - Finasteride affects water intake of C57BL/J6 mice upon ethanol exposure ***A*** and ***B,*** Fluid intake (g/kg) and water intake (g/kg) over the course of whole experiment, respectively. ***C, D*** *and* ***E,*** Fluid intake (g/kg) at 4, 6 and 8 hours post-injection, respectively. ***F, G*** *and* ***H,*** Water intake (%) at 4, 6 and 8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, Mann-Whitney test for panel *C, E* and *G,* unpaired student's t test for panels *F* and *H*, and unpaired student's t test with Welch's correction for panels *D.* Statistical significance indicated as ** $p < 0.01$ compared to VEH. VEH: n = 10; FIN: n = 10.

5.2.3. Alcohol consumption of α4^{Q246M} mice after finasteride treatment

Ethanol consumption in male α4^{Q246M} wild type and homozygous mutant mice was assessed following finasteride treatment using the limited access two-bottle choice protocol described in *Section 5.2.2*. The mean ± SEM values and statistical analysis results of this experiment are contained in *Table 5.6* (control parameters), *Table 5.7* (results), *Table 5.8* (ethanol intake statistics), *Table 5.9* (ethanol preference statistics), *Table 5.10* (fluid intake statistics) and *Table 5.11* (water intake statistics) in *Section 5.5*.

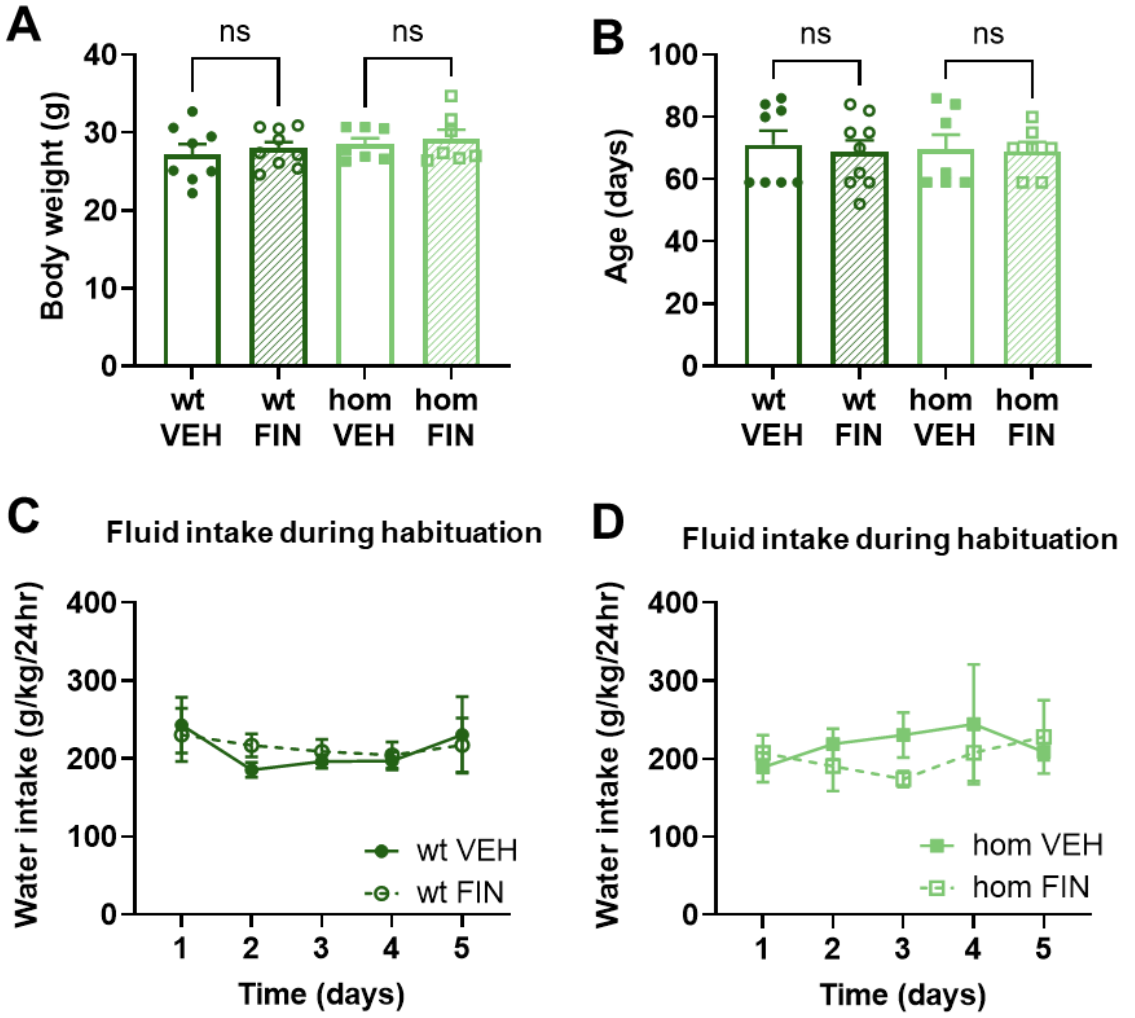


Figure 5.8 Control parameters of the alcohol two bottle choice limited access paradigm for α4^{Q246M} wild type and mutant mice 2 hours post-injection

A, Body weight (in grams) of animals at the start of the experiment. ***B,*** Age (in days) of animals at the start of the experiment. ***C*** *and* ***D,*** Water intake (g/kg/24 hrs) of wild type (*C*) and homozygous mutant (*D*) mice during the habituation period. Data are shown as mean ± SEM. Statistical tests used are ordinary one-way ANOVA with comparisons using Tukey's test for panels *A* and *B*, and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *C* and *D*. wt VEH: n = 8; wt FIN: n = 9, hom VEH: n = 7; hom FIN: n = 7.

The animals had no difference in their body weight (*Figure 5.8 A*) and were age-matched (*Figure 5.8 B*). Fluid intake during habituation was unaffected between treatment groups in either wild type (*Figure 5.8 C*) or homozygous mutant (*Figure 5.8 D*) mice.

Analysis of ethanol intake data over the 6-hour period revealed no significant differences between vehicle- and finasteride-treated mice in either genotype (*Figure 5.9 A* and *B*). Ethanol consumption across all four groups substantially decreased after the initial two hours. While no statistically significant difference was observed between the treatment groups over the entire 6-hour period, a trend was detected, with finasteride-treated mice, both wild type and mutant, showing reduced intakes (*Figure 5.9 C*).

Analysis of the first two time periods (2-4 and 4-6 hours) detected no significant effect of finasteride (*Figure 5.9 D* and *E*). However, two-way ANOVA revealed a significant treatment effect on ethanol intake at 6-8 hours (*Figure 5.9 F*). Finasteride reduced intake in both wild type and mutant mice, however, post-hoc analysis found no significant differences between treatment groups.

Two-way RM ANOVA of ethanol preference over the entire experiment revealed a significant effect of treatment in both wild type and homozygous mutant animals (*Figure 5.10 A* and *B*). Finasteride led to an overall reduction in ethanol preference in both genotypes. While the average preference of wild type animals did not differ significantly between vehicle and finasteride treatments, there was a trend towards reduced ethanol preference with finasteride (*Figure 5.10 C*). In contrast, finasteride significantly decreased average ethanol preference in mutant animals compared to vehicle-treated mutants (*Figure 5.10 C*). Additionally, two-way ANOVA of average preference indicated a significant treatment effect (*Figure 5.10 C*).

Analysis of specific time points showed that finasteride had the most significant effect at 6-8 hours (*Figure 5.10 F*), whereas at the other two time points (2-4 and 4-6 hours), the effect was not significant (*Figure 5.10 D* and *E*).

Fluid intake in both wild type and homozygous mutant animals was unaffected by treatment (*Figure 5.11 A and B*). Similar to ethanol intake, fluid consumption decreased over time. Total fluid intake over the 6-hour period was not impacted by finasteride treatment in either genotype (*Figure 5.11 C*). Moreover, post-hoc analyses of specific

time points revealed no significant differences between treatment groups in either wild type or mutant mice (*Figure 5.11 D, E and F*).

Two-way RM ANOVA detected no significant effect of treatment on water intake in wild type or homozygous mutant mice over the entire 6-hour experiment (*Figure 5.12 A and B*). However, it is noteworthy that vehicle- and finasteride-treated animals exhibited different trends in both genotypes. Water consumption increased over the course of the experiment for finasteride-treated mice, whereas it decreased for vehicle-treated animals. Total water intake over 6 hours was not affected by finasteride in either genotype (*Figure 5.12 C*).

Furthermore, analysis of specific time points revealed that treatment did not affect water consumption during the initial and middle 2-hour periods (*Figure 5.12 D* and *E*). However, a significant impact on water intake was observed in the final 2 hours (6-8 hours post-injection; *Figure 5.12 F*). Post-hoc analysis indicated a significant effect of finasteride in mutant animals, with a trend towards increased water intake in finasteride-treated wild type animals as well.

Therefore, given that ethanol intake is the same between the two treatment groups in the last two hours of the experiment for both genotypes (*Figure 5.9 A* and *B*), the decreased ethanol preference of finasteride-treated animals in this period can be attributed to the increased water intake.

Lastly, we did not observe any significant differences between vehicle-treated wild type and vehicle-treated homozygous mutant animals (*Figure 5.9 C, Figure 5.10 C, Figure 5.11 C* and *Figure 5.12 C*).

It was hypothesised that finasteride would have a greater effect on wild type animals compared to homozygous mutants, as the latter lack neurosteroid sensitivity in α4-containing $GABA_A$ receptor subpopulations. However, finasteride treatment did not differentially affect the genotypes, suggesting that other receptor subtypes may also be important.

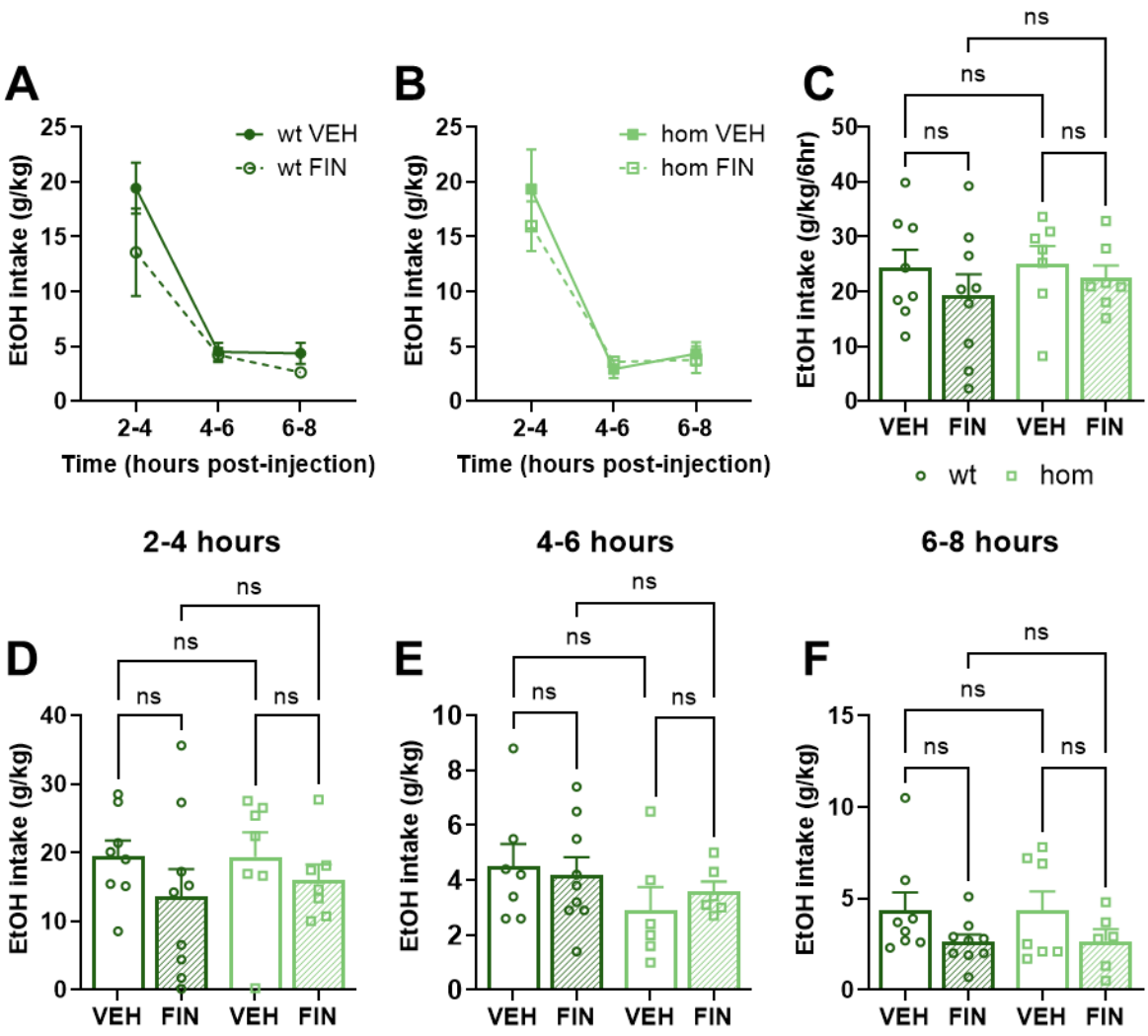


Figure 5.9 Finasteride does not influence ethanol intake of α4^{Q246M} wild type and mutant mice at 2-8 hours post-injection

A and ***B,*** Ethanol intake (g/kg) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. C, Total ethanol intake (g/kg) of mice over the 6-hour time period. ***D, E*** *and* ***F,*** Ethanol intake (g/kg) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C*, *D, E* and *F*. wt VEH: n = 8; wt FIN: n = 9, hom VEH: n = 7; hom FIN: n = 7.

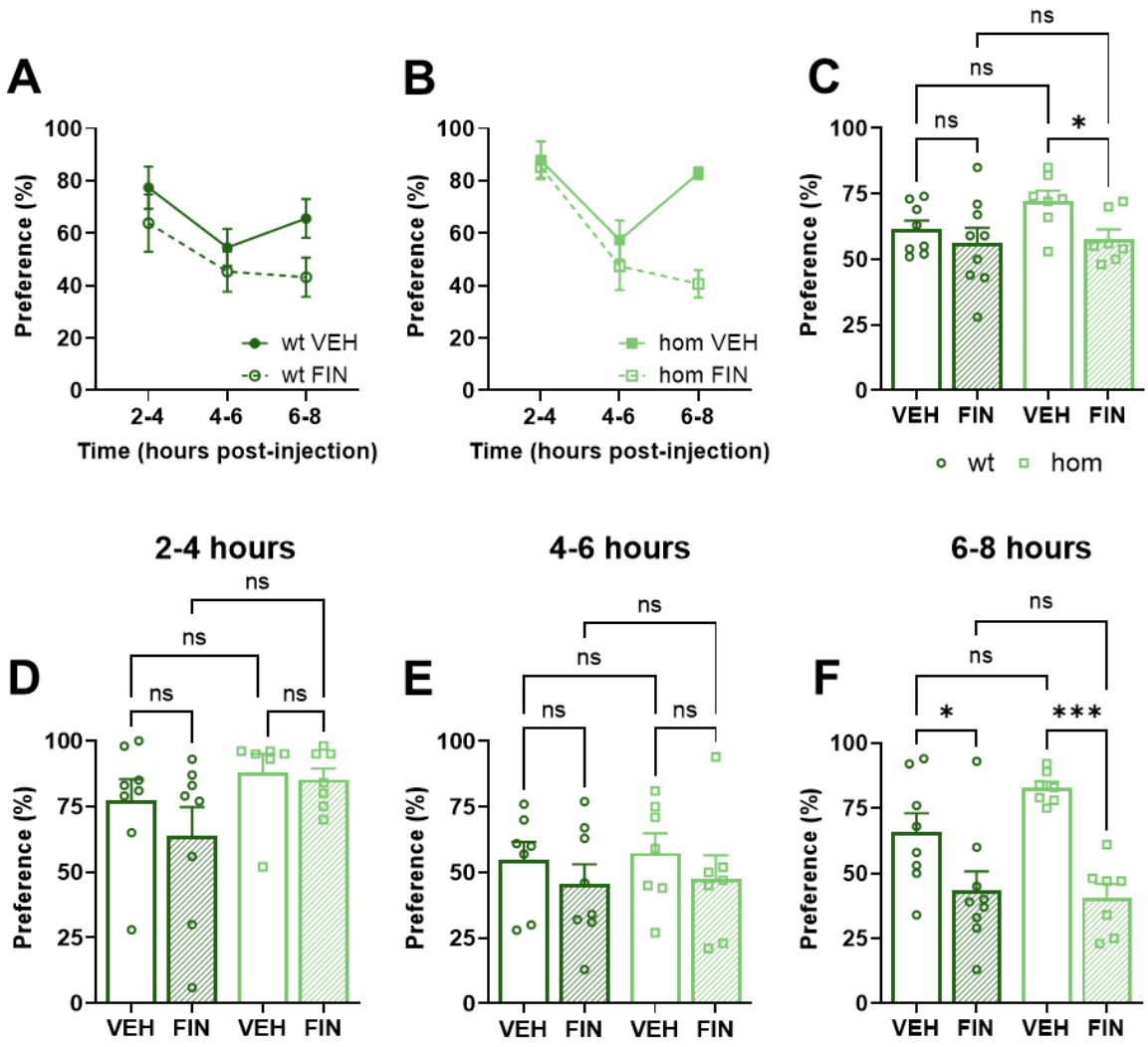


Figure 5.10 Finasteride alters ethanol preference of α4^{Q246M} wild type and mutant mice at 6-8 hours post-injection

A and ***B,*** Ethanol preference (%) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. ***C,*** Average preference (%) over the 6-hour time period. ***D, E*** *and* ***F,*** Ethanol preference (%) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C*, *D, E* and *F*. Statistical significance indicated as * $p < 0.05$, *** $p < 0.01$. wt VEH: n = 8; wt FIN: n = 9, hom VEH: n = 7; hom FIN: n = 7.

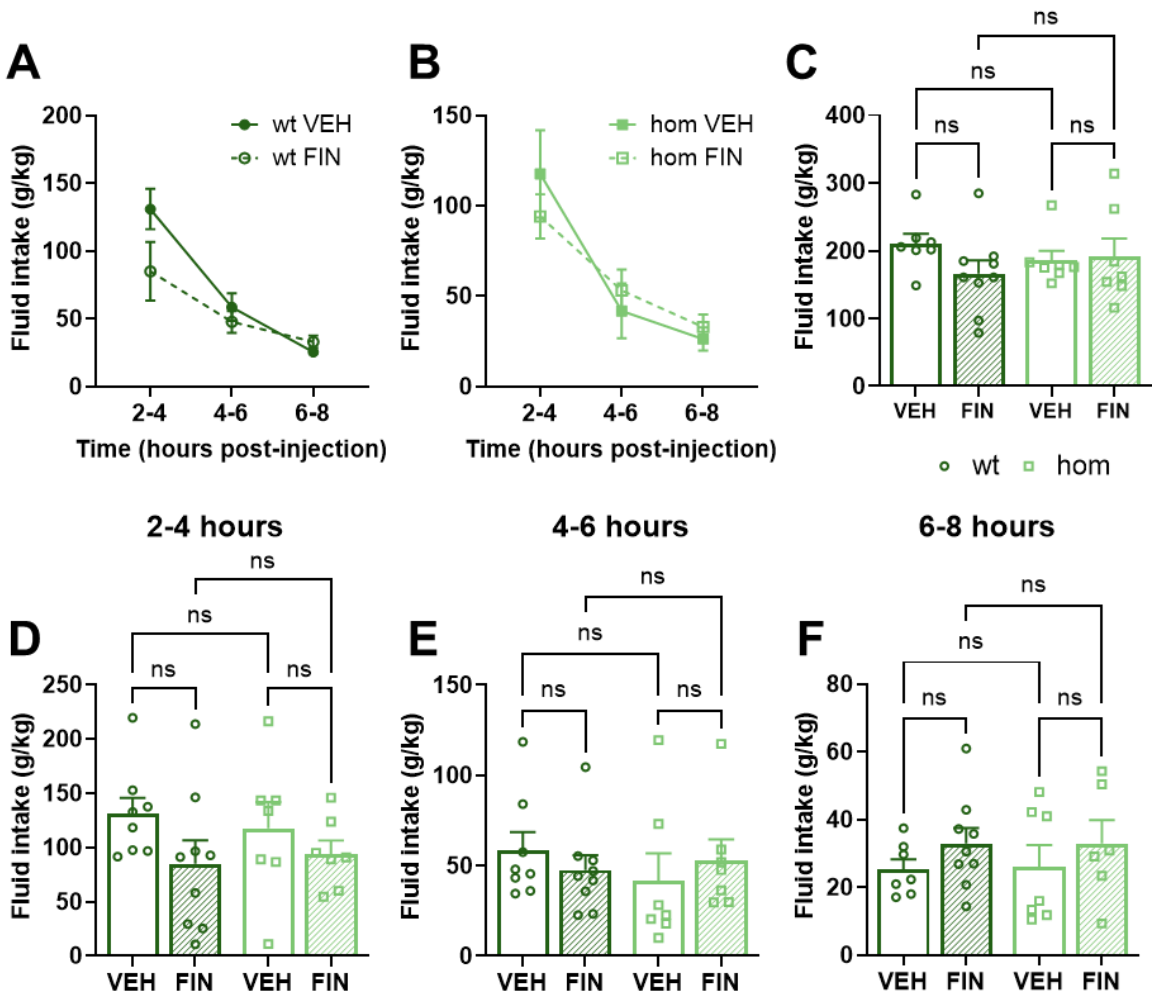


Figure 5.11 Finasteride does not impact upon fluid intake of α4^{Q246M} wild type and mutant mice at 2-8 hours post-injection

A and ***B,*** Fluid intake (g/kg) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. ***C,*** Total fluid intake (g/kg) over the 6-hour time period. ***D, E*** *and* ***F,*** Fluid intake (g/kg) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C*, *D, E* and *F*. wt VEH: n = 8; wt FIN: n = 9, hom VEH: n = 7; hom FIN: n = 7.

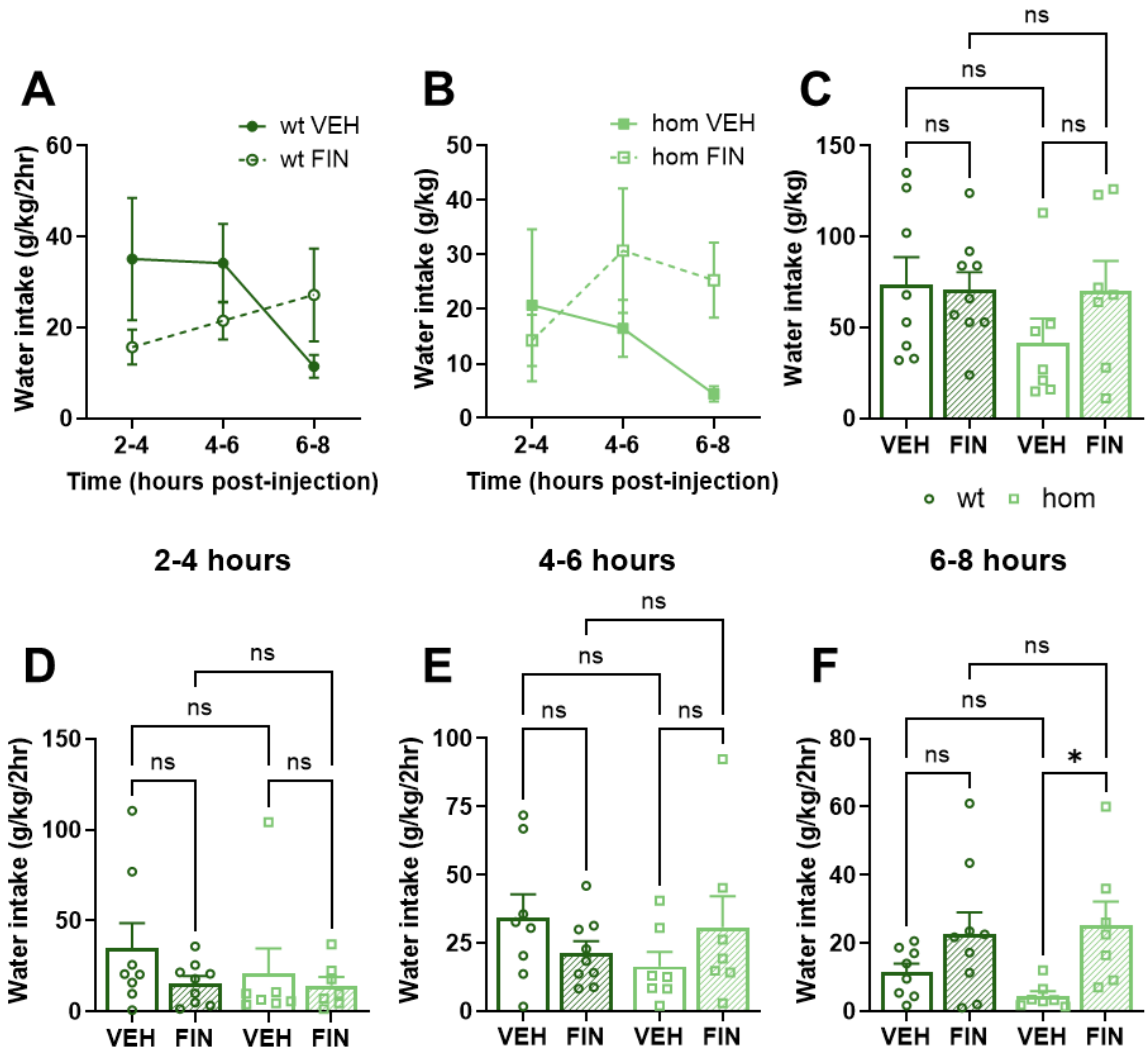


Figure 5.12 Finasteride treatment increases water intake at 6-8 hours post-injection

A and ***B,*** Water intake (g/kg) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. ***C,*** Total water intake (g/kg) over the 6-hour time period. ***D, E and F,*** Water intake (g/kg) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C, D, E* and *F*. wt VEH: n = 8; wt FIN: n = 9, hom VEH: n = 7; hom FIN: n = 7.

5.2.4 Alcohol consumption of α2^{Q241M} mice after finasteride treatment

The limited access two-bottle choice protocol, as detailed in *Section 5.2.2*, was employed to assess ethanol consumption in male α2^{Q241M} wild type and homozygous mutant mice following finasteride treatment. The mean ± SEM values and statistical analysis results of this experiment are contained in *Table 5.12* (control parameters), *Table 5.13* (results), *Table 5.14* (ethanol intake statistics), *Table 5.15* (ethanol preference statistics), *Table 5.16* (fluid intake statistics) and *Table 5.17* (water intake statistics) in *Section 5.5*.

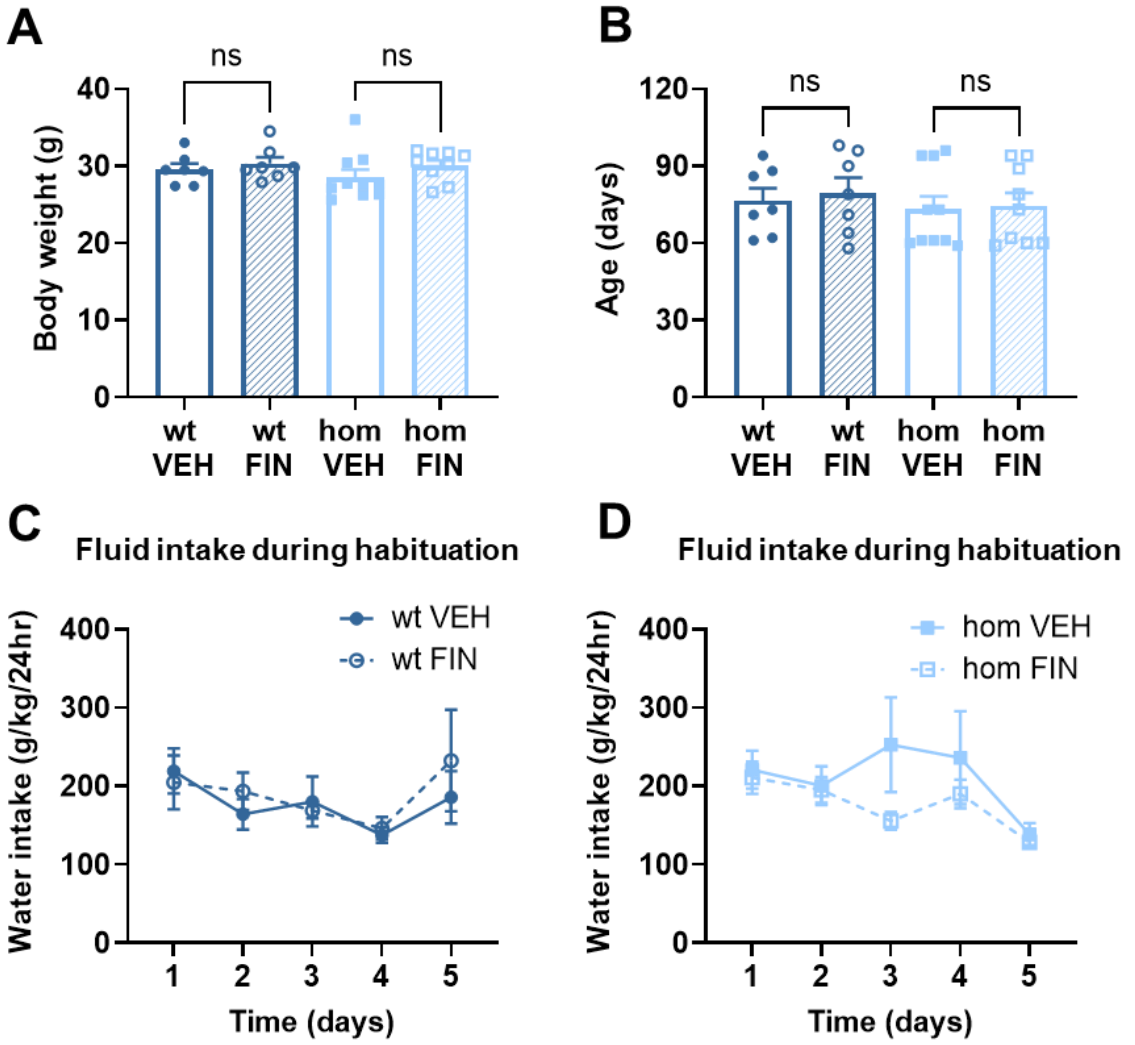


Figure 5.13 Control parameters of the alcohol two bottle choice limited access paradigm for α2^{Q241M} wild type and mutant mice 2 hours post-injection

A, Body weight (in grams) of animals at the start of the experiment. ***B,*** Age (in days) of animals at the start of the experiment. ***C*** *and* ***D,*** Water intake (g/kg/24 hrs) of wild type (*C*) and homozygous mutant (*D*) mice during the habituation period. Data are shown as mean ± SEM. Statistical tests used are ordinary one-way ANOVA with comparisons using Tukey's test for panels *A* and *B*, and two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *C* and *D*. wt VEH: n = 7; wt FIN: n = 7, hom VEH: n = 10; hom FIN: n = 9.

The animals exhibited no differences in body weight (*Figure 5.13 A*) and were age-matched (*Figure 5.13 B*). Fluid intake during habituation remained unaffected between treatment groups in both wild type (*Figure 5.13 C*) and homozygous mutant (*Figure 5.13 D*) mice.

Ethanol intake data over the 6-hour period showed no significant differences between vehicle- and finasteride-treated mice of either genotype (*Figure 5.14 A and B*). After the initial two hours, ethanol consumption markedly decreased across all four groups. Although the difference between treatment groups was not statistically significant over the 6-hour period, a trend indicated that finasteride-treated mice, both wild type and mutant, had slightly lower ethanol intakes (*Figure 5.14 C*). Additionally, there was a trend towards reduced ethanol consumption in homozygous mutant mice compared to wild type mice.

Analysis of ethanol intake at specific time points revealed no significant effect of finasteride in either genotype (*Figure 5.14 D, E* and *F*). However, finasteride had a differential impact on wild type and homozygous mutant mice at 4-6 hours post-injection. Interestingly, wild type mice treated with finasteride consumed more ethanol compared to mutant animals treated with finasteride (*Figure 5.14 E*).

Two-way RM ANOVA of ethanol preference throughout the experiment highlighted a significant treatment effect in both wild type and homozygous mutant animals (*Figure 5.15 A and B*). Finasteride administration resulted in an overall decrease in ethanol preference across both genotypes. Although the average ethanol preference among homozygous mutants did not show a significant difference between vehicle and finasteride treatments, there was a tendency towards reduced ethanol preference with finasteride (*Figure 5.15 C*). Conversely, in wild type animals, finasteride significantly reduced average ethanol preference compared to vehicle-treated mice (*Figure 5.15 C*). Furthermore, two-way ANOVA analysis of average preference confirmed a significant treatment effect (*Figure 5.15 C*).

Ethanol preference analysis at specific time points revealed that finasteride exerted its strongest effect during the initial 2 hours (*Figure 5.15 D*), with diminishing effects observed during the middle 2 hours (*Figure 5.15 E*) and the smallest impact at 6-8 hours

post-injection (*Figure 5.15 F*). Post-hoc analyses indicated significant differences only for homozygous mutants at 2-4 hours and for both genotypes at 4-6 hours post-injection.

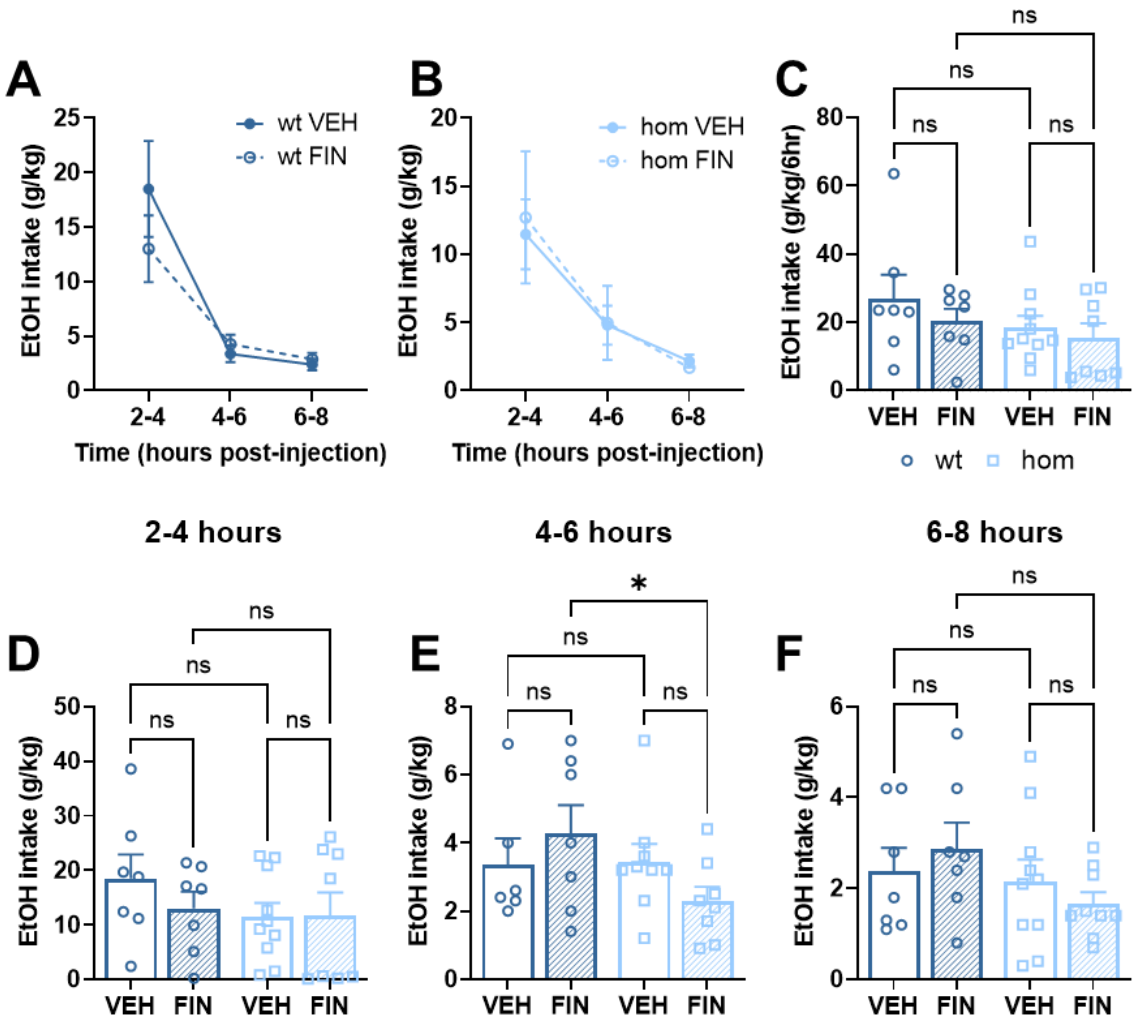


Figure 5.14 Finasteride does not influence ethanol intake of α2^{Q241M} wild type and mutant mice at 2-8 hours post-injection

A and ***B,*** Ethanol intake (g/kg) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. ***C,*** Total ethanol intake (g/kg) over the 6-hour experimental period. ***D, E*** *and* ***F,*** Ethanol intake (g/kg) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C, D, E* and *F*. Statistical significance indicated as * $p < 0.05$. wt VEH: n = 7; wt FIN: n = 7, hom VEH: n = 10; hom FIN: n = 9.

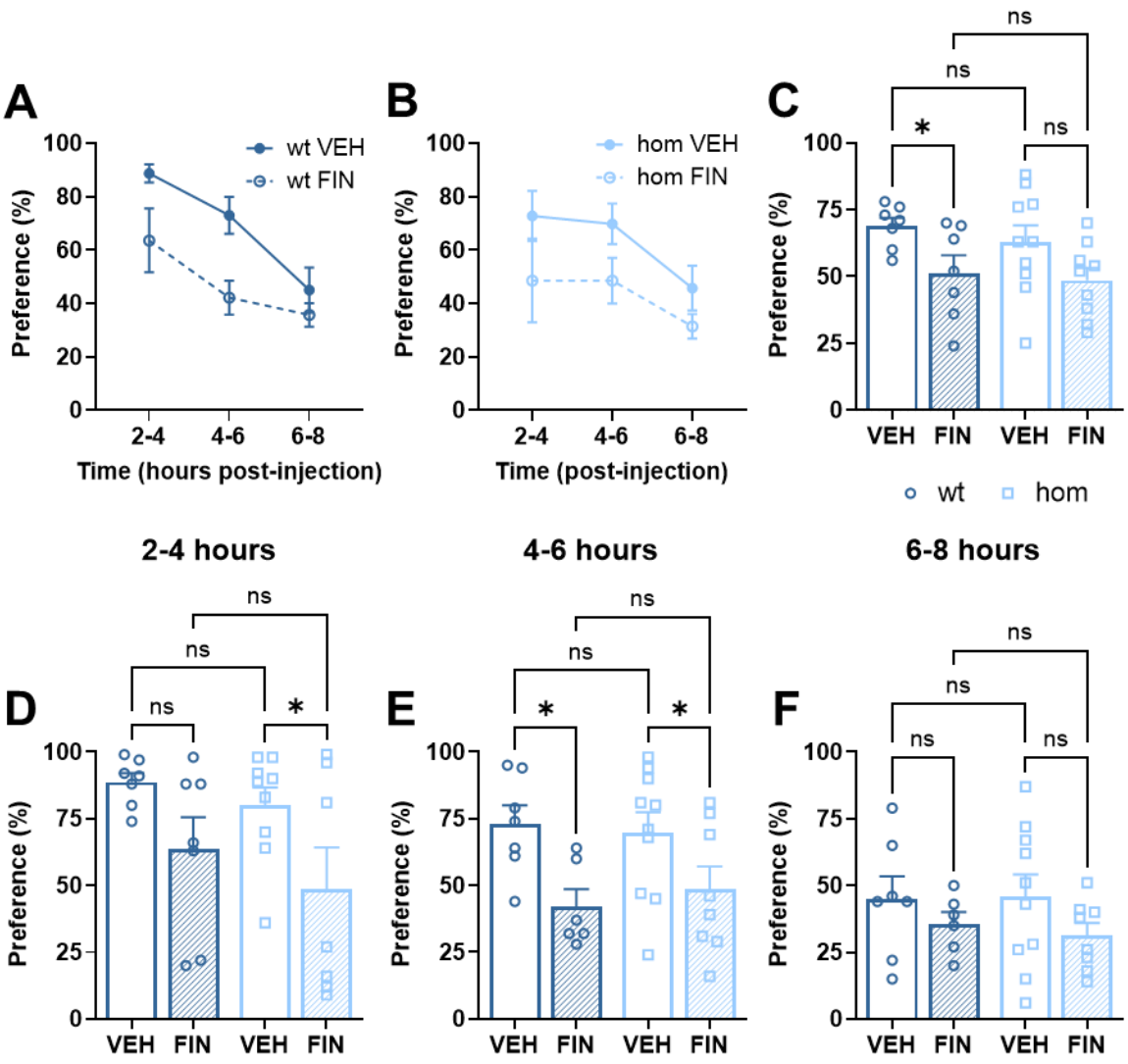


Figure 5.15 Finasteride transiently alters ethanol preference of α2^{Q241M} wild type and mutant mice at 4-6 hours post-injection

A and ***B,*** Ethanol preference (%) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. ***C,*** Average ethanol preference (%) over the 6-hour experimental period. ***D, E*** *and* ***F,*** Ethanol preference (%) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C*, *D, E* and *F*. Statistical significance indicated as * $p < 0.05$. wt VEH: n = 7; wt FIN: n = 7, hom VEH: n = 10; hom FIN: n = 9.

Total fluid intake in both wild type and homozygous mutant animals remained unaffected by treatment (*Figure 5.16 A* and *B*). Similar to ethanol intake, fluid consumption decreased progressively over the course of the experiment. The total fluid intake over the 6-hour period did not show any impact from finasteride treatment in either genotype (*Figure 5.16 C*). Furthermore, detailed analysis of specific time points found no significant differences between treatment groups in either wild type or mutant mice (*Figure 5.16 D, E* and *F*).

Two-way RM ANOVA revealed no significant treatment effect on water intake in wild type or homozygous mutant mice throughout the 6-hour experiment (*Figure 5.17 A* and *B*). However, it is notable that there were distinct trends observed between vehicle- and finasteride-treated animals in wild type mice. Water consumption declined over the experiment for animals treated with finasteride and increased for mice treated with vehicle (*Figure 5.17 A*). Finasteride-treated homozygous mutants maintained consistent intake, while vehicle-treated mutants decreased their water intake over the duration of the study (*Figure 5.17 B*). Total water intake over the 6-hour period remained unaffected by finasteride treatment in either genotype (*Figure 5.17 C*).

Additionally, analysis of specific time points indicated that treatment did not influence water consumption during the initial 2 hours (*Figure 5.17 D*), despite both genotypes showing a trend towards increased intake following finasteride administration. However, a significant impact on water intake was evident by finasteride between 4 to 6 hours post-injection (*Figure 5.17 E*). Post-hoc analysis revealed a significant effect of finasteride in wild type animals, with a tendency towards increased water intake observed in finasteride-treated mutant mice as well. During the final two hours of the experiment, finasteride did not affect water intake in either genotype (*Figure 5.17 F*).

During the initial 2-hour period, we observed no significant difference in total fluid consumption for wild type mice, as finasteride administration reduced ethanol intake but concurrently increased water intake, resulting in no net effect on total fluid consumption.

The differences in ethanol preference across various time points can be linked to the decreased ethanol intake and increased water consumption in animals treated with finasteride in both genotypes.

Similar to the $\alpha4^{Q246M}$ knock-in mouse model, it was hypothesised that finasteride would exert a more pronounced effect on wild type animals compared to homozygous mutants, given that neurosteroid sensitivity in α2-containing $GABA_A$ receptors is removed in mutant animals. Indeed, we observed a greater reduction in ethanol preference induced by finasteride in wild type mice compared to homozygous mutants. This observation underscores importance of neurosteroid-sensitive α2-$GABA_A$ receptors in modulating ethanol drinking behaviours.

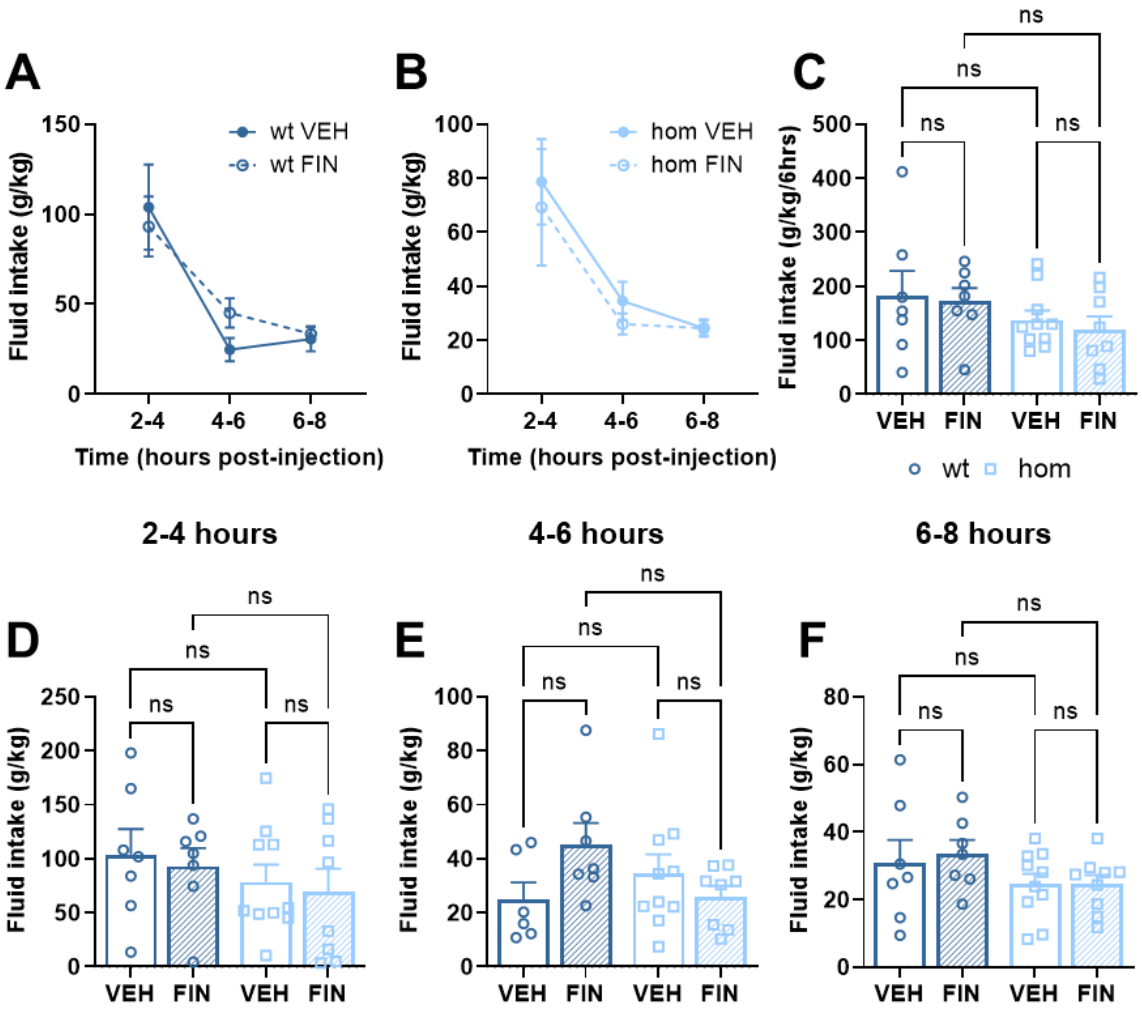


Figure 5.16 Finasteride does not influence fluid intake of $\alpha2^{Q241M}$ wild type and mutant mice at 2-8 hours post-injection

A and ***B,*** Fluid intake (g/kg) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. ***C,*** Total fluid intake (g/kg) over the 6 hour experimental period. ***D, E*** *and* ***F,*** Fluid intake (g/kg) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C, D, E* and *F*. All comparisons are non-significant. wt VEH: n = 7; wt FIN: n = 7, hom VEH: n = 10; hom FIN: n = 9.

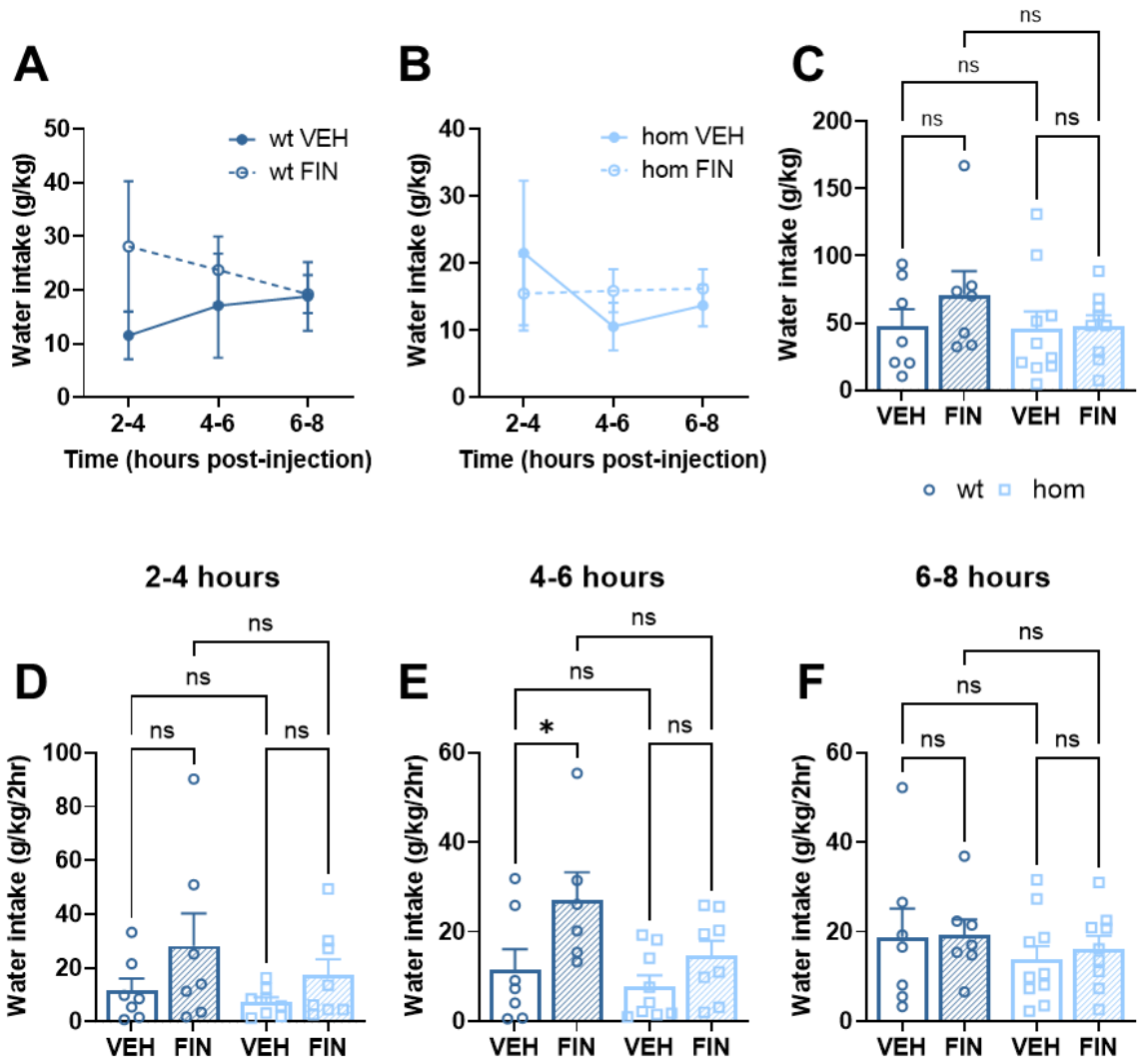


Figure 5.17 Finasteride affects water intake of α2^{Q241M} wild type mice at 4-6 hours post-injection

A and ***B,*** Water intake (g/kg) of wild type (*A*) and homozygous mutant (*B*) mice over the course of whole experiment, respectively. ***C,*** Total water intake (g/kg) over the 6 hour experimental period. ***D, E*** *and* ***F,*** Water intake (g/kg) at 2-4, 4-6 and 6-8 hours post-injection, respectively. Data are shown as mean ± SEM. Statistical tests used are two-way repeated measures ANOVA with comparisons using Šidák's test (to compare treatment differences) for panels *A* and *B*, ordinary two-way ANOVA with uncorrected Fisher's LSD test for panels *C, D, E* and *F*. All comparisons are non-significant. wt VEH: n = 7; wt FIN: n = 7, hom VEH: n = 10; hom FIN: n = 9.

5.2.5 Blood ethanol concentration measurements of all mouse lines

We investigated the impact of finasteride on blood ethanol concentration across three mouse lines: C57BL/J6, $\alpha4^{Q246M}$, and $\alpha2^{Q241M}$. Blood samples were collected from the trunk following a 6-hour limited access period to ethanol. Blood ethanol concentrations were quantified using a colorimetric assay detailed in *Section 2.6.1*. The mean ± SEM values and statistical analysis of this experiment are contained in *Table 5.18* in *Section 5.5*.

Administration of finasteride did not result in a significant difference in blood ethanol concentration (BEC) in C57BL/J6 animals; however, a strong trend (P = 0.062) towards decreased BEC was observed due to finasteride treatment (*Figure 5.18 A*).

Unexpectedly, pre-treatment with finasteride did not induce any alteration in blood ethanol concentration in either wild type or homozygous mutant $\alpha4^{Q246M}$ mice (*Figure 4.18 B*).

Analysis of blood ethanol concentration in the $\alpha2^{Q241M}$ knock-in mouse line detected a significant interaction between treatment and genotype. Finasteride administration led to a significant reduction in wild type mice, as confirmed by post-hoc analyses (*Figure 5.18 C*). In contrast, finasteride did not affect blood ethanol concentration in $\alpha2^{M/M}$ animals (*Figure 5.18 C*). This finding reinforces the notion that neurosteroid modulation of α2-containing receptors plays an important role in mediating some of ethanol's effects.

Ethanol consumption during the final 2-hour period showed a significant correlation with blood ethanol concentration; higher intake levels corresponded to higher BEC levels (*Figure 5.18 D*). Likewise, the overall preference for ethanol was a good predictor for BEC levels (*Figure 5.18 E*). Higher preference for ethanol corresponded to higher concentrations of ethanol in the blood.

Collectively, these findings suggest that pre-treatment with finasteride modifies ethanol consumption patterns, resulting in changes in blood ethanol concentration. Moreover, the neurosteroid sensitivity of α2-$GABA_A$ receptors may play a role in regulating these processes.

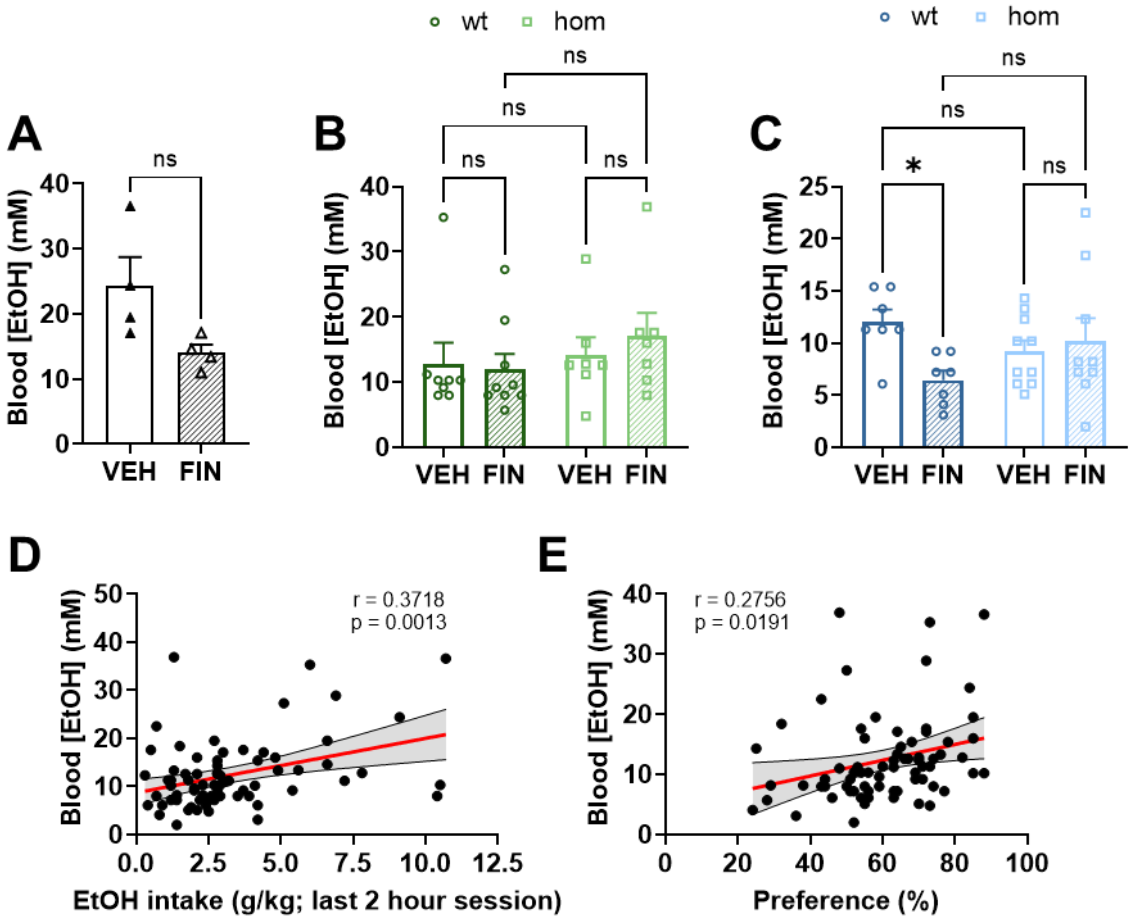


Figure 5.18 The effect of finasteride on blood ethanol concentration of mice

A, B and ***C,*** Blood ethanol concentration (BEC; mM) of C57BL/J6 (*A*), $\alpha4^{Q246M}$ (*B*) and $\alpha2^{Q241M}$ (*C*) wild type and homozygous mutant mice. ***D*** *and* ***E,*** Significant correlation between BEC (mM) and ethanol intake (g/kg) in the last 2 hours (*D*), and between BEC and preference (E; %). All data points from all 3 different mouse lines were pooled together (n = 72). Linear regression analysis was used to fit the data (red line). 95 % confidence intervals are shown in grey. Data are shown as mean ± SEM for panels *A, B* and *C*. Statistical tests used are unpaired t-test for panel *A* and ordinary two-way ANOVA with comparisons using uncorrected Fisher's LSD test for panels *B* and *C*. Statistical significance indicated as * $p < 0.05$.

C57BL/J6: VEH: n = 4, FIN: n = 4

$\alpha4^{Q246M}$: wt VEH: n = 8; wt FIN: n = 9, hom VEH: n = 7, hom FIN: n = 7

$\alpha2^{Q241M}$: wt VEH: n = 7, wt FIN: n = 7, hom VEH: n = 10, hom FIN: n = 9

5.3 Discussion

Previously, we investigated the impact of eliminating neurosteroid sensitivity in specific $GABA_A$ receptor populations on ethanol consumption. Our findings indicated that neurosteroid modulation of both α4- and α2-containing receptors is likely to influence ethanol drinking behaviours. In our prior experiment, we primarily assessed ethanol consumption over 24-hour periods across two weeks. The most significant difference in consumption between wild type and mutant mice was observed on the first day of ethanol access. Moreover, it has been suggested that mice consume approximately half of their daily ethanol intake within the first hour of access (Quadir et al., 2021). Consequently, we elected to focus on the initial six-hour period, which encompasses half of the dark cycle when mice exhibit peak activity. These observations collectively led us to hypothesise that our study would effectively capture the majority of ethanol consumption in mice. Similar protocols, such as drinking in the dark (DID), have been shown to result in substantial ethanol intake and a physiologically relevant elevation in blood ethanol concentrations (Rhodes et al., 2005).

Despite the well-established link between ethanol consumption and neurosteroids, it is surprising that few studies have examined the effect of blocking neurosteroid synthesis via finasteride on the ethanol consumption of C57BL/J6 mice. To our knowledge, no study has employed two bottle choice paradigms to investigate these effectsOther researchers have either utilised other rodent models (rats) or different protocols, such as lickometer chambers (Ford et al., 2005, Ford et al., 2008a, Ford et al., 2008b, Milivojevic and Covault, 2012). Therefore, in addition to our two knock-in mouse models, we extended our study to include C57BL/J6 mice.

Dosing protocols for finasteride, specifically the timing of injection, exhibit considerable variability in the literature, with timings ranging from 15 minutes to 20 hours prior to behavioural testing. Consequently, we first conducted an exploratory study to determine the optimal injection time points for our purposes. Our 24-hour experiment revealed that the effects of finasteride are relatively acute, manifesting within the first 8 hours post-injection. Based on these findings, we optimised our protocol. This refined protocol was subsequently employed to assess the ethanol intake of all three mouse lines studied in this chapter.

5.3.1 The effect of finasteride pre-treatment on ethanol consumption in C57BL/J6 mice

We observed a trend towards decreased overall ethanol intake (37 % reduction) in the finasteride-treated group compared to the vehicle-treated group. Throughout the assessment periods, finasteride-treated mice consistently showed lower ethanol consumption, although these reductions did not reach statistical significance. Notably, a pronounced trend was evident at 4-6 hours post-injection (P = 0.066), indicating a potential effect during this specific timeframe.

Finasteride significantly reduced ethanol preference of C57BL/J6 mice, both at individual time points and overall (44 % decrease). Total fluid intake was unaffected by the administration of finasteride. The lack of change in total fluid intake was due to a significant increase in water consumption among finasteride-treated mice. It appears that mice compensate for the reduction in ethanol consumption by increasing their water intake, a phenomenon documented by others (Ford et al., 2008a). The observed increase in water intake with finasteride treatment is considered to be ethanol-specific, as finasteride did not affect water consumption in non-alcohol exposed rodents (Milivojevic and Covault, 2012). The combined effect of decreased ethanol intake and increased water intake in finasteride-treated mice accounts for the substantial reduction in ethanol preference.

These findings align with previous studies using lickometer chambers, which have shown that acute administration of finasteride decreases both ethanol intake and preference (Ford et al., 2005, Ford et al., 2008b). Moreover, these results provide additional support for the hypothesis that endogenous neurosteroids are pivotal in modulating ethanol consumption behaviours.

We measured blood ethanol concentrations (BEC) of mice following the limited access paradigm and observed a strong trend towards a reduction in BEC after pre-treatment with finasteride (P = 0.062). The BEC of vehicle-treated mice fell within the range known to produce ataxia and intoxicating effects (Cronise et al., 2005), suggesting that these animals likely experienced ethanol intoxication during the drinking session.

Previous studies reported that a single finasteride injection 2 hours prior to ethanol administration did not alter blood ethanol concentrations (Gorin et al., 2005). However,

there are significant methodological differences between our study and that by Gorin et al. (2005). Firstly, they utilised Withdrawal Seizure-Prone and Withdrawal Seizure-Resistant selective mouse lines, whereas we employed C57BL/6J mice. Additionally, they administered ethanol via injection, while we assessed voluntary ethanol consumption. Lastly, they measured BEC two hours post-injection, whereas we evaluated BEC eight hours post-injection.

Some reports have suggested that acute finasteride pre-treatment can induce sedative effects (Gabriel et al., 2004, Khisti et al., 2004), potentially confounding the interpretation of results. However, our observation of increased water intake indicates that there was no generalised behavioural depression. Furthermore, other rodent studies have not observed any effects of finasteride in open field (Frye and Walf, 2002) or forced swim test (Hirani et al., 2002) paradigms when the pre-treatment period was extended up to 2 hours. Therefore, by administering finasteride 2 hours prior to ethanol access, we likely minimised any confounding sedative effects.

5.3.2 The effect of finasteride pre-treatment on ethanol consumption in α4^{Q246M} mice

We observed no statistically significant effect of finasteride treatment on ethanol consumption in either α4^{Q246M} wild type or homozygous mutant animals. However, there was a trend towards reduced ethanol intake in both genotypes. Notably, homozygous mutants exhibited a smaller reduction (11 %) in ethanol consumption following finasteride pre-treatment compared to wild type animals (21 % reduction). This potentially suggests that neurosteroid modulation of α4-containing $GABA_A$ receptors plays a role in regulating ethanol consumption in mice. The effect of finasteride was most pronounced at 6-8 hours post-injection. This finding is somewhat unexpected, as our previous experiments showed the greatest changes in ethanol consumption during the initial 2 hours of access. Wild type animals consumed ethanol in amounts similar to those of our C57BL/6J mice under the same paradigm.

Pre-treatment with finasteride resulted in an overall decrease in ethanol preference in both wild type and mutant animals. Unexpectedly, the effect was more statistically significant in homozygous mutants, with the most pronounced reduction occurring

during the last 2 hours of the experiment. These late-stage effects predominantly drive the overall reduction observed in homozygous mutants.

Finasteride appears to differentially affect wild type and mutant animals. During the initial time period, it had a more pronounced effect on wild type mice (wt: 17 % decrease, hom: 3 % decrease); this effect was similar between the groups in the middle period (wt: 18 % decrease, hom: 18 % decrease) and became stronger in the mutants during the later hours (wt: 35 % decrease, hom: 50 % decrease). A strong trend (P = 0.077) suggests that finasteride affects the genotypes differently during the initial 2 hours, with finasteride-treated homozygous mutants showing higher ethanol preferences compared to finasteride-treated wild type animals.

The larger difference in ethanol preference in mutants is surprising, given that their ethanol intake was minimally affected by finasteride. This difference in preference is primarily driven by a substantial increase (4.5-fold) in water intake in finasteride-treated $\alpha4^{M/M}$ mice compared to vehicle-treated $\alpha4^{M/M}$ animals. Comparatively only a 2-fold increase was observed between treatment groups in wild type animals. Consistent with our findings in C57BL/6J mice, the largest decrease in ethanol preference following finasteride treatment coincided with the most significant reduction in ethanol intake and a substantial increase in water consumption. This suggests that the mice compensated for the decreased ethanol intake by increasing their water consumption.

Analysing the data from specific time periods reveals that vehicle-treated wild type and mutant mice exhibit similar behaviours during the initial two periods (2-4 and 4-6 hours). However, during the last 2 hours, mutant mice display higher ethanol preferences compared to wild type mice, which is due to the difference in their water consumption. This increase complicates the interpretation of our results. Without this increase, we would conclude that finasteride reduces ethanol preference in both genotypes, suggesting that neurosteroid modulation of α4 receptors is likely not crucial in regulating ethanol drinking in this limited access paradigm, or at least not for the entire duration of the experiment.

Previous studies have shown that α4 subunit expression significantly decreases one hour after ethanol exposure (Liang et al., 2007). Therefore, we speculate that finasteride has a stronger effect on ethanol preference in wild type animals compared to mutants during the initial time period because α4 subunit expression was likely not significantly altered,

or at least not for the entirety of the 2-hour period. However, ethanol exposure decreases the expression of the α4 subunit, thereby diminishing the differential effect of finasteride between genotypes.

Administration of finasteride also led to a more pronounced reduction in ethanol consumption in wild type animals compared to mutants during the initial two hours (wt: -30 %, hom: -17 %). In contrast, the effects of finasteride were less divergent at the subsequent time points (4-6 hours: -6 % vs 10 %, 6-8 hours: -40 % vs -37 %). This pattern mirrors our observations with ethanol preference and may be explained by alterations in α4 subunit expression. The effect of finasteride between genotypes might have been more pronounced if we had examined a shorter time period, such as 30 minutes or 1 hour. However, animals may not consume measurable volumes of ethanol during such short intervals. This is one possibility though additional factors could also be at play.

Taken together, our data suggests that neurosteroid modulation of α4-$GABA_A$ receptors may be crucial immediately after ethanol exposure for a brief time period. However, due to changes induced in subunit expression by ethanol, their role may diminish later on.

We measured blood ethanol concentrations for $\alpha4^{Q246M}$ wild type ($\alpha4^{Q/Q}$) and homozygous mutant ($\alpha4^{M/M}$) mice following the limited access paradigm. Unlike our findings with C57BL/6J mice, we observed no effect of finasteride on BEC in either genotype. The reason for this discrepancy remains unclear. We employed identical protocols, timing, room conditions and husbandry practices.

One potential explanation could be genetic drift within the $\alpha4^{Q246M}$ mouse line over time, which might account for the differences observed between $\alpha4^{Q/Q}$ wild type mice and the results obtained from C57BL/6J mice. Although we have maintained rigorous backcrossing and breeder refreshment protocols, we have not conducted whole genome sequencing in the past 2 years to definitively rule out genetic changes. Nevertheless, even if a genetic drift has occurred, we believe that the appropriate control for homozygous mutant animals is their wild type littermates.

5.3.3 The effect of finasteride pre-treatment on ethanol consumption in α2^{Q241M} mice

We observed no statistically significant effect of finasteride treatment on ethanol consumption in either α2^{Q241M} wild type or homozygous mutant animals, despite both genotypes showing reduced total ethanol intakes following finasteride administration. The extent of decrease in wild type animals (25 %) was higher compared to mutants (16 %), suggesting a potential role for neurosteroid modulation of α2-GABA$_A$ receptors in ethanol consumption.

Pre-treatment with finasteride affected the average ethanol preference over the 6-hour period in both a2$^{Q/Q}$ and a2$^{M/M}$ animals. This effect was significant and more pronounced in wild type mice compared to mutant animals, where it did not reach statistical significance. These findings also underscore the importance of neurosteroid sensitivity of α2-GABA$_A$ receptors in regulating ethanol drinking behaviours.

We observed a reduction in ethanol preference at every time point. However, the largest difference induced by finasteride was detected at 4-6 hours post-injection. This pattern, consistent with our findings in the other two mouse lines, coincided with the most substantial increase in water intake.

Collectively, these findings suggest that neurosteroid modulation of α2-GABA$_A$ receptors is crucial in regulating ethanol self-administration.

We assessed the blood ethanol concentration of both α2^{Q241M} wild type and homozygous mutant mice after the limited access ethanol experiment. Wild type mice, similar to C57BL/J6, showed a significant reduction in BEC, whereas BEC of mutant mice was unchanged. This effect can be attributed to the differential impact of finasteride between genotypes on ethanol intake and preference. This finding further strengthens the importance of α2-GABA$_A$ receptors in mediating the effects of ethanol via neurosteroid modulation.

5.3.4 Limitations

A potential limitation of these experiments is the use of finasteride, which inhibits only Type II 5-α reductase, whereas dutasteride inhibits both Type I and Type II enzymes

(Nickel, 2004). Consequently, dutasteride is more effective, reducing neurosteroid levels by approximately 20 % more than finasteride (Clark et al., 2004). Using dutasteride could therefore have provided a more substantial effect. Indeed, dutasteride has been trialled in a randomised, placebo-controlled clinical study, demonstrating that a daily dose of 1 mg significantly reduced the number of heavy drinking days and drinks per week in men seeking treatment (Covault et al., 2024).

5.4 Conclusions

1. Voluntary ethanol consumption in C57BL/J6 mice is affected by finasteride mainly between 2- and 8-hours post-injection.

2. Pre-treatment of C57BL/J6 mice with finasteride leads to a significant reduction in ethanol preference, and subsequently results in a substantial decrease in blood ethanol concentrations, supporting the hypothesis that neurosteroids play a pivotal role in modulating ethanol consumption.

3. Administration of finasteride significantly decreases ethanol preference in both α4 wild type and homozygous mutant mice within the limited access paradigm. However, there is a trend towards more pronounced effects in wild type animals than in mutants during the initial 2-hour period, suggesting a potential involvement of neurosteroid modulation of α4-$GABA_A$ receptors in ethanol consumption for a brief time period after ethanol exposure.

4. Finasteride treatment significantly reduces average preference in α2 wild type animals, but not in $\alpha2^{M/M}$ mutants. Moreover, it markedly decreases blood ethanol concentrations in wild type mice, whereas no such effect is observed in mutants. These findings underscore the critical role of neurosteroid sensitivity of α2-$GABA_A$ receptors in modulating ethanol drinking behaviours.

5.5 Appendix

The tables in this section contain the following abbreviations: BEC – blood ethanol concentration, FIN – finasteride, Hab – habituation, PI – post-injection, Pref – preference, VEH – vehicle.

Table 5.1 C57BL/J6 - control parameters for limited access two bottle choice experiments

	Body weight (g)			Fluid intake – habituation (g/kg/24hr)		
	Mean ± SEM		P value	Mean ± SEM		P value
	VEH	FIN	VEH vs FIN	VEH	FIN	VEH vs FIN
Protocol A	24.2 ± 0.4	24.9 ± 0.5	0.299	156.5 ± 11.5	191.5 ± 14.8	0.091
Protocol B	22.5 ± 0.6	23.8 ± 0.7	0.198	326.9 ± 46.1	324.2 ± 43.3	0.966
Protocol C	23.4 ± 0.5	23.9 ± 0.5	0.530	250.0 ± 40.5	263.9 ± 13.8	0.533

Table 5.2 C57BL/J6 – Protocol A results

Protocol A (C57BL/J6)	EtOH intake (g/kg/2hr)			EtOH preference (%)		
	Mean ± SEM		P value	Mean ± SEM		P value
	VEH (n = 6)	FIN (n = 6)	VEH vs FIN	VEH (n = 6)	FIN (n = 6)	VEH vs FIN
20-22 hours PI	7.2 ± 3.1	8.2 ± 3.1	0.822	70 ± 11	75 ± 9	0.744
22-24 hours PI	5.7 ± 2.2	3.5 ± 1.0	0.589	75 ± 13	48 ± 8	0.093
24-26 hours PI	3.3 ± 0.7	2.8 ± 0.8	0.593	66 ± 8	42 ± 7	0.052

Table 5.3 C57BL/J6 – Protocol B results

Protocol B (C57BL/J6)	EtOH intake (g/kg/2hr)			EtOH preference (%)		
	Mean ± SEM		P value	Mean ± SEM		P value
	VEH (n = 6)	FIN (n = 6)	VEH vs FIN	VEH (n = 6)	FIN (n = 6)	VEH vs FIN
4-6 hours PI	7.7 ± 3.0	2.7 ± 1.0	0.893	75 ± 7	25 ± 9	**0.009**
6-8 hours PI	4.5 ± 1.7	1.6 ± .05	0.875	62 ± 11	23 ± 7	0.076
8-10 hours PI	4.1 ± 1.3	2.7 ± 0.7	0.996	58 ± 11	29 ± 8	0.235
10-12 hours PI	3.4 ± 0.6	1.8 ± 0.4	0.570	50 ± 9	31 ± 8	0.615
12-14 hours PI	4.3 ± 1.0	2.3 ± 0.6	0.813	43 ± 5	28 ± 6	0.375
14-16 hours PI	1.3 ± 0.3	1.5 ± 0.5	0.999	60 ± 9	67 ± 14	0.999
16-18 hours PI	1.1 ± 0.3	1.2 ± 0.4	0.999	51 ± 10	53 ± 13	0.999
18-20 hours PI	1.1 ± 0.2	1.1 ± 0.4	0.999	37 ± 9	36 ± 14	0.999
20-22 hours PI	1.3 ± 0.3	1.2 ± 0.4	0.999	70 ± 3	52 ± 10	0.828
22-24 hours PI	1.1 ± 0.3	1.2 ± 0.4	0.999	48 ± 14	63 ± 12	0.999
24-26 hours PI	1.0 ± 0.3	2.0 ± 0.5	0.801	43 ± 9	71 ± 11	0.659
26-28 hours PI	4.7 ± 2.9	1.6 ± 0.4	0.993	29 ± 11	19 ± 5	0.999

Table 5.4 C57BL/J6 – Protocol C results

Protocol C C57BL/J6	Ethanol intake (g/kg/2hr)			Ethanol preference (%)		
	Mean ± SEM		P value	Mean ± SEM		P value
	VEH (n = 10)	FIN (n = 10)	VEH vs FIN	VEH (n = 10)	FIN (n = 10)	VEH vs FIN
2-4 hours PI	16.8 ± 4.4	12.3 ± 4.1	0.393	80 ± 5	49 ± 11	**0.029**
4-6 hours PI	5.2 ± 1.4	2.1 ± 0.4	0.066	66 ± 8	35 ± 7	**0.007**
6-8 hours PI	5.5 ± 1.1	3.4 ± 0.6	0.111	61 ± 7	34 ± 6	**0.009**
Protocol C C57BL/J6	**Fluid intake (g/kg/2hr)**			**Water intake (g/kg)**		
	Mean ± SEM		P value	Mean ± SEM		P value
	VEH (n = 10)	FIN (n = 10)	VEH vs FIN	VEH (n = 10)	FIN (n = 10)	VEH vs FIN
2-4 hours PI	88.6 ± 24.2	87.2 ± 20.3	0.739	12.8 ± 2.7	34.1 ± 5.3	**0.002**
4-6 hours PI	57.2 ± 13.5	52.8 ± 5.8	0.773	12.4 ± 3.0	26.8 ± 3.4	**0.005**
6-8 hours PI	48.2 ± 8.7	55.8 ± 7.9	0.631	20.5 ± 5.3	30.3 ± 3.8	0.162

Table 5.5 C57BL/J6 – Protocol A, B and C statistics

Limited ethanol access protocols - C57BL/J6				
	Source of variation	**P value**	**P value summary**	**F (DFn, DFd)**
Protocol A Fluid intake Figure 5.1 C	Time	<0.0001	****	F (1.775, 17.31) = 21.10
	Treatment	0.105	ns	F (1, 10) = 3.179
	Time x Treatment	0.113	ns	F (4, 39) = 2.001
Protocol A EtOH intake Figure 5.2 A	Time	0.103	ns	F (1.528, 15.28) = 2.794
	Treatment	0.749	ns	F (1, 10) = 0.1081
	Time x Treatment	0.732	ns	F (2, 20) = 0.3165
Protocol A EtOH pref. Figure 5.2 B	Time	0.194	ns	F (1.956, 19.56) = 1.788
	Treatment	0.074	ns	F (1, 10) = 3.990
	Time x Treatment	0.224	ns	F (2, 20) = 1.615
Protocol B Fluid intake Figure 5.3 C	Time	0.055	ns	F (1.670, 15.70) = 3.696
	Treatment	0.999	ns	F (1, 10) = 4.720e-007
	Time x Treatment	0.898	ns	F (5, 47) = 0.3214
Protocol B EtOH intake Figure 5.4 A	Time	0.310	ns	F (1.150, 10.64) = 1.188
	Treatment	0.036	*	F (1, 10) = 5.827
	Time x Treatment	0.601	ns	F (4, 37) = 0.6945
Protocol B EtOH pref. Figure 5.4 B	Time	0.327	ns	F (2.607, 25.42) = 1.198
	Treatment	0.004	**	F (1, 10) = 13.89
	Time x Treatment	0.077	ns	F (4, 39) = 2.294
Protocol C Fluid intake Figure 5.5 C	Time	0.134	ns	F (2.513, 40.71) = 2.030
	Treatment	0.680	ns	F (1, 18) = 0.1763
	Time x Treatment	0.933	ns	F (5, 81) = 0.2603
Protocol C EtOH intake Figure 5.6 A	Time	0.001	***	F (1.214, 21.85) = 12.97
	Treatment	0.229	ns	F (1, 18) = 1.554
	Time x Treatment	0.840	ns	F (2, 36) = 0.1755
Protocol C EtOH pref. Figure 5.6 B	Time	0.007	**	F (1.938, 34.88) = 5.940
	Treatment	0.003	**	F (1, 18) = 11.49
	Time x Treatment	0.907	ns	F (2, 36) = 0.09764
Protocol C Fluid intake Figure 5.7 A	Time	0.037	*	F (1.457, 26.23) = 4.213
	Treatment	0.964	ns	F (1, 18) = 0.002059
	Time x Treatment	0.903	ns	F (2, 36) = 0.1027
Protocol C Water intake Figure 5.7 B	Time	0.248	ns	F (1.622, 28.38) = 1.460
	Treatment	0.001	**	F (1, 18) = 14.70
	Time x Treatment	0.313	ns	F (2, 35) = 1.201

Table 5.6 α4^{Q246M} - control parameters for limited access two bottle choice experiment (Protocol C) and statistical analysis of control parameters

α4^{Q246M}	Age (days)		Body weight (g)		Fluid intake – habituation (g/kg/24hr)	
	Mean ± SEM		Mean ± SEM		Mean ± SEM	
	VEH	FIN	VEH	FIN	VEH	FIN
Wt	71 ± 5	69 ± 4	27.2 ± 1.3	28.0 ± 0.8	210.1 ± 9.9	215.6 ± 4.4
Hom	70 ± 5	69 ± 3	28.5 ± 0.8	29.2 ± 1.2	218.3 ± 9.4	201.6 ± 9.1

	Tukey's multiple comparisons test			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Body weight (Fig. 5.8 A)	0.880	0.504	0.284	0.620
Age (Fig. 5.8 B)	0.989	0.604	0.195	0.490

	Source of variation	P value	P value summary	F (DFn, DFd)
Hab. - Water intake WT Figure 5.8 C	Time	0.397	ns	F (2.351, 35.27) = 0.9802
	Treatment	0.823	ns	F (1, 15) = 0.05208
	Time x Treatment	0.863	ns	F (4, 60) = 0.3202
Hab. - Water intake HOM Figure 5.8 D	Time	0.805	ns	F (2.267, 25.51) = 0.2522
	Treatment	0.563	ns	F (1, 12) = 0.3541
	Time x Treatment	0.696	ns	F (4, 45) = 0.5560

	Šídák's multiple comparisons test P values				
	VEH vs FIN				
	Day 1	Day 2	Day 3	Day 4	Day 5
Hab. - Water intake WT Figure 5.8 C	0.999	0.399	0.962	0.998	0.999
Hab. - Water intake HOM Figure 5.8 D	0.982	0.959	0.421	0.997	0.998

Table 5.7 α4^{Q246M} – Protocol C results

α4^{Q246M}	EtOH intake (g/kg/2hr)		EtOH preference (%)		Fluid intake (g/kg/2hr)		Water intake (g/kg/2hr)	
	Mean ± SEM		Mean ± SEM		Mean ± SEM		Mean ± SEM	
	VEH	FIN	VEH	FIN	VEH	FIN	VEH	FIN
Wt: 2-4 hours PI	19.4 ± 2.3	13.6 ± 4.0	77 ± 8	64 ± 11	130.8 ± 14.9	84.9 ± 21.6	35.1 ± 13.5	15.7 ± 3.9
Wt: 4-6 hours PI	4.5 ± 0.8	4.2 ± 0.6	55 ± 7	45 ± 8	58.5 ± 10.2	47.6 ± 8.1	34.2 ± 8.6	21.5 ± 4.2
Wt: 6-8 hours PI	4.4 ± 1.0	2.6 ± 0.4	66 ± 7	43 ± 8	25.4 ± 2.9	32.9 ± 4.5	11.4 ± 2.5	22.6 ± 6.4
Hom: 2-4 hours PI	19.4 ± 3.6	16.0 ± 2.3	88 ± 7	85 ± 4	117.6 ± 24.1	94.1 ± 12.3	20.7 ± 13.9	14.3 ± 4.7
Hom: 4-6 hours PI	2.9 ± 0.8	3.6 ± 0.4	57 ± 7	47 ± 9	41.7 ± 15.1	53.1 ± 11.5	16.5 ± 5.2	30.7 ± 11.4
Hom: 6-8 hours PI	4.3 ± 1.1	2.7 ± 0.6	83 ± 2	41 ± 5	26.1 ± 6.3	32.9 ± 6.9	4.5 ± 1.4	25.3 ± 6.9

Table 5.8 $\alpha4^{Q246M}$ – Protocol C - ethanol intake statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
EtOH intake 2-8 hrs WT Figure 5.9 A	Time	<0.0001	****	F (1.125, 16.31) = 26.79
	Treatment	0.165	ns	F (1, 15) = 2.129
	Time x Treatment	0.376	ns	F (2, 29) = 1.013
EtOH intake 2-8 hrs HOM Figure 5.9 B	Time	<0.0001	****	F (1.248, 21.21) = 33.86
	Treatment	0.507	ns	F (1, 34) = 0.4505
	Time x Treatment	0.586	ns	F (2, 34) = 0.5433
Total EtOH intake Figure 5.9 C	Treatment	0.278	ns	F (1, 27) = 1.227
	Genotype	0.565	ns	F (1, 27) = 0.3386
	Treatment x Genotype	0.719	ns	F (1, 27) = 0.1327
EtOH intake 2-4 hrs Figure 5.9 D	Treatment	0.169	ns	F (1, 27) = 1.994
	Genotype	0.725	ns	F (1, 27) = 0.1266
	Treatment x Genotype	0.710	ns	F (1, 27) = 0.1418
EtOH intake 4-6 hrs Figure 5.9 E	Treatment	0.798	ns	F (1, 24) = 0.06695
	Genotype	0.134	ns	F (1, 24) = 2.410
	Treatment x Genotype	0.502	ns	F (1, 24) = 0.4654
EtOH intake 6-8 hrs Figure 5.9 F	Treatment	0.045	*	F (1, 26) = 4.454
	Genotype	0.995	ns	F (1, 26) = 4.255e-005
	Treatment x Genotype	0.962	ns	F (1, 26) = 0.002363

	Šídák's multiple comparisons test P values		
	2-4 hrs	4-6 hrs	6-8 hrs
	VEH vs FIN	VEH vs FIN	VEH vs FIN
EtOH intake 2-8 hrs WT (Fig. 5.9 A)	0.541	0.989	0.343
EtOH intake 2-8 hrs HOM (Fig. 5.9 B)	0.829	0.863	0.982

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Total EtOH intake (Fig. 5.9 C)	0.880	0.504	0.284	0.620
EtOH intake 2-4 hrs (Fig. 5.9 D)	0.989	0.604	0.195	0.490
EtOH intake 4-6 hrs (Fig. 5.9 E)	0.137	0.533	0.750	0.538
EtOH intake 6-8 hrs (Fig. 5.9 F)	0.976	0.970	0.113	0.183

Table 5.9 $\alpha4^{Q246M}$ – Protocol C - ethanol preference statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Preference 2-8 hrs WT Figure 5.10 A	Time	0.045	*	F (1.883, 25.42) = 3.587
	Treatment	0.048	*	F (1, 15) = 4.647
	Time x Treatment	0.712	ns	F (2, 27) = 0.3443
Preference 2-8 hrs HOM Figure 5.10 B	Time	<0.0001	****	F (1.571, 27.50) = 15.34
	Treatment	0.001	**	F (1, 35) = 12.58
	Time x Treatment	0.008	**	F (2, 35) = 5.615
Average preference Figure 5.10 C	Treatment	0.040	*	F (1, 27) = 4.669
	Genotype	0.179	ns	F (1, 27) = 1.901
	Treatment x Genotype	0.319	ns	F (1, 27) = 1.031
Preference 2-4 hrs Figure 5.10 D	Treatment	0.349	ns	F (1, 25) = 0.9126
	Genotype	0.069	ns	F (1, 25) = 3.599
	Treatment x Genotype	0.520	ns	F (1, 25) = 0.4251
Preference 4-6 hrs Figure 5.10 E	Treatment	0.235	ns	F (1, 25) = 1.481
	Genotype	0.758	ns	F (1, 25) = 0.09694
	Treatment x Genotype	0.960	ns	F (1, 25) = 0.002596
Preference 6-8 hrs Figure 5.10 F	Treatment	<0.0001	****	F (1, 27) = 25.37
	Genotype	0.261	ns	F (1, 27) = 1.320
	Treatment x Genotype	0.135	ns	F (1, 27) = 2.373

	Šídák's multiple comparisons test P values		
	2-4 hrs	**4-6 hrs**	**6-8 hrs**
	VEH vs FIN	VEH vs FIN	VEH vs FIN
Preference 2-8 hrs WT (Fig. 5.10 A)	0.711	0.779	0.145
Preference 2-8 hrs HOM (Fig. 5.10 B)	0.987	0.797	**0.0002**

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	**FIN**	**WT**	**HOM**
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Average preference (Fig. 5.10 C)	0.106	0.796	0.402	**0.041**
Preference 2-4 hrs (Fig. 5.10 D)	0.397	0.077	0.241	0.840
Preference 4-6 hrs (Fig. 5.10 E)	0.803	0.853	0.410	0.386
Preference 6-8 hrs (Fig. 5.10 F)	0.071	0.781	**0.015**	**0.0001**

Table 5.10 $\alpha4^{Q246M}$ – Protocol C - fluid intake statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Fluid intake 2-8 hrs WT Figure 5.11 A	Time	<0.0001	****	F (1.406, 20.39) = 24.34
	Treatment	0.192	ns	F (1, 15) = 1.869
	Time x Treatment	0.078	ns	F (2, 29) = 2.795
Fluid intake 2-8 hrs HOM Figure 5.11 B	Time	0.000	***	F (1.208, 21.13) = 15.51
	Treatment	0.880	ns	F (1, 35) = 0.02311
	Time x Treatment	0.421	ns	F (2, 35) = 0.8881
Total fluid intake Figure 5.11 C	Treatment	0.339	ns	F (1, 26) = 0.9490
	Genotype	0.989	ns	F (1, 26) = 0.0002083
	Treatment x Genotype	0.215	ns	F (1, 26) = 1.613
Fluid intake 2-4 hrs Figure 5.11 D	Treatment	0.082	ns	F (1, 27) = 3.256
	Genotype	0.917	ns	F (1, 27) = 0.01099
	Treatment x Genotype	0.564	ns	F (1, 27) = 0.3404
Fluid intake 4-6 hrs Figure 5.11 E	Treatment	0.983	ns	F (1, 27) = 0.0004556
	Genotype	0.619	ns	F (1, 27) = 0.2533
	Treatment x Genotype	0.326	ns	F (1, 27) = 0.9996
Fluid intake 6-8 hrs Figure 5.11 F	Treatment	0.187	ns	F (1, 25) = 1.840
	Genotype	0.946	ns	F (1, 25) = 0.004736
	Treatment x Genotype	0.939	ns	F (1, 25) = 0.005965

	Šídák's multiple comparisons test P values		
	2-4 hrs	4-6 hrs	6-8 hrs
	VEH vs FIN	VEH vs FIN	VEH vs FIN
Fluid intake 2-8 hrs WT (Fig. 5.11 A)	0.274	0.802	0.458
Fluid intake 2-8 hrs HOM (Fig. 5.11 B)	0.793	0.915	0.864

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Total fluid intake (Fig. 5.11 C)	0.396	0.358	0.114	0.841
Fluid intake 2-4 hrs (Fig. 5.11 D)	0.635	0.734	0.087	0.417
Fluid intake 4-6 hrs (Fig. 5.11 E)	0.304	0.725	0.473	0.496
Fluid intake 6-8 hrs (Fig. 5.11 F)	0.919	0.995	0.296	0.396

Table 5.11 $\alpha4^{Q246M}$ – Protocol C - water intake statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Water intake 2-8 hrs WT Figure 5.12 A	Time	0.5649	ns	F (1.900, 28.50) = 0.5670
	Treatment	0.3899	ns	F (1, 15) = 0.7841
	Time x Treatment	0.0952	ns	F (2, 30) = 2.547
Water intake 2-8 hrs HOM Figure 5.12 B	Time	0.5100	ns	F (1.365, 16.38) = 0.5762
	Treatment	0.1960	ns	F (1, 12) = 1.875
	Time x Treatment	0.2512	ns	F (2, 24) = 1.464
Total water intake Figure 5.12 C	Treatment	0.3503	ns	F (1, 27) = 0.9033
	Genotype	0.2377	ns	F (1, 27) = 1.458
	Treatment x Genotype	0.2518	ns	F (1, 27) = 1.371
Water intake 2-4 hrs Figure 5.12 D	Treatment	0.2030	ns	F (1, 27) = 1.703
	Genotype	0.4330	ns	F (1, 27) = 0.6335
	Treatment x Genotype	0.5203	ns	F (1, 27) = 0.4243
Water intake 4-6 hrs Figure 5.12 E	Treatment	0.9173	ns	F (1, 27) = 0.01098
	Genotype	0.5820	ns	F (1, 27) = 0.3104
	Treatment x Genotype	0.0883	ns	F (1, 27) = 3.128
Water intake 6-8 hrs Figure 5.12 F	Treatment	0.0041	**	F (1, 27) = 9.847
	Genotype	0.6773	ns	F (1, 27) = 0.1770
	Treatment x Genotype	0.3554	ns	F (1, 27) = 0.8841

	Šídák's multiple comparisons test P values		
	2-4 hrs	4-6 hrs	6-8 hrs
	VEH vs FIN	VEH vs FIN	VEH vs FIN
Water intake 2-8 hrs WT (Fig.5.12 A)	0.494	0.516	0.428
Water intake 2-8 hrs HOM (Fig.5.12 B)	0.965	0.637	0.068

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Total water intake (Fig. 5.12 C)	0.109	0.979	0.871	0.163
Water intake 2-4 hrs (Fig. 5.12 D)	0.321	0.918	0.158	0.662
Water intake 4-6 hrs (Fig. 5.12 E)	0.116	0.393	0.227	0.216
Water intake 6-8 hrs (Fig. 5.12 F)	0.351	0.713	0.114	**0.010**

Table 5.12 $\alpha2^{Q241M}$ - control parameters for limited access two bottle choice experiment (Protocol C) and statistical analysis of control parameters

$\alpha2^{Q241M}$	Age (days)		Body weight (g)		Fluid intake – habituation (g/kg/24hr)	
	Mean ± SEM		Mean ± SEM		Mean ± SEM	
	VEH	FIN	VEH	FIN	VEH	FIN
Wt	76 ± 5	79 ± 6	29.6 ± 0.7	30.3 ± 0.8	177.4 ± 13.4	189.2 ± 14.8
Hom	73 ± 5	74 ± 5	28.5 ± 1.0	30.1 ± 0.7	209.8 ± 19.8	176.0 ± 15.0

	Tukey's multiple comparisons test P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Body weight (Fig. 5.13 A)	0.823	0.998	0.943	0.525
Age (Fig. 5.13 B)	0.972	0.912	0.982	0.998

	Source of variation	P value	P value summary	F (DFn, DFd)
Hab. - Water intake Wt Figure 5.13 C	Time	0.145	ns	F (1.986, 23.34) = 2.103
	Treatment	0.644	ns	F (1, 12) = 0.2248
	Time x Treatment	0.793	ns	F (4, 47) = 0.4211
Hab. - Water intake Hom Figure 5.13 D	Time	0.031	*	F (2.032, 34.55) = 3.841
	Treatment	0.334	ns	F (1, 17) = 0.9870
	Time x Treatment	0.291	ns	F (4, 68) = 1.269

	Šídák's multiple comparisons test P values				
	VEH vs FIN				
	Day 1	Day 2	Day 3	Day 4	Day 5
Hab. - Water intake Wt Figure 5.13 C	0.999	0.891	0.999	0.993	0.979
Hab. - Water intake Hom Figure 5.13 D	0.999	0.999	0.543	0.960	0.971

Table 5.13 $\alpha2^{Q241M}$ – Protocol C results

$\alpha2^{Q241M}$	EtOH intake (g/kg/2hr)		EtOH preference (%)		Fluid intake (g/kg/2hr)		Water intake (g/kg/2hr)	
	Mean ± SEM		Mean ± SEM		Mean ± SEM		Mean ± SEM	
	VEH	FIN	VEH	FIN	VEH	FIN	VEH	FIN
Wt: 2-4 hours PI	18.5 ± 4.4	13.0 ± 3.1	89 ± 3	64 ± 12	103.9 ± 23.6	93.1 ± 16.6	11.5 ± 4.5	28.1 ± 12.1
Wt: 4-6 hours PI	3.4 ± 0.8	4.3 ± 0.9	73 ± 7	42 ± 6	24.8 ± 6.4	45.2 ± 8.1	11.3 ± 4.7	27.0 ± 6.3
Wt: 6-8 hours PI	2.4 ± 0.5	2.9 ± 0.6	45 ± 8	36 ± 4	30.7 ± 6.9	33.5 ± 4.1	18.8 ± 6.4	19.3 ± 3.5
Hom: 2-4 hours PI	11.5 ± 2.6	11.6 ± 4.3	73 ± 9	49 ± 16	78.7 ± 15.8	69.3 ± 21.6	7.2 ± 1.9	17.2 ± 5.9
Hom: 4-6 hours PI	3.4 ± 0.5	2.3 ± 0.4	70 ± 8	49 ± 9	34.5 ± 7.1	26.0 ± 3.9	7.8 ± 2.5	14. 6 ± 3.4
Hom: 6-8 hours PI	2.2 ± 0.5	1.7 ± 0.3	46 ± 8	31 ± 5	24.5 ± 3.2	24.5 ± 2.7	13.7 ± 3.1	16.2 ± 2.9

Table 5.14 $\alpha2^{Q241M}$ – Protocol C - ethanol intake statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
EtOH intake 2-8 hrs WT Figure 5.14 A	Time	0.001	***	F (1.025, 11.79) = 22.62
	Treatment	0.495	ns	F (1, 12) = 0.4945
	Time x Treatment	0.295	ns	F (2, 23) = 1.290
EtOH intake 2-8 hrs HOM Figure 5.14 B	Time	0.001	***	F (1.440, 23.77) = 12.15
	Treatment	0.911	ns	F (1, 17) = 0.01297
	Time x Treatment	0.928	ns	F (2, 33) = 0.07442
Total EtOH intake Figure 5.14 C	Treatment	0.660	ns	F (1, 29) = 0.1971
	Genotype	0.444	ns	F (1, 29) = 0.6025
	Treatment x Genotype	0.412	ns	F (1, 29) = 0.6925
EtOH intake 2-4 hrs Figure 5.14 D	Treatment	0.465	ns	F (1, 28) = 0.5500
	Genotype	0.252	ns	F (1, 28) = 1.366
	Treatment x Genotype	0.442	ns	F (1, 28) = 0.6091
EtOH intake 4-6 hrs Figure 5.14 E	Treatment	0.835	ns	F (1, 26) = 0.04411
	Genotype	0.148	ns	F (1, 26) = 2.224
	Treatment x Genotype	0.119	ns	F (1, 26) = 2.604
EtOH intake 6-8 hrs Figure 5.14 F	Treatment	0.994	ns	F (1, 29) = 5.218e-005
	Genotype	0.136	ns	F (1, 29) = 2.355
	Treatment x Genotype	0.291	ns	F (1, 29) = 1.159

	Šídák's multiple comparisons test P values		
	2-4 hrs	4-6 hrs	6-8 hrs
	VEH vs FIN	VEH vs FIN	VEH vs FIN
EtOH intake 2-8 hrs WT (Fig. 5.14 A)	0.700	0.834	0.897
EtOH intake 2-8 hrs HOM (Fig. 5.14 B)	0.995	0.999	0.752

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Total EtOH intake (Fig. 5.14 C)	0.260	0.969	0.407	0.768
EtOH intake 2-4 hrs (Fig. 5.14 D)	0.169	0.791	0.318	0.977
EtOH intake 4-6 hrs (Fig. 5.14 E)	0.932	**0.036**	0.360	0.177
EtOH intake 6-8 hrs (Fig. 5.14 F)	0.746	0.078	0.481	0.419

Table 5.15 $\alpha2^{Q241M}$ – Protocol C - ethanol preference statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Preference 2-8 hrs WT Figure 5.15 A	Time	0.000	***	F (1.838, 31.24) = 11.01
	Treatment	0.001	**	F (1, 34) = 12.02
	Time x Treatment	0.369	ns	F (2, 34) = 1.026
Preference 2-8 hrs HOM Figure 5.15 B	Time	0.044	*	F (1.784, 26.76) = 3.657
	Treatment	0.020	*	F (1, 17) = 6.608
	Time x Treatment	0.859	ns	F (2, 30) = 0.1528
Average preference Figure 5.15 C	Treatment	0.008	**	F (1, 29) = 8.215
	Genotype	0.442	ns	F (1, 29) = 0.6087
	Treatment x Genotype	0.774	ns	F (1, 29) = 0.08399
Preference 2-4 hrs Figure 5.15 D	Treatment	0.010	*	F (1, 26) = 7.693
	Genotype	0.256	ns	F (1, 26) = 1.352
	Treatment x Genotype	0.760	ns	F (1, 26) = 0.09497
Preference 4-6 hrs Figure 5.15 E	Treatment	0.003	**	F (1, 27) = 10.86
	Genotype	0.845	ns	F (1, 27) = 0.03923
	Treatment x Genotype	0.552	ns	F (1, 27) = 0.3632
Preference 6-8 hrs Figure 5.15 F	Treatment	0.123	ns	F (1, 27) = 2.541
	Genotype	0.811	ns	F (1, 27) = 0.05856
	Treatment x Genotype	0.739	ns	F (1, 27) = 0.1131

	Šídák's multiple comparisons test P values		
	2-4 hrs	**4-6 hrs**	**6-8 hrs**
	VEH vs FIN	VEH vs FIN	VEH vs FIN
Preference 2-8 hrs WT (Fig. 5.15 A)	0.229	**0.023**	0.729
Preference 2-8 hrs HOM (Fig. 5.15 B)	0.513	0.230	0.403

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	**FIN**	**WT**	**HOM**
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Average preference (Fig. 5.15 C)	0.450	0.734	**0.046**	0.058
Preference 2-4 hrs (Fig. 5.15 D)	0.539	0.322	0.102	**0.033**
Preference 4-6 hrs (Fig. 5.15 E)	0.766	0.592	**0.016**	**0.048**
Preference 6-8 hrs (Fig. 5.15 F)	0.945	0.699	0.416	0.148

Table 5.16 $\alpha 2^{Q241M}$ – Protocol C - fluid intake statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Fluid intake 2-8 hrs WT Figure 5.16 A	Time	0.000	***	F (1.230, 14.14) = 23.23
	Treatment	0.839	ns	F (1, 12) = 0.04320
	Time x Treatment	0.479	ns	F (2, 23) = 0.7607
Fluid intake 2-8 hrs HOM Figure 5.16 B	Time	0.002	**	F (1.116, 17.86) = 12.27
	Treatment	0.539	ns	F (1, 17) = 0.3924
	Time x Treatment	0.891	ns	F (2, 32) = 0.1154
Total fluid intake Figure 5.16 C	Treatment	0.977	ns	F (1, 29) = 0.0008296
	Genotype	0.313	ns	F (1, 29) = 1.056
	Treatment x Genotype	0.731	ns	F (1, 29) = 0.1201
Fluid intake 2-4 hrs Figure 5.16 D	Treatment	0.609	ns	F (1, 28) = 0.2673
	Genotype	0.219	ns	F (1, 28) = 1.579
	Treatment x Genotype	0.973	ns	F (1, 28) = 0.001160
Fluid intake 4-6 hrs Figure 5.16 E	Treatment	0.391	ns	F (1, 27) = 0.7605
	Genotype	0.494	ns	F (1, 27) = 0.4813
	Treatment x Genotype	0.043	*	F (1, 27) = 4.502
Fluid intake 6-8 hrs Figure 5.16 F	Treatment	0.739	ns	F (1, 29) = 0.1131
	Genotype	0.079	ns	F (1, 29) = 3.314
	Treatment x Genotype	0.739	ns	F (1, 29) = 0.1134

	Šídák's multiple comparisons test P values		
	2-4 hrs	4-6 hrs	6-8 hrs
	VEH vs FIN	VEH vs FIN	VEH vs FIN
Fluid intake 2-8 hrs WT (Fig. 5.16 A)	0.977	0.210	0.981
Fluid intake 2-8 hrs HOM (Fig. 5.16 B)	0.980	0.672	0.999

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Total fluid intake (Fig. 5.16 C)	0.334	0.637	0.836	0.776
Fluid intake 2-4 hrs (Fig. 5.16 D)	0.357	0.406	0.715	0.719
Fluid intake 4-6 hrs (Fig. 5.16 E)	0.321	0.057	0.060	0.344
Fluid intake 6-8 hrs (Fig. 5.16 F)	0.298	0.142	0.661	0.999

Table 5.17 α2^{Q241M} – Protocol C - water intake statistics

	Source of variation	P value	P value summary	F (DFn, DFd)
Water intake 2-8 hrs WT Figure 5.17 A	Time	0.9775	ns	F (1.915, 22.98) = 0.0197
	Treatment	0.2981	ns	F (1, 12) = 1.183
	Time x Treatment	0.5163	ns	F (2, 24) = 0.6797
Water intake 2-8 hrs HOM Figure 5.17 B	Time	0.5388	ns	F (1.312, 22.31) = 0.4947
	Treatment	0.9083	ns	F (1, 17) = 0.01365
	Time x Treatment	0.5539	ns	F (2, 34) = 0.6012
Total water intake Figure 5.17 C	Treatment	0.3375	ns	F (1, 29) = 0.9513
	Genotype	0.3422	ns	F (1, 29) = 0.9324
	Treatment x Genotype	0.4112	ns	F (1, 29) = 0.6953
Water intake 2-4 hrs Figure 5.17 D	Treatment	0.0641	ns	F (1, 26) = 3.739
	Genotype	0.2784	ns	F (1, 26) = 1.226
	Treatment x Genotype	0.6354	ns	F (1, 26) = 0.2302
Water intake 4-6 hrs Figure 5.17 E	Treatment	0.0110	*	F (1, 26) = 7.508
	Genotype	0.0631	ns	F (1, 26) = 3.769
	Treatment x Genotype	0.2975	ns	F (1, 26) = 1.130
Water intake 6-8 hrs Figure 5.17 F	Treatment	0.7133	ns	F (1, 29) = 0.1377
	Genotype	0.3174	ns	F (1, 29) = 1.035
	Treatment x Genotype	0.7939	ns	F (1, 29) = 0.06952

	Šídák's multiple comparisons test P values		
	2-4 hrs	4-6 hrs	6-8 hrs
	VEH vs FIN	VEH vs FIN	VEH vs FIN
Water intake 2-8 hrs WT (Fig.5.17 A)	0.556	0.925	0.999
Water intake 2-8 hrs HOM (Fig.5.17 B)	0.948	0.628	0.914

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
Total water intake (Fig. 5.17 C)	0.926	0.218	0.243	0.914
Water intake 2-4 hrs (Fig. 5.17 D)	0.661	0.272	0.111	0.297
Water intake 4-6 hrs (Fig. 5.17 E)	0.526	0.051	**0.018**	0.214
Water intake 6-8 hrs (Fig. 5.17 F)	0.367	0.602	0.944	0.630

Table 5.18 Mean blood ethanol concentrations (BEC) and statistical analysis for all animal lines

C57BL/J6	Mean ± SEM		P value	
	VEH	FIN	VEH vs FIN	
BEC (mM)	24.4 ± 4.3	14.0 ± 1.3	0.062	

$\alpha4^{Q246M}$	WT		HOM	
	Mean ± SEM		Mean ± SEM	
	VEH	FIN	VEH	FIN
BEC (mM)	12.8 ± 3.2	12.0 ± 2.3	14.1 ± 2.8	17.0 ± 3.6

$\alpha2^{Q241M}$	WT		HOM	
	Mean ± SEM		Mean ± SEM	
	VEH	FIN	VEH	FIN
BEC (mM)	12.0 ± 1.2	6.4 ± 0.9	9.2 ± 2.2	10.2 ± 2.2

	Source of variation	P value	P value summary	F (DFn, DFd)
BEC (mM) $\alpha4^{Q246M}$ Figure 5.15 B	Treatment	0.732	ns	F (1, 27) = 0.1196
	Genotype	0.297	ns	F (1, 27) = 1.129
	Treatment x Genotype	0.537	ns	F (1, 27) = 0.3918
BEC (mM) $\alpha2^{Q241M}$ Figure 5.15 C	Treatment	0.142	ns	F (1, 29) = 2.280
	Genotype	0.748	ns	F (1, 29) = 0.1055
	Treatment x Genotype	0.036	*	F (1, 29) = 4.829

	Uncorrected Fisher's LSD multiple comparison P values			
	VEH	FIN	WT	HOM
	Wt vs Hom	Wt vs Hom	VEH vs FIN	VEH vs FIN
BEC – $\alpha4^{Q246M}$ Figure 5.15 B	0.763	0.237	0.837	0.517
BEC – $\alpha2^{Q241M}$ Figure 5.15 C	0.191	0.088	**0.021**	0.602

Chapter 6 General Discussion

6.1 Role of neurosteroids in alcohol consumption of C57BL/J6 mice

Multiple lines of evidence indicate a significant interaction between neurosteroids and ethanol. This conclusion is substantiated by findings from a variety of experimental approaches and methodologies (Bowen et al., 1999, Engel et al., 2001, Finn et al., 2004a, Ford et al., 2008a, Ford et al., 2005, Gabriel et al., 2004, Hodge et al., 2001, Janak and Michael Gill, 2003, Morrow et al., 2001a, Nie and Janak, 2003, Ramaker et al., 2014, Torres and Ortega, 2003, Torres and Ortega, 2004, VanDoren et al., 2000). Despite the well-established association between ethanol consumption and neurosteroids, only a few studies have investigated the effect of inhibiting neurosteroid synthesis with finasteride on ethanol consumption in C57BL/6J mice (Ford et al., 2008a, Ford et al., 2005, Ford et al., 2008b, Milivojevic and Covault, 2012). To our knowledge, no prior study has utilised a two bottle choice paradigm to examine these effects. Previous research has either employed alternative rodent models (e.g. rats) or different experimental protocols, such as lickometer chambers.

Our results corroborate those of previous studies, demonstrating that inhibition of neurosteroid synthesis results in a reduction in both ethanol consumption and preference in C57BL/6J mice. Moreover, we show that our limited access paradigm produces physiologically relevant blood ethanol concentrations, and that finasteride exhibits a strong trend towards reducing these levels. Consequently, these findings further support the hypothesis that neurosteroids play a crucial role in modulating ethanol consumption behaviours.

We also observed a 'front-loading' behaviour, with animals consuming most of the ethanol within the initial 2-hour period. This aligns with previous studies and supports the notion that ethanol-induced hyperlocomotion drives this behaviour (Wilcox et al., 2014). Similar 'front-loading' patterns have been reported with ethanol and other drugs of abuse, such as cocaine, in several studies (Linsenbardt and Boehm, 2012, Barkley-Levenson and Crabbe, 2012, Griffin et al., 2009, Rhodes et al., 2007).

6.2 Role of neurosteroid modulation of different $GABA_A$ receptor subtypes in the effects of ethanol

The understanding that ethanol mediates its effects through $GABA_A$ receptors has evolved over decades, with converging evidence from various scientific disciplines supporting this conclusion. Ethanol exerts its GABA-mimetic effects through multiple mechanisms, including direct interaction, presynaptic modulation, alteration of neural circuits regulating GABAergic signalling, and elevation of neurosteroids, which are potent modulators of $GABA_A$ receptors (Förstera et al., 2016, Gatta et al., 2022).

Traditionally, the behavioural significance of a gene has been investigated through the study of global gene knock-out animals. This approach has been applied to various $GABA_A$ receptor subunits, including α2, α4, and δ (reviewed in (Boehm et al., 2004)). However, the elimination of any $GABA_A$ receptor subunit can induce compensatory changes in other $GABA_A$ receptor subunits (e.g., upregulation of expression, differential localisation) or in other channels (Brickley et al., 2001), potentially complicating the interpretation of behavioural findings (Ponomarev et al., 2006).

Therefore, our two novel knock-in mouse lines, $\alpha4^{Q246M}$ and $\alpha2^{Q241M}$, which lack only neurosteroid sensitivity, with otherwise no major effects on receptor function, provide a unique opportunity to elucidate the roles of α4- and α2-containing receptor isoforms in the neurosteroid-mediated modulation of ethanol effects.

6.3 Role of neurosteroid modulation of α4-$GABA_A$ receptors in the effects of ethanol

The α4 and δ subunits of $GABA_A$ receptors play crucial roles in the neurobiology of alcoholism and are thought to be expressed in a single receptor isoform (Olsen and Sieghart, 2009). These subunits predominantly form extrasynaptic receptors, mediating tonic inhibition (Stell et al., 2003). Chronic ethanol exposure upregulates α4 and δ subunit expression, enhancing tonic inhibitory currents and contributing to the neuroadaptive changes associated with alcohol dependence (Liang et al., 2006). Studies

employing knock-out and knock-in models have demonstrated that these subunits modulate ethanol sensitivity and consumption (Mihalek et al., 2001, Nie et al., 2011, Rewal et al., 2009). Furthermore, neurosteroids exert a strong GABA-modulatory effect on δ-containing $GABA_A$ receptors (Belelli et al., 2002, Wohlfarth et al., 2002, Brown et al., 2002). Targeting α4 and δ subunit-containing $GABA_A$ receptors may thus represent a promising therapeutic strategy for the treatment of alcoholism, offering potential for reducing alcohol intake and mitigating withdrawal symptoms.

Using the intermittent access (to ethanol) paradigm, we observed that mutant mice carrying the Q246M mutation in α4-$GABA_A$ receptors exhibited reduced ethanol intake compared to their wild type littermates, with the most pronounced difference occurring on the first day. These findings imply that α4-type $GABA_A$ receptors are crucial in alcohol drinking behaviour by mediating the indirect effects of ethanol through neurosteroids. The reduced ethanol intake in mutant mice from the onset of alcohol access suggests that neurosteroids may need to act on α4-$GABA_A$ receptors for ethanol to exert its reinforcing effects. Supporting this notion, Rewal et al. (2009) demonstrated that regional knockdown of the α4 subunit in the nucleus accumbens shell, a region integral to the reinforcing effects of drugs, results in decreased alcohol consumption.

Our findings from the intermittent access paradigm indicated that the most significant difference in ethanol consumption between wild type and homozygous mutant mice occurred within the first 24 hours. Consequently, our experiments with finasteride using the limited access paradigm focused on the initial hours of the first day of ethanol access.

Unexpectedly, no differences in ethanol intake were observed between wild type and homozygous mutant mice in the limited access paradigm (6 hours of ethanol access). The underlying cause of this finding remains unclear. We speculate that this may in part be due to ethanol altering α4 subunit expression patterns. Ethanol dynamically regulates α4 subunit expression: acute ethanol administration, either via intraperitoneal injection or oral gavage, decreases α4 subunit expression in various brain regions—including the dentate gyrus, CA1 (Gonzalez et al., 2012, Liang et al., 2007), nucleus accumbens (Liang and Olsen, 2014), and thalamus (Werner et al., 2016)—within 15 minutes to 1 hour of exposure. This reduction in ethanol-sensitive receptors may rapidly counteract increased central nervous system inhibition and behavioural responses induced by ethanol. Conversely, increased α4 subunit expression occurs in the thalamus after 4 hours

(Werner et al., 2016) and in the dentate gyrus, CA1, and nucleus accumbens after 48 hours (Liang et al., 2014, Liang et al., 2007). The interval between 1 hour and 48 hours for α4 subunit expression in the hippocampus and nucleus accumbens remains unexplored, leaving a gap in our understanding of intermediate temporal receptor dynamics. The initial decrease in α4 expression in the thalamus is independent of neurosteroid action, whereas the subsequent increase is mediated by neurosteroids, as pre-treatment with finasteride blocks this ethanol-induced upregulation (Werner et al., 2016). Finasteride does not affect basal neurosteroid levels or $GABA_A$ receptor subunit expression.

In our study, finasteride administration resulted in a more pronounced decrease in ethanol consumption in wild type animals compared to mutants during the initial two hours of the limited access paradigm. This differential effect suggests that finasteride may exert a stronger influence on ethanol drinking behaviours in wild type animals when α4 subunit expression is minimally altered. This is likely due to the time required for ethanol to reach the brain (approximately 20 minutes; Ferraro et al. (1991)) and for receptor internalisation processes to occur, allowing neurosteroid modulation to play a pivotal role. However, ethanol exposure decreases α4 subunit expression for a period of time (at least for a couple of hours), potentially reducing the differential impact of finasteride across genotypes during the limited access paradigm. These findings underscore the critical role of neurosteroid modulation of α4-$GABA_A$ receptors immediately following ethanol exposure, although this effect may temporarily diminish due to ethanol-induced changes in subunit expression until subsequent upregulation occurs.

We hypothesise that the most pronounced difference observed on day 1 in our intermittent access paradigm is due to ethanol-induced upregulation of α4 subunit expression. While the exact timing of this upregulation across the whole brain remains uncertain, its occurrence after 4 hours in the thalamus and substantial increase in other regions after 48 hours, suggests a comparable temporal pattern across those areas. Therefore, the lack of observable difference in the limited access paradigm may be attributed to not reaching the period of upregulation, which was captured in the intermittent access protocol. Since α4 subunit upregulation is dependent on neurosteroid action, removing the neurosteroid binding site from α4-$GABA_A$ receptors

could potentially prevent ethanol-induced α4 subunit upregulation, thereby contributing to the reduced ethanol intake observed in homozygous mutant mice.

Ethanol-induced upregulation of the α4 subunit reverses and gradually returns to baseline levels by day 7 (Liang et al., 2007) after initial exposure to ethanol. Additionally, chronic ethanol exposure reduces brain neurosteroid levels (Janis et al., 1998, Snelling et al., 2014). These factors may partially explain why the difference in ethanol intake between wild type and mutant animals diminishes after the first week in the intermittent access paradigm.

Both wild type and mutant animals consumed more ethanol during the 6-hour limited access protocol compared to the first 24 hours of the intermittent access paradigm. This may be attributed to the injection administered before ethanol access. Single intraperitoneal injections are known to elevate corticosterone levels (Drude et al., 2011) and heart rates (Meijer et al., 2006), indicative of acute stress. Additionally, mild stress exposure increases ethanol consumption in rodents (Minnick et al., 1995). Therefore, these factors likely account for the differences observed between the two protocols.

Our functional experiments demonstrated that ethanol leads to an increase in mIPSC amplitude, frequency, decay kinetics and charge transfer in both wild type and mutant animals. Our electrophysiological data using the knock-in $\alpha4^{Q246M}$ mouse line suggest that certain ethanol effects are mediated through neurosteroid modulation of α4-type receptors. Acutely, the neurosteroid sensitivity of these receptors is crucial for ethanol-induced presynaptic changes, as evidenced by alterations in mIPSC frequency. Chronically, this sensitivity may contribute to ethanol-induced modifications in tonic currents.

Our acute functional data align with our limited access two bottle choice paradigm results, showing minimal effects through neurosteroid modulation of α4-type $GABA_A$ receptors acutely. Additionally, we did not observe potentiation of tonic currents by acute ethanol application. The potentiation of tonic currents by ethanol is contentious, with some studies reporting changes (Wei et al., 2004, Liang et al., 2006, Fleming et al., 2007), while others do not (Borghese et al., 2006). Our data is inconclusive on this matter, possibly due to the internalisation of α4-containing receptors. Ethanol might potentiate

these extrasynaptic receptors, but the reduced number of receptors could diminish this effect. Indeed, Liang et al. (2007) found no potentiation of tonic currents in the dentate gyrus one hour after ethanol exposure.

However, we observed a trend toward increased tonic inhibition in animals that had participated in the two bottle choice intermittent access protocol. α4 subunit expression increases in the hippocampus, cerebral cortex, and thalamus after six, but not after two, weeks of ethanol exposure (Matthews et al., 1998, Grobin et al., 2000, Werner et al., 2016). However, social isolation for 30 days, in the absence of other stressors, upregulates α4 and δ subunit expression, leading to increased tonic current in the dentate gyrus (Serra et al., 2006). Our animals in the intermittent access paradigm were singly housed for at least 21 days, suggesting that single housing may enhance α4 and δ subunit expression, leading to more pronounced potentiation of tonic currents by ethanol compared to naïve mice.

Additionally, ethanol exhibits higher potency in isolated animals compared to group-housed ones (Serra et al., 2006). Ethanol potentiated mIPSCs in CA1 neurons at 25 mM in isolated rats, whereas in group-housed rats, this effect was observed only at 100 mM. Consistent with this, we observed larger percentage increases in ethanol-induced effects in our two-bottle choice mice compared to naïve animals.

The timing of upregulation due to social isolation remains unclear, as it was only shown at 30 days. Social isolation also decreases hippocampal and cerebrocortical concentrations of allopregnanolone (Serra et al., 2006, Agís-Balboa et al., 2007). Furthermore, social isolation does not affect overall ethanol consumption but decreases the preference for ethanol, indicating a reduced response to rewarding stimuli (Pisu et al., 2011). Singly housed animals consumed slightly less ethanol during the first two weeks compared to group-housed animals (Pisu et al., 2011). Therefore, even if α4 subunit expression was upregulated during the second week of our intermittent access experiments, the decrease in neurosteroid levels due to both single housing and ethanol likely explains why no differences were observed between wild type and mutant mice. Additionally, social isolation may in part explain why wild type mice reduce their ethanol intake and preference during the second week.

In conclusion, we believe that neurosteroid modulation of α4-containing receptors likely plays a crucial role in mediating the effects of ethanol. The exact temporal significance remains unclear; however, our data suggest that this modulation is important both acutely and chronically. While our functional experiments have not fully explained the behavioural phenotype, further investigations into other brain areas are necessary to correlate the behavioural and functional findings.

6.4 Role of neurosteroid modulation of α2-$GABA_A$ receptors in the effects of ethanol

Human genetic studies have demonstrated that polymorphisms in the *GABRA2* gene, which encodes the $GABA_A$ α2 subunit, are associated with ethanol dependence (Bierut Laura et al., 2010, Lappalainen et al., 2005, Enoch et al., 2006, Ittiwut et al., 2012, Covault et al., 2004, Edenberg et al., 2004, Li et al., 2014). Evidence also indicates that these haplotypic variations in *GABRA2* can alter the subjective effects of ethanol, as measured by self-assessment of ethanol-related sensations and mood (Roh et al., 2011, Haughey et al., 2008). Studies using α2-subunit mutant mice show that alcohol consumption is influenced by these genetic manipulations, even at socially relevant ethanol concentrations (Blednov et al., 2011, Boehm et al., 2004, Newman et al., 2016, Dixon et al., 2012, Olsen et al., 2014). These findings suggest a direct relationship between ethanol and α2-subunit-containing $GABA_A$ receptors, possibly mediated by intermediate modulators. For instance, inhibiting neurosteroid synthesis, which is activated by ethanol, attenuates differences between risk and protective haplotypes, suggesting that ethanol may indirectly enhance transmission at α2-subunit-containing receptors by increasing neurosteroid levels (Pierucci-Lagha et al., 2005).

Using the intermittent access paradigm, animals rendered insensitive to neurosteroids at α2-containing $GABA_A$ receptors exhibited reduced ethanol intake and preference compared to their wild type littermates during the first week, with the most pronounced difference on the first day. These findings suggest that α2-containing $GABA_A$ receptors are crucial in mediating the indirect effects of ethanol on alcohol drinking behaviour through neurosteroids.

We observed similar trends in our limited access experiments. Homozygous mutant mice exhibited slightly reduced ethanol intake and preference compared to wild type animals. Additionally, pre-treatment with finasteride decreased ethanol preference and blood ethanol concentration in wild type mice, but not in mutant animals. These findings underscore the importance of neurosteroid sensitivity at α2-$GABA_A$ receptors in regulating ethanol drinking behaviours.

Interestingly, Newman et al. (2016) did not observe differences in ethanol intake during the DID protocol between $\alpha2^{Q/Q}$ and $\alpha2^{M/M}$ mice. However, they found that despite similar ethanol intake, $\alpha2^{M/M}$ mice had lower blood ethanol concentrations. They reported significantly higher BEC values for both wild type and mutant animals compared to our study. Methodological differences may account for this discrepancy. Newman et al. used a single ethanol bottle, while we provided both a water bottle and an ethanol bottle. It has been shown that the supply of a water bottle can reduce BECs by approximately 40 % (Rhodes et al., 2007), which aligns with the observed difference between the two studies.

Our functional experiments demonstrated that acute neurosteroid modulation of α2-type $GABA_A$ receptors does not contribute to ethanol-induced increases in mIPSC amplitude and frequency in the dentate gyrus. However, ethanol significantly prolonged mIPSCs by approximately 25 % in wild type cells compared to 3 % in mutant cells, highlighting the importance of neurosteroid sensitivity in α2-containing receptors. We observed a more pronounced role of neurosteroid modulation of α2-$GABA_A$ receptors following chronic ethanol exposure, consistent with studies showing increased α2 subunit expression after ethanol administration (Lindemeyer et al., 2017). Overall, our electrophysiological findings suggest that ethanol's effects in the dentate gyrus depend partly on the neurosteroid sensitivity of α2-containing $GABA_A$ receptors, particularly evident following chronic rather than acute exposure.

In summary, we posit that neurosteroid modulation of α2-containing receptors is pivotal in mediating ethanol's effects, though the precise temporal dynamics require further clarification. Our findings suggest this modulation is significant both acutely and chronically. While our functional experiments did not fully elucidate the behavioural phenotype, additional studies in other brain regions are warranted to correlate behavioural outcomes with functional observations.

6.5 Potential therapeutic treatments for alcohol use disorder

Despite alcohol abuse being a significant sociological and economic issue for centuries, no singular effective therapy has been discovered. The complexity of alcohol dependence's pathophysiology complicates the search for a universal drug solution. Only a limited number of approved pharmacotherapies are available, and their effectiveness in clinical settings is restricted, underscoring the continued necessity for more efficacious treatments to tackle the multifaceted challenges of alcohol dependence. This section explores the potential therapeutic roles of neurosteroids and subtype selective $GABA_A$ receptor drugs in the treatment of alcohol use disorders.

Potentiating neurosteroids

Allopregnanolone has garnered attention for its potential in treating alcohol use disorder by modulating the GABAergic system, thereby exerting anxiolytic, sedative and potentially anti-craving effects (Gatta et al., 2022). Allopregnanolone is proposed as a protective factor in healthy individuals, maintaining CNS inhibition, behavioural control, and HPA axis homeostasis (Morrow, 2007). It mitigates neuroimmune activation via TLR4 receptors, potentially preventing excessive brain excitability and systemic inflammatory responses that could lead to alcohol use disorders (Balan et al., 2019, Murugan et al., 2019).

Chronic stress or ethanol exposure depletes neuroactive steroids, disrupting HPA axis and CRH regulation, and impairing $GABA_A$ receptor function. Neuroinflammation markers increase, with tolerance developing to acute alcohol or stress effects on allopregnanolone production. This leads to prominent alcohol dependence symptoms like anxiety, dysphoria, and craving. Binge drinking exacerbates these issues, while repeated withdrawals worsen adaptations (Breese and Knapp, 2016, Olsen and Liang, 2017). Allopregnanolone may restore and normalise GABA inhibition, CRF signalling, and reduce neuroimmune activation post-ethanol exposure.

Changes in neurosteroid production are observed in neuropsychiatric disorders commonly associated with alcohol use disorder. FDA approval of brexanolone, an allopregnanolone formulation, for postpartum depression is promising, as it offers

sustained effects without tolerance or dependence risks seen with the benzodiazepines with which they have some overlapping effects (Powell et al., 2020). This supports investigating neurosteroids as potential treatments for AUD, anxiety, epilepsy, and depression, highlighting their role in neuroregulation.

Inhibitory neurosteroids

Another class of neurosteroids, known as inhibitory neurosteroids, also hold promise for potentially treating alcohol use disorder. The mechanism by which dehydroepiandrosterone (DHEA) reduces ethanol intake is still being explored. Studies indicate DHEA affects extrasynaptic $GABA_A$ receptors (Hulin et al., 2011). Behavioural studies confirm DHEA's ability to dose-dependently reduce ethanol consumption in rats (Hulin et al., 2011), and studies confirmed that DHEA crosses the blood-brain barrier without the need for conversion to sex hormones like testosterone or estradiol to reduce ethanol intake (Worrel et al., 2011, Gurkovskaya et al., 2009).

Behaviourally, DHEA resembles other negative modulators under reinforcement schedules, such as flumazenil (Amato et al., 2010). However, instead of exhibiting anxiogenic effects like other negative modulators, it produces anxiolytic effects, which may be beneficial for treating alcohol abuse (Hulin et al., 2011). Additionally, there is minimal evidence suggesting that DHEA and 7-keto DHEA (a metabolite of DHEA) have proconvulsant properties (Amato et al., 2012). However, DHEA's androgenic effects can lead to adverse effects like acne and hirsutism (Van Vollenhoven et al., 1999, Hartkamp et al., 2008). However, 7-keto DHEA, which does not convert to sex hormones (Lardy et al., 1995), similarly reduces ethanol intake, offering a potentially safer alternative.

$GABA_A$ receptor subtype selective drugs

Gaboxadol, also referred to as THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), is a GABAergic drug that selectively targets extrasynaptic $GABA_A$ receptors with δ-subunits. It has been explored for its potential therapeutic applications in treating conditions such as insomnia and alcohol use disorder (Vashchinkina et al., 2012, Ramaker et al., 2012, Lundahl et al., 2007, Wafford and Ebert, 2006).

THIP dose-dependently reduces ethanol intake, altering both consumption and motivation phases of operant self-administration and shifting drinking patterns (Ramaker et al., 2012). Furthermore, behavioural experiments showed no acute reinforcement with THIP in intravenous self-administration sessions, both in mice and in baboons (Vashchinkina et al., 2012). Additionally, conditioning sessions revealed no place preference, but rather persistent aversion. These findings indicate that THIP could be a promising treatment for alcohol use disorder. However, the clinical development of gaboxadol was discontinued after phase III trials revealed an inconsistent efficacy profile and the emergence of psychiatric side effects (Dresler et al., 2014). Developing compounds similar to THIP, but with an improved safety profile, could pave the way for new therapeutic approaches to treat alcohol use disorders.

Another δ-subunit selective compound, DS2 (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide), may serve as an alternative. However, DS2 has limited brain penetrability, suggesting that its *in vivo* effects are likely due to the modulation of peripheral immune cells (Neumann et al., 2019). Enhancing the bioavailability of DS2 could potentially be beneficial for treating AUD.

There is ongoing interest in developing $GABA_A$ receptor subtype-selective compounds, in contrast to classical benzodiazepines which act as nonselective positive allosteric modulators at GABA receptors containing α1-, α2-, α3-, and α5-subunits with a γ2 subunit (Rudolph and Knoflach, 2011). While classical benzodiazepines are effective in treating anxiety and epilepsy, they are associated with sedation, cognitive impairment, and risks of abuse and dependence (Rudolph and Knoflach, 2011). Recently, a novel compound, PF-06372865, has been identified as a α2/α3-selective ligand, demonstrating a favourable safety profile and progressing to human preclinical trials (Owen et al., 2019). This compound shows promise for treating anxiety and epilepsy. Given the significant role of α2-type $GABA_A$ receptors in alcoholism, PF-06372865 could also be explored as a potential treatment for alcohol use disorder.

6.6 Future directions

This study has examined the critical role of neurosteroid modulation of $GABA_A$ receptors in influencing alcohol consumption behaviours and has offered insights into the contributions of different $GABA_A$ receptor subtypes to these effects. However, several areas warrant further investigation.

Given the significant involvement of both α2 and α4 subunits in the chronic effects of ethanol, future research should prioritise exploring these mechanisms. Moreover, considering deficits in the reinforcing effects of ethanol observed in $\alpha4^{M/M}$ and $\alpha2^{M/M}$ mice, it is imperative to address whether these mutations alter alcohol-reinforcement behaviour compared to wild type controls, utilising conditioned place preference protocols.

Furthermore, this study focused on a single brain region, necessitating an expansion to other brain areas also implicated in alcohol use disorder, such as CA1, ventral tegmental area, nucleus accumbens, amygdala and thalamus. It is plausible that altered neuronal signalling in these regions underpins the behavioural phenotype. Whilst differences between genotypes suggest effects via specific $GABA_A$ receptor populations, electrophysiological recordings in the presence of finasteride should also be considered to validate whether the observed effects are indeed attributable to the endogenous neurosteroid modulation of $GABA_A$ receptors.

Lastly, while this study primarily assessed ethanol consumption behaviourally, future investigations should encompass other ethanol-related effects such as anxiety, sedation, hypnosis, and motor coordination. This broader approach will elucidate whether neurosteroid modulation of $GABA_A$ receptors influences these aspects of ethanol's portfolio of effects.

6.7 Concluding statement

Neurosteroids, such as allopregnanolone, are vital endogenous regulators of $GABA_A$ receptors, influencing diverse physiological processes and implicated in various central nervous system disorders, including alcohol use disorder. This study underscores the pivotal role of neurosteroids in modulating ethanol drinking behaviour in male C57BL/J6 mice. Additionally, using $\alpha2^{Q241M}$ and $\alpha4^{Q246M}$ knock-in mice, we demonstrate the essential contribution of neurosteroid potentiation of these receptor subtypes to mediating ethanol's effects. Our findings indicate that this modulation is significant both acutely and chronically.

Functional experiments conducted in this study suggest that ethanol's actions in the dentate gyrus are partly dependent upon the neurosteroid sensitivity of α2- and α4-containing $GABA_A$ receptors. These receptors serve as targets for ethanol-induced enhancement of both phasic and tonic currents, particularly following chronic rather than acute exposure.

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