Investigation of Muscarinic and Metabotropic (Glutamate) Response Properties of Adult and Immature Olfactory Cortical Brain Slices In Vitro.

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Abstract

Muscarinic cholinergic- and metabotropic glutamate receptor-evoked responses of adult and immature olfactory cortical neurones were studied in brain slices using intracellular electrophysiological recordings.

An attempt was made to study the ontogeny of the cortical slow post-stimulus afterdepolarization (sADP) induced by muscarinic cholinergic or metabotropic glutamate agonists in immature (14-22 days) and adult (greater than 40 day old) rats. In adult neurones, 10 μM oxotremorine-M (OXO-M, a muscarinic agonist) or 1S,3R-ACPD (10-50 μM, a metabotropic glutamate receptor agonist) displayed postsynaptic excitatory and presynaptic inhibitory effects. In contrast, immature rat olfactory neurones responded to 10 μM OXO-M with spontaneous epileptiform activity which was blocked by pharmacological agents such as atropine (1 μM), pirenzepine (a muscarinic M₁ receptor antagonist; 300 nM), tetrodotoxin (1 μM) or the glutamate receptor antagonists DL-APV (100 μM) or CNQX (20 μM). Inhibition of either GABAA or GABAB receptor activity with bicuculline (10 μM) or CGP52432 (1 μM) respectively, actively promoted bursting behaviour, working synergistically with lower doses of OXO-M. Conversely, augmenting GABAA or GABAB receptor function with pentobarbitone (100 μM) or baclofen (10 μM), respectively, blocked the bursting. The induced bursting was accompanied by a dramatic prolongation of evoked postsynaptic potentials (PSPs), exhibiting recurrent superimposed spike discharges, not seen in control. 1S,3R-ACPD (10-50 μM) induced typical adult-type responses in immature
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cells, i.e. presynaptic inhibition and postsynaptic excitation, with no evidence of
epileptiform behaviour.

During experiments designed to investigate the possible involvement of release
of calcium from intracellular stores in the generation of the cholinergic/metabotropic
 glutamate agonist-induced sADP and its underlying tail current ($I_{ADP}$) in adult guinea-
 pig olfactory neurones, it was found that the sADP induced by OXO-M (10 μM) or
1S,3R ACPD (10-50 μM), could be reversibly blocked by 0.5-3 mM caffeine, a
compound known to promote release of calcium from intracellular stores. However,
when this inhibitory action was investigated further using compounds with more
specific effects on intracellular calcium release, i.e. ryanodine (10 μM), thapsigargin (3
μM) or dantrolene (10 μM), no consistent inhibition of $I_{ADP}$ was observed. In addition,
other possible effects of caffeine were discounted as being important by use of more
specific pharmacological tools, i.e. IBMX (100 μM; an inhibitor of phosphodiesterase
activity) or adenosine (100 μM; to reverse any inhibition of adenosine receptors
imposed by caffeine). In caesium-loaded cells, a direct block of voltage-sensitive
calcium entry by 3 mM caffeine was found, but this could not account for the full
inhibition of $I_{ADP}$ in this system; a direct blockade of the proposed $I_{ADP}$ $K^+$ channels by
caffeine was therefore proposed.

In conclusion, the muscarinic response profile of immature rat olfactory
neurones was found to differ from adult neurones, since they displayed bursting
behaviour in response to muscarinic (but not metabotropic) activation, thought to be
generated through a local network mechanism. Furthermore, release of calcium from
intracellular stores was unlikely to play an important role in sADP or $I_{ADP}$ generation.
Caffeine, a known modulator of intracellular calcium release, however, blocked $I_{ADP}$
through a mechanism which did not involve intracellular calcium stores, most likely having a direct action on calcium entry and/or the proposed $I_{ADP} K^+$ conductance.
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CHAPTER 1

INTRODUCTION
1.1. Foreword

The olfactory cortical area is concerned with the processing of olfactory signals to the brain from sensory receptors in the nose, receiving input via the olfactory nerves and the olfactory bulbs located at the very front of the brain of most mammals (e.g. Kandel & Schwartz, 1985). It is one of the first regions of the brain used to investigate neuronal functions in vitro in the form of a slice preparation (Yamamoto & McIlwain, 1966) and over the years has been used extensively for both extracellular (Williams et al., 1985; Das et al., 1992) and intracellular (Scholfield, 1978; Hoffman & Haberly, 1991) investigations of basic cortical electrophysiology and pharmacology (Williams & Constanti, 1988; Constanti et al., 1993; Libri et al., 1997). More recently it has been shown in our laboratory that olfactory cortical pyramidal neurones in vitro are strongly excited by agonists of muscarinic cholinergic or metabotropic glutamate receptors (Constanti et al., 1993; Libri et al., 1994; Libri et al., 1997). The olfactory cortical area also has a complex network of synaptic connections between the principal cell types and interneurones (Hasselmo & Bower, 1992; Hasselmo & Bower, 1993) which can be incorporated into useful models of cortical memory (Haberly & Bower, 1989), displaying features such as long term potentiation (LTP) (Kanter & Haberly, 1990) and various fast and slow excitatory and inhibitory synaptic events (Satou et al., 1982; Tseng & Haberly, 1988; Libri et al., 1997). Olfactory cortical cells can show a variety of afterpotentials in response to a long depolarizing stimulus, e.g. a slow afterhyperpolarization (sAHP; Constanti & Sim, 1987) or a slow post-stimulus afterdepolarization (sADP), thought to be a result of a calcium-dependent decrease in a novel potassium conductance (Constanti & Bagetta, 1991). This unusual phenomenon has been shown to display a rather complicated pharmacology, and has strong
implications for neuronal excitability, possibly also contributing to the generation and maintenance of epileptiform behaviour in this region (Constanti et al., 1993; Libri et al., 1997). In other brain areas, hyperpolarizing and depolarizing afterpotentials have been shown to be dependent upon calcium entry into the recorded cells, with subsequent activation of calcium-induced calcium release (CICR) mechanisms (Torres et al., 1996; Li & Hatton, 1997). It is not, however, clear whether such mechanisms are also involved in the cortical sADP generation process.

It now appears that the olfactory cortical region is crucially involved in the formation and propagation of kindling-induced epileptogenesis (Hoffman & Haberly, 1996; Löscher & Ebert, 1996) and slice preparations show spontaneous epileptiform bursting in response to various experimental manipulations such as reducing extracellular magnesium (Libri et al., 1996) or exposure to the chemoconvulsant agent 4-aminopyridine (4-AP; Galvan et al., 1982). In fact, it is now suggested that the olfactory cortex may play an important role in some human epileptiform syndromes (such as temporal lobe epilepsy), particularly considering the olfactory ‘hallucinations’ often seen during certain types of seizure (Devinsky et al., 1998), and seizures which occur upon olfactory stimulation (Komárek, 1994).

The original aim of the present work was to characterise the developmental features of the sADP and whether it was dependent on intracellular calcium release mechanisms. Quite unexpectedly our attention was diverted by a novel epileptiform bursting behaviour seen in immature, but not adult rat olfactory slices in response to muscarinic receptor activation. This thesis represents an account of both these sets of experiments carried out using the single microelectrode current clamp/voltage clamp technique in rat and guinea-pig olfactory cortical neurones in vitro.
1.2 Anatomy of the olfactory cortex

The mammalian olfactory cortex is a phylogenetically old structure, situated bilaterally on the underside of the brain, identified in both rodents (e.g. Löscher & Ebert, 1996) and humans (Zatorre et al., 1992) as being located towards the rostral part of the cortical region near its junction with the temporal cortex, and immediately behind the two olfactory bulbs (Fig. 1.1). The cortex is joined on each side to the olfactory bulbs by a layer of afferent fibres, originating from the mitral cell bodies, called the lateral olfactory tract (LOT) which forms a broad distribution across the cortical surface layer, in such a way that small regions of the cortex receive fibres from large areas of the olfactory bulb and small regions of the olfactory bulb project onto large areas of the cortex (Litaudon et al., 1997).

![Diagram of the brain showing the position of the olfactory cortex](image)

Fig. 1.1. Underside of the brain of a guinea-pig showing the position of the olfactory cortex towards the front of the inferior surface (underlying the lateral olfactory tracts on both sides; modified from Lottman, 1963).
This region of cortex can be subdivided into anatomical divisions, of which there are three prepiriform regions, the olfactory tubercle and the periamygdaloid area (Fig. 1.2; Halliwell, 1975). Most of the recordings in this series of experiments were taken from the periamygdaloid section of the brain slices.

Fig. 1.2. Representation of the surface of the olfactory cortex showing the anatomical subdivisions; PP = prepiriform cortex, OT = olfactory tubercle and PAM = periamygdaloid cortex. The grey area is the lateral olfactory tract (taken from Halliwell, 1975).

The olfactory cortex receives inputs from several regions of the brain, including serotonergic projections from the dorsal and median raphe nuclei (Datiche et al., 1995), catecholaminergic innervation from the ventral tegmental area and the dorsal part of the locus coeruleus (Datich & Cattarelli, 1995) and dopaminergic inputs from the ventral tegmental area and the substantia nigra (Loughlin & Fallon, 1984). Of particular interest to the present work is the general cholinergic input into the cerebral cortex, specifically the olfactory cortex. The main inputs into the frontal cortex originate in nuclei of the basal forebrain (for review see Fibiger, 1982), identified by a combination of retrograde tracing and acetylcholinesterase staining techniques as the nucleus of the diagonal band, nucleus basalis of the substantia inominata and cells of the medullary laminae of the globus pallidus (Mesulam & Van-Hoesen, 1976). Of these inputs, the most important functional projections to the olfactory cortex appear to be from the nucleus of the diagonal band (Luskin & Price, 1982).
1.2.1 Laminar arrangement of the olfactory cortical region

The olfactory cortex can be categorised into a series of several layers according to different cell and fibre types (e.g. Haberly & Shepherd 1973; Hasselmo & Bower; 1992; Hasselmo & Bower, 1993; Hoffman & Haberly, 1996). The laminar system is usually named according to the nomenclature of Haberly & Shepherd (1973), where the most superficial layer (Ia) is composed of the afferent LOT fibres originating in the olfactory bulb (Fig. 1.3). When these fibres reach the olfactory cortex, they form synapses onto both superficial and deep pyramidal neurones (see Haberly & Shepherd, 1973). Layers Ib, II and III contain the intrinsic (association) fibres which form a network connecting the different types of cells present within the olfactory cortex (Hasselman & Bower, 1992).

Fig. 1.3 Schematic representation of the laminar arrangement of the olfactory cortex as seen in the transverse slice preparation used in this study (see methods). A stimulating electrode, used to evoke synaptic EPSP/IPSP complexes was within the intrinsic fibre layer Ib/II. Most recordings were taken...
from deep pyramidal cells located in layer III. At the synaptic connections, + denotes excitation while - indicates inhibition (modified from Haberly & Shepherd, 1973).

So-called superficial pyramidal neurones are located in layer II, and display a discreet pattern of electrophysiological responses, while ‘deep’ pyramidal neurones are located in layer III, and show a different pattern of behaviour. Layer III also contains a population of non-pyramidal neurones, probably interneurones, which are of many different morphologies (e.g. bipolar, multipolar), but similar electrophysiology (Libri et al., 1994). Underlying layer III is an area often labelled as olfactory layer IV, the endopiriform nucleus, which is now known to be particularly susceptible to kindling-induced epileptogenesis (Löscher & Ebert, 1996), and has been implicated in the generation and spread of epileptiform activity throughout the olfactory cortex (Hoffman & Haberly, 1996).

1.2.2 Cortical cell types

There are three principal cell types present in the olfactory cortex, differing in morphology and location within the olfactory cortical layers, as well as responsiveness to stimuli. In the so-called densely packed cell layer (layer II), there is an abundance of superficial pyramidal neurone somata, which are classically pyramidal in shape (Stevens, 1969), but with relatively small cell bodies (15-30 μm diameter; Shepherd, 1974), and short apical dendritic trunks (Haberly, 1983). Such neurones display a characteristic response pattern to intracellular depolarizing stimuli, resulting in a clear spike fractionation and a strong accommodation of firing (Libri et al., 1994).
The deeper layer III contains the two other principle cell types, the deep pyramidal and the non-pyramidal neurones. The deep pyramidal neurones have typical pyramidal shaped somata of larger diameter to the superficial neurones (20-40 \( \mu \text{m} \) diameter; Shepherd, 1974), and extend a single long apical dendrite towards the surface layers of the cortex. The non-pyramidal neurones tend to be either bipolar or multipolar in shape (15-40 \( \mu \text{m} \) diameter; Shepherd, 1974), with dendritic trees generally confined to the deep layer III (Tseng & Haberly, 1989). The basic electrophysiology of these cells is very similar, displaying a characteristic action potential firing pattern in response to short depolarizing stimuli, although the non-pyramidal neurones tend to display a fast spike-afterhyperpolarization which is notably absent in the deep pyramidal neurones. Neither cell type shows spike fractionation, or strong spike accommodation in response to short or long depolarizing stimuli, respectively (Libri et al., 1994).

1.2.3 Synaptic arrangement

The olfactory cortex has a complicated network of synaptic connections, including those of afferent fibres, principally originating in the olfactory bulb, and a series of association fibres connecting the cells within the olfactory cortical layers (e.g. Haberly & Bower, 1989; Hasselmo & Bower, 1992). The afferent fibres tend to form synapses onto both the superficial and deep pyramidal neurones (Haberly & Bower, 1984) while the association fibres form both excitatory and inhibitory connections onto all three cortical cell types (Fig. 1.3; Haberly & Shepherd, 1973). It is noteworthy that the afferent inputs from the olfactory bulb only synapse with the principle cell types, i.e. the superficial and deep pyramidal neurones. This is a similar situation to that seen in
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the olfactory bulb, however is in contrast to other brain areas such as the cerebellum, hippocampus and hypothalamus, where afferent inputs appear to form a diffuse pattern of connectivity to all cell types. The central inputs, however, synapse onto the principle cell types as well as the interneurones; the two different inputs therefore seem to make use of different arrays of neuronal circuitry within the cortex, the olfactory afferent inputs having a primary function in olfactory processing, while the central inputs play a more 'modulatory' role (Shepherd, 1974). The unusual network arrangement of afferent and intrinsic fibres within the olfactory cortex has been postulated to be an extremely useful model for the study of processes involved in learning and memory, in particular the ability of cells to provide feedback and feed-forward excitation and inhibition onto themselves and surrounding neurones (Haberly & Bower, 1989); in addition, there are several reports of long term potentiation (LTP) of synaptic activity within the olfactory association fibre networks (Stripling et al., 1988; Kanter & Haberly, 1990), a process heavily implicated in the formation and laying down of memory traces (e.g. Eccles, 1983). In olfactory cortical brain slices, intracellular and extracellular recordings have demonstrated that electrical stimulation of the afferent LOT or association fibres results in a characteristic excitatory and fast/slow inhibitory postsynaptic potential (EPSP/IPSP) complex (intracellular recordings: Satou et al., 1982; Tseng & Haberly, 1988; Libri et al., 1997; field potential extracellular recordings: Williams et al., 1985; Williams & Constanti, 1988; Das et al., 1992). The excitatory postsynaptic potential (EPSP) is mediated by glutamate, released from synaptic terminals, acting on NMDA and non-NMDA type glutamate receptors. This precedes an early fast inhibitory postsynaptic potential (IPSP) resulting from released γ-aminobutyric acid (GABA) acting on GABA_A receptors, which is followed by a late slow IPSP resulting from
GABA acting on GABA\textsubscript{B} receptors (Malcangio \textit{et al.}, 1995). The EPSP and the fast (GABA\textsubscript{A}, chloride-mediated) IPSP recorded in the adult olfactory cortex are typically reduced upon depolarization of the membrane potential, while the slow (GABA\textsubscript{B}, potassium-mediated) IPSP becomes more prominent on membrane depolarization. The GABA\textsubscript{A} fast IPSP was demonstrated to reverse at around -50 mV membrane potential whereas the GABA\textsubscript{B} slow IPSP disappears (without showing clear reversal) at around -90 mV (Libri \textit{et al.}, 1996).

\textbf{1.3 Effects of muscarinic cholinergic receptor activation.}

\textbf{1.3.1 Muscarinic receptor subtypes.}

The various subtypes of the muscarinic acetylcholine receptor were originally classified pharmacologically as either M\textsubscript{1} or M\textsubscript{2}, depending on their differential sensitivity to pirenzepine, a selective antagonist of the M\textsubscript{1} receptor (Wolfe, 1989). Through molecular cloning techniques, 5 subtypes of the muscarinic receptor have now been identified, named M\textsubscript{1}-M\textsubscript{5}, based upon their order of discovery. Further to this, the muscarinic receptor subtypes can be generally grouped according to their functional coupling to either mobilization of intracellular calcium (M\textsubscript{1}, M\textsubscript{3} & M\textsubscript{5}) or their inhibition of adenylate cyclase activity (M\textsubscript{2} & M\textsubscript{4}). The M\textsubscript{1}, M\textsubscript{3} and M\textsubscript{5} subtypes also have the potential to couple to phospholipases A2, C and D, tyrosine kinase and calcium influx, while the M\textsubscript{2} and M\textsubscript{4} subtypes have an additional role in augmentation of phospholipase A2 (for review, see Felder, 1995). Studies using immunoprecipitation techniques (using M\textsubscript{1} or M\textsubscript{2} specific antisera) indicate that the olfactory cortex shows a
tendency to express the $M_1$, and to a certain extent the $M_2$ subtypes (Wall et al. 1991; Li et al., 1991), which will have implications in the responses to muscarinic receptor activation of cells from this region.

1.3.2 General excitatory effects of muscarinic receptor activation.

For over 30 years it has been known that mAChR activation in the cerebral cortex results in a slow neuronal excitation (Krnjević & Phillis, 1963a,b). The early studies of Krnjević & Phillis, using extracellular recordings from cat cerebral cortex in vivo revealed that direct iontophoretic application of acetylcholine onto most cortical neurones resulted in initiation of a strong excitatory response, consisting of spontaneous repetitive firing in the 10 Hz frequency range, with notably long latency and prolonged duration (Krnjević & Phillis, 1963a). Later evidence indicated that the discharge was specific to muscarinic rather than nicotinic receptor activation (Krnjević & Phillis, 1963b). With the advent of intracellular recording, also performed on cat cerebral cortex neurones in vivo, it was revealed that iontophoretic application of muscarinic agonists resulted in a depolarization of the membrane potential, associated with an increase in membrane input resistance, and an intense neuronal spike discharge activity (Krnjević et al., 1971). This depolarization and spike discharge was originally attributed to a muscarinic reduction of the resting potassium ($K^+$) conductance of the cortical neurones in conjunction with an inhibition of the delayed rectifier $K^+$ current of the action potential. It has been shown more recently that muscarinic receptor activation in a variety of neuronal systems can lead to a suppression of a number of different types of $K^+$ currents, principally the $M$-current ($I_M$) (Adams et al., 1982; Constanti & Galvan, 1983; Womble & Moises, 1992), the post-stimulus slow afterhyperpolarization
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(Constanti & Sim, 1987; Washburn & Moises, 1992) and its underlying calcium-activated potassium current ($I_{AHP}$) (McCormick & Prince, 1986; Constanti & Sim, 1987; Benson et al., 1988), a fast transient outward current ($I_A$) (Nakajima et al., 1986) and a fast inwardly rectifying current ($I_{K,IR}$) (Brown, 1990). In addition, muscarinic activation has also been shown to modulate background 'leak' conductances ($I_{K,leak}$) (Halliwell, 1990) and also inward calcium currents (Toselli & Lux, 1989; Schultz et al., 1990).

More recently it has been reported that the depolarization induced by acetylcholine in layer V pyramidal neurones in the medial prefrontal cortex may be predominantly due to muscarinic activation of a non-selective cation current rather than a traditional suppression of potassium currents (Haj-Dahmane & Andrade, 1996).

Other excitatory events resulting from muscarinic receptor activation have been studied in various different types of neurones. It has already been mentioned that muscarinic agonists can suppress the slow calcium-dependent afterhyperpolarization (sAHP) following a burst of action potentials. This is often accompanied by a decrease in the spike accommodation displayed by such cells, a factor which is itself excitatory (e.g. Constanti & Sim, 1986; Washburn & Moises, 1992) which may be due to a suppression of a fast spike-afterhyperpolarization. A further excitatory event is the muscarinic induction, in certain neurones, of a slow post-stimulus afterdepolarization (sADP) which actually replaces the sAHP in responsive cells. The first indications of the presence of a muscarinic agonist-induced sADP in cortical neurones were apparent in the recordings of Krsjević et al., (1971), although at that time no further studies were performed on the phenomenon. Since this time there have been several reports of sADPs elicited by muscarinic agonists in various systems including hippocampal neurones (Costa et al., 1991), septal neurones (Hasuo & Gallagher, 1990) and cells from
various cortical regions including the association cortex (Andrade, 1991; Araneda & Andrade, 1991), the sensorimotor cortex (Schwindt et al., 1988) and the olfactory cortex (Constanti & Bagetta, 1991; Constanti et al., 1993; Libri et al., 1994).

The slow onset and recovery of neuronal excitatory events in response to muscarinic activation suggests that there are various intracellular transduction mechanisms involved, rather than direct opening/closing of ion channels, as in the case of nicotinic acetylcholine receptor activation (Betz, 1990). There is a large body of evidence which suggests that the muscarinic receptor subtype responsible for the depolarizing action of cholinergic agonists in neurones of different regions is generally M₁ (e.g. Benson et al., 1988; Uchimura & North, 1990; Hsu et al., 1996); receptors of this subgroup are usually linked to their signal transduction mechanism by a pertussis toxin-insensitive G-protein (Wess, 1993), coupled to activation of intracellular protein kinase C (PKC) and release of inositol 1,4,5 trisphosphate and calcium (see later). In contrast, the M₂ subtype, which modulates adenylate cyclase activity (and hence a decrease in intracellular concentration of cyclic adenosine monophosphate [cAMP]) has been linked with presynaptic muscarinic activation in some systems (Felder, 1995). These intracellular signalling mechanisms are also likely to be implicated in the neuronal responses induced by exposure to muscarinic agonists in the olfactory cortex.

1.3.3 Effects of muscarinic agonists on synaptic transmission.

Considering the widely distributed cholinergic innervation throughout the brain, it seems likely that as well as mediating general postsynaptic changes in neuronal excitability, acetylcholine receptors should play a role in modulation of synaptic transmission. Indeed, acetylcholine has been proposed as a major neurotransmitter in
the processes of learning and the formation of memories, particularly taking into account the behavioural pharmacology of cholinomimetics and cholinergic antagonists, as well as the behavioural neuroscience of specific brain conditions in which cholinergic nuclei are lesioned, or major cholinergic pathways are transected (for review see Blokland, 1996).

There has been much research into the effects of cholinergic agents on evoked synaptic transmission in various neuronal systems recorded in vitro. In general, application of muscarinic agonists results in a depression of synaptic transmission, as reported in brain slices taken from, for example, the basolateral amygdala (Washburn & Moises, 1992). Early studies in slices of the olfactory cortex showed that bath application of muscarine reversibly depressed field potentials (known as ‘N-waves’ or ‘surface negative field potentials’) elicited by orthodromic stimulation of the LOT, an effect reversible by application of atropine or pirenzepine, and not mimicked by application of nicotinic agonists (Williams et al., 1985). Later work confirmed that several muscarinic agonists suppressed the evoked N-wave in a dose-dependent manner (Williams & Constanti, 1988), particularly the potent muscarinic agonist oxotremorine-M (OXO-M) (Bagetta & Constanti, 1990).

Suppression of LOT-evoked synaptic transmission by muscarinic agonists was generally blocked by application of the non-specific muscarinic antagonist atropine or the muscarinic M₁ receptor subtype specific antagonists pirenzepine (Williams et al., 1985) or telenzepine (Bagetta & Constanti, 1990), indicating that this inhibitory effect of muscarinic agonists was most likely mediated via a presynaptic muscarinic M₁-type receptor. Indeed, further pharmacological studies revealed that application of the muscarinic M₂ (cardiac) receptor specific antagonists methoctramine or AF-DX 116
(Bagetta & Constanti, 1990), the \( M_3 \) (smooth muscle/glandular) receptor specific antagonists hexahydro-sila-difenidol (HHSiD) or its p-fluoro- analogue (p-F-HHSiD) (Bagetta & Constanti, 1991) or the \( M_4 \) receptor specific antagonist himbacine (Das et al., 1992) indicated that none of these muscarinic receptor subtypes were likely to be involved in the muscarinic receptor-induced suppression of evoked synaptic transmission in the olfactory cortex; however, contrasting studies in other CNS systems suggest that presynaptic inhibition may be mediated by the \( M_2 \) or \( M_3 \) receptor subtypes (Hsu et al., 1995; Bellingham & Berger, 1996)

A similar action of muscarinic agonists has been confirmed in intracellular recordings. Both EPSPs and IPSPs have been shown to be reversibly reduced by > 80% in olfactory cortex upon muscarinic receptor activation (Libri et al., 1996) and in other neuronal systems such as the hippocampus (Burgard et al., 1993; Milburn & Prince, 1993) and the visual cortex (Veknin & Teyler, 1991). This activity most likely results from a muscarinic receptor-mediated reduction in activity of voltage gated calcium channels in the presynaptic terminal taking place via a G-protein linked transduction mechanism (for review see Miller, 1990). It is interesting to note, however, that in the olfactory cortex, muscarinic suppression of evoked synaptic transmission appears to be specific to the association fibre networks in the deeper layers, and does not apparently occur on the afferent (LOT) fibres in the surface layer (Hasselmo & Bower, 1992), a factor which is thought to have implications in the storing and recall of olfactory memories. An interesting theory of the function of acetylcholine in memory has now been formed which suggests that the cholinergic-suppression of synaptic transmission in the olfactory cortex and regions CA1 and CA3 of the hippocampus (areas implicated in so called ‘associative’ memory) actually aids the formation of new memories by
preventing interference between neuronal networks responsible for formation of new memories (based on afferent fibre activity), and those responsible for recall of previously stored memory patterns (for review see Hasselmo & Bower, 1993). It is certainly clear that the correct function of cholinergic systems in the brain is important for learning and memory, since the loss of cholinergic innervation in neurodegenerative diseases such as Alzheimer’s disease, is associated with profound impairment of memory formation and function (Sitaram, 1984).

1.4 Effects of metabotropic glutamate receptor activation

Metabotropic glutamate receptors (mGluRs) are a large family of G-protein-linked receptors with diverse properties in terms of intracellular transduction coupling, pharmacology and central neuronal distribution (for review see Bockaert et al., 1993). At least eight different subtypes of mGluRs (mGluR1-mGluR8) have been so far cloned and characterized according to their agonist selectivity, intracellular effector mechanisms and sequence similarity (for review see Pin & Duvoisin, 1995). Group I mGluRs (mGluR1/mGluR5) are coupled to stimulation of phosphoinositide (PI) hydrolysis and intracellular calcium mobilization, and are potently activated by the cyclic glutamate analogue 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), but are insensitive to L-2-amino-4-phosphonobutyrate (L-AP4). Group II mGluRs (mGluR2/mGluR3) are negatively coupled to adenyl cyclase and cyclic AMP formation, and are also potently activated by 1S,3R-ACPD, but are not activated by L-AP4. Finally, group III mGluRs (mGluR4/ mGluR6/mGluR7/mGluR8) are also negatively coupled to adenyl cyclase activity, but are activated by L-AP4, and not 1S,3R-ACPD (for review see Schoepp, 1994; Pin & Duvoisin, 1995).
Metabotropic glutamate receptor activation in neurones of the olfactory cortex results in a very similar response profile to that seen following muscarinic activation in this system. Thus, in responding neurones (Constanti et al., 1993), mGluR agonists produce a slow neuronal excitation consisting of a depolarization and repetitive firing activity, an increase in input resistance and replacement of the post-stimulus sAHP (and its underlying outward tail current $I_{AHP}$) with a sADP (and underlying inward tail current $I_{ADP}$; Constanti & Libri, 1992; Libri et al., 1996). This postsynaptic excitation is associated with an inhibition of evoked excitatory synaptic transmission by up to 80% (Collins, 1993; Libri et al., 1996; 1997). A detailed study of the pharmacological profile of mGluR actions in the olfactory cortex indicated that the postsynaptic excitatory effects of mGluR activation were most likely mediated by group I mGluRs (linked to PI hydrolysis), whereas at least two pharmacologically distinct mGluRs (group II and III, negatively linked to cyclic AMP formation) may be involved, as presynaptic inhibitory autoreceptors, in the modulation of olfactory cortical synaptic transmission (Libri et al., 1997).

1.5 The post-stimulus afterdepolarization (sADP).

As mentioned above, the first indication that a post-stimulus afterdepolarization in response to muscarinic activation was present in cortical neurones came from the pioneer intracellular experiments of Krmjević and colleagues in cat brain in vivo (Krmjević et al., 1971). Although there was a clear presence of a sADP lasting several seconds or minutes following a depolarizing stimulus in the presence of iontophoretically applied acetylcholine, it took a considerable length of time before any further investigation of this phenomenon was attempted. A prominent post-stimulus
sADP was found during intracellular recordings taken from sensorimotor cortex slices in the presence of muscarine (>5 μM). The sADP replaced a prominent sAHP in these cells, and at the peak of the depolarization, repetitive firing was observed (Schwindt et al., 1988); however, the ionic mechanisms underlying the sADP in these neurones were not investigated at the time. The first voltage clamp study of the sADP was made in slices of olfactory cortex in the presence of the agonist OXO-M (10 μM; Constanti and Bagetta, 1991) using a single microelectrode current/voltage clamp recording technique (Fig. 1.4.). Firstly it was discovered that the sADP was predominantly expressed in a discreet population of neurones within the olfactory cortex, located in layer III of this region, which were then termed ‘responding neurones’ (Constanti et al., 1993). It was later confirmed by a combination of electrophysiological techniques and intracellular neurobiotin staining that the responding neurones fell into two categories, while the non-responding cells were of only one type. Those neurones displaying a strong muscarinic depolarization and excitation generally showed a prominent (greater than 10 mV amplitude) sADP, and were found to be the deep pyramidal neurones (‘type I’ responding neurones), whereas those displaying a less prominent depolarization and a weak sADP (less than 5 mV amplitude) were found to be interneurones located in layers II-III, and were labelled ‘type II’ cells. Finally, a third population of neurones showed no muscarinic depolarization or sADP. These were shown to be superficial pyramidal neurones located in layer II and were labelled ‘type III’ non-responding cells (Constanti et al., 1993; Libri et al., 1994). Interestingly, it was also found that a prominent sADP of similar amplitude and time course could be elicited in responding olfactory neurones by application of a similar depolarizing stimulus in the presence of trans-ACPD, a racemic metabotropic glutamate receptor agonist (Constanti & Libri, 1992).
Fig. 1.4 Recordings of the sADP and its underlying tail current $I_{ADP}$ obtained from guinea-pig olfactory cortical neurones in vitro. A shows a typical sAHP following a 1.6 second (~0.6 nA) stimulus under control conditions, which under the influence of OXO-M (10 µM) is replaced by a sADP, with superimposed repetitive firing. The arrow indicates injection of constant negative current to bring the cell below the threshold for firing of action potentials (taken from Constanti et al., 1993). B (taken from Constanti & Bagetta, 1991) shows the outward tail current underlying the sAHP, which is replaced by a slow inward tail current underlying the sADP in the presence of OXO-M. Both tail currents were measured using the ‘hybrid’ voltage clamp technique (DCC indicates recording in discontinuous current clamp mode, and VC indicates voltage clamp mode), and similar stimulus parameters were used as in A.

1.5.1 General characteristics of the sADP and its underlying tail current $I_{ADP}$.

The sADP elicited by a long depolarizing current pulse (~1.6 seconds, 1-2 nA) was found to be 10-15 mV in amplitude in responding neurones (when measured at a
membrane potential of -70 mV), the peak amplitude often being difficult to measure under these conditions due to the appearance of a self-perpetuating spike discharge of ~10 Hz frequency superimposed upon the peak of the sADP (lasting between 30 seconds and several minutes; Constanti & Bagetta, 1991; Constanti & Libri, 1992; Constanti et al., 1993). The membrane current underlying the sADP ($I_{ADP}$) was revealed using a ‘hybrid clamp’ technique (Pennefather et al., 1985; Lancaster & Adams, 1986) in which a manual switch to voltage clamp (usually at -70 mV holding potential) was made immediately following a depolarizing stimulus. This current, in the layer III pyramidal cells, was found to be an inward tail current of ~0.3 nA, ~2 seconds time to peak, and overall duration of ~50 seconds when induced by 10 μM OXO-M (Constanti et al., 1993; Libri et al., 1997) or 10 μM 1S,3R ACPD (Constanti & Libri, 1992; Libri et al., 1997). It was also found that both the sADP and $I_{ADP}$ amplitude were affected by the width and intensity of the given depolarizing stimulus, with shorter stimuli or stimuli of lower amplitude resulting in a smaller sADP and $I_{ADP}$. Further to this, $I_{ADP}$ could be induced in the presence of OXO-M under voltage clamp conditions (in the presence of TTX) by briefly stepping the membrane potential more positive, with $I_{ADP}$ amplitude, as in the case of current and ‘hybrid’ clamp experiments, being dependent on the width (stimulus width ranging from 1-20 s) and amplitude (ranging from +10 mV to +40 mV step, while voltage clamped at -70 mV) of the depolarizing command (Constanti et al., 1993).

When first characterised, the $I_{ADP}$ was shown to be associated with a clear decrease in input conductance at its peak, a factor which suggested that it might result from a ‘switching off’ and subsequent reactivation of a background $K^+$ current in the presence of mAChR agonists (Constanti & Bagetta, 1991). A reduction in a resting current was also reported to underlie a slow afterdepolarization in cells from layer V of the
neocortex under mGluR activation (Greene et al., 1994). In neurones from both regions, it was found that sADP and \( I_{\text{ADP}} \) amplitude decreased with hyperpolarization and increased with depolarization of membrane potential, disappearing (but not reversing) at potentials approaching the potassium equilibrium potential (\( E_K \)). Raising extracellular potassium concentration also diminished the sADP response, both factors pointing towards a potassium conductance mechanism being involved in generation of \( I_{\text{ADP}} \) (Constanti et al., 1993; Greene et al., 1994; see also section 1.5.4 below).

1.5.2 Role of calcium in cortical sADP generation.

The sADP and \( I_{\text{ADP}} \) in the layer III olfactory cortical neurones were found to be reduced by administration of \( \text{Cd}^{2+} \), or by removal of extracellular calcium from the bathing medium, confirming that calcium entry into the cell was an essential component of \( I_{\text{ADP}} \) induction (Constanti & Bagetta, 1991). The induced sADP in layer V neocortical neurones was also reduced by removal of extracellular \( \text{Ca}^{2+} \) (being replaced by \( \text{Mn}^{2+} \)) or by intracellular application of the calcium chelator 5,5'-dimethyl-bis-(o-aminophenoxy)-\( N,N,N',N'\)-tetraacetic acid (BAPTA) (Greene et al., 1994). However, intracellular application of the calcium chelators EGTA or BAPTA, or extracellular exposure to the membrane permeant acetoxyethyl ester derivative of BAPTA, BAPTA-AM, failed to reduce the sADP or \( I_{\text{ADP}} \) recorded in olfactory neurones, a factor which was ascribed to the time course of intracellular calcium loading being too fast for the chelators to buffer the calcium effectively (Constanti et al., 1993). Nevertheless, facilitating entry of calcium into neurones of either olfactory cortex or neocortex by pre-treatment with tetraethylammonium (TEA) or tetrabutylammonium (TBA) (to prolong spike duration) resulted in an increase in sADP amplitude (Constanti et al., 1993; Greene et al., 1994).
1.5.3 Proposed mechanism of sADP and $I_{ADP}$ induction.

The mechanism of induction of the sADP in olfactory cortical neurones is clearly complicated, requiring a number of different cellular conditions to be satisfied. The presence of either a mAChR or mGluR agonist is clearly essential for appearance of the sADP, these receptors being linked to G-protein coupled second messenger systems. The presence of extracellular calcium (and indeed calcium entry into the cell) is also an essential component in this mechanism. Previous work also suggests that a potassium conductance underlies the sADP, but unlike the sAHP, which is a calcium-activated potassium conductance, and therefore associated with an increase in conductance (Lancaster & Adams, 1986), the sADP phenomenon is associated with a decrease in conductance. The implication of all of these factors is that under the conditions of mAChR or mGluR activation there may be a background potassium conductance in operation, either activated or somehow 'primed' by mAChR or mGluR agonists to become sensitive to intracellular calcium accumulation. Deactivation of this conductance following entry into the cell of a large bolus of calcium (provided by a prolonged depolarizing stimulus) is followed by a slow reactivation, as the calcium level in the cell gradually falls back towards resting value, returning membrane potential to its pre-stimulus level. A schematic diagram of the proposed sADP induction mechanism is shown in Fig. 1.5.
It is as yet unclear if this proposed potassium conductance is always present under resting conditions and made sensitive to calcium by the presence of mAChR or mGluR agonists, or whether the conductance is activated by the presence of these agents. Traditional potassium channel blockers like TEA, TBA and 4-AP block induction of the sADP (Constanti & Bagetta, 1991; also see 1.5.5 below), but there is currently no specific agent to block this novel conductance mechanism, which would allow an investigation of
this possibility (deactivation of a background K⁺ current by a specific \( I_{ADP} \) channel blocker would be expected to depolarize the cell in the absence of any further stimulus).

1.5.4 Comparison of the cortical sADP with ADPs from other brain regions.

There have been many reports of afterdepolarizing responses in a variety of different neuronal systems, some displaying very similar characteristics to the cortical sADP, and others having quite different properties. A sADP has been described in several neurone types which displays very similar characteristics to those seen in olfactory neurones; e.g. a clear muscarinic receptor-induced sADP was seen in neurones from layer V of the sensorimotor cortex which replaced the sAHP and resulted in continuous self-perpetuating repetitive firing activity. This sADP was calcium dependent, but thought to be due either to a non-specific cation current, or an increase in chloride conductance (Schwindt et al., 1988). A sADP was also reported in cells from the dorsolateral septal nucleus, however this differed from the sADP in cortical neurones in that the presence of mAChR or mGluR agonists was not necessary for induction of the underlying depolarizing current, although application of muscarine to these cells was seen to potentiate the sADP current. In this system the sADP was again attributed to a calcium-dependent increase in non-specific cation (possibly sodium) conductance (Hasuo & Gallagher, 1990; Hasuo et al., 1990). Cells from bullfrog sympathetic ganglia have also been demonstrated to have a long lasting sADP resulting from a calcium-dependent inactivation of a potassium current (Tokimasa, 1985), while mouse sympathetic ganglion neurones display a slow sADP which appears to be a calcium-dependent chloride current (De Castro et al., 1997). In neither the bullfrog or the mouse sympathetic ganglion cells was mAChR activation necessary to induce the
sADP, and it was unknown if application of such an agonist would potentiate the depolarizing response.

As well as the slow afterdepolarizations in these neuronal systems, there are numerous reports of *fast* afterdepolarizations in many different cell types. The nomenclature of such events varies from system to system, often being labelled as afterdepolarizations (ADPs) or depolarizing afterpotentials (DAPs). The most characterised of these DAP responses is in long myelinated axons of both the peripheral and central nervous systems. This particular DAP recorded in non mammalian species (specifically frog and lizard peripheral myelinated axons) displays a variable time course (generally up to 100 ms) and appears to be insensitive to manipulation of extracellular calcium and potassium concentrations, while at the same time being sensitive to external sodium concentration and the presence of tetrodotoxin (Barrett & Barrett, 1982). DAP amplitudes tended to be of lower amplitude in the lizard (David *et al.*, 1995) and frog (Bowe *et al.*, 1987) axons when compared to that measured in rat axons. In these studies, and a further study conducted solely in rat myelinated spinal cord axons (Blight & Someya, 1985) it was found that depolarization of the recorded neurones reduced the amplitude of the DAP. This body of evidence indicates that the mechanism of induction is very different to the cortical sADP, more than likely depending on the capacitance properties of the recorded axons. A study of DAPs in non-myelinated axons (of the vagus nerve), however, indicated that in such neurones the DAP response is sensitive to extracellular potassium concentration, and very strongly dependent on entry of calcium into the cells (Jirounek *et al.*, 1991), suggesting a more similar mechanism of induction to the cortical sADP.
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Of more interest to the current study are DAP responses recorded in mammalian central nervous system neurones. DAPs have been reported in pyramidal cells of the hippocampal regions CA1 (Costa et al., 1991) and CA3 (Gähwiler, 1984), and in other neurone types, including guinea-pig trigeminal motoneurones (Chandler et al., 1994; Kobayashi et al., 1997), immature rat dentate granule neurones (Zhang et al., 1993) and rat supraoptic nucleus neurones (Smith & Armstrong, 1993; Li & Hatton, 1997). It is interesting to note that cholinergic neurones of the nucleus of the diagonal band, an area known to have a substantial cholinergic input to the olfactory cortex (Mesulam & Van-Hoesen, 1976), also display a DAP response (Gorelova & Reiner, 1996), although of smaller amplitude and shorter duration. There is a possible functional significance to there being afterdepolarizing responses in both of these systems. All of the above described DAP responses are present in the absence of mAChR or mGluR agonists, although they are mostly dependent on entry of calcium into cells. This suggests that although the overall mechanism of induction of fast DAPs is different from that of the cortical sADP, there are important similarities which can be exploited during the study of this phenomenon. DAP responses have been associated with the release of calcium from internal stores by a calcium-induced calcium release (CICR) mechanism (see later) in supraoptic nucleus neurones (Li & Hatton, 1997), a potential avenue for continued research of the cortical sADP phenomenon.

It should be noted that a further sADP type was recently discovered in layer V pyramidal neurones of the sensorimotor cortex. Application of GABA to these neurones in a brain slice preparation resulted in the sAHP being replaced by a sADP response, which was insensitive to external calcium in the bathing solution, although it was sensitive to TTX. It was also found that sectioning of the apical dendrites of these cells reduced the
amplitude of the evoked sADP by up to 75 %, indicating that this was largely a dendritic phenomenon (Cerne & Spain, 1997).

A recent report also investigated the ionic mechanism of a muscarinic agonist-induced sADP phenomenon in rat medial prefrontal cortex which shows very similar characteristics to those seen in the olfactory cortex, the response comprising of a prolonged post-stimulus afterdepolarization, with a repetitive discharge superimposed, and an underlying slow inward tail current revealed under voltage clamp (Haj-Dahmane & Andrade, 1998). These authors reported a similar calcium-dependence of this phenomenon, although the $I_{ADP}$ in this system appeared to show a very much smaller amplitude and shorter duration. Furthermore, an alternative mechanism of induction was implied (based upon experiments showing a clear dependence of $I_{ADP}$ amplitude upon the extracellular sodium concentration, and the fact that the $I_{ADP}$ remained unaffected by intracellular perfusion of caesium, which blocks outward potassium conductances). The authors proposed that the sADP in the neocortex involved a calcium-activated inward cation conductance, with strong instantaneous rectifying properties, which could apparently explain the profound decrease in conductance observed at the peak of the depolarizing tail current (c.f. Constanti & Bagetta, 1991). It is quite possible that the mechanism of induction of the neocortical sADP is different to that of the sADP in the olfactory cortex, or that the former response results from a mixture of a decrease in potassium conductance and an increase in cation non-selective conductance (as suggested by Greene et al., 1994). Further experimentation is clearly needed to address this issue.
1.5.5 Pharmacology of afterdepolarizing responses.

In view of the varied mechanisms underlying induction of depolarizing afterpotentials in different systems, it is not surprising that the pharmacology of these phenomena is also less than straightforward. There is good evidence that the mAChR and mGluR-induced sADPs in olfactory cortical neurones are mediated by the \( M_1 \) muscarinic receptor subtype (inhibited by 50 nM pirenzepine) (Constanti & Bagetta, 1991) or group II or III metabotropic glutamate receptor subtypes (Libri et al., 1997). It was also reported that activation of \( 5\)-hydroxytryptamine_2 receptors or \( \alpha_1 \)-adrenoceptors in the association cortex could induce a post-stimulus sADP, possibly by converging on a similar set of intracellular mechanisms as the muscarinic system (Araneda & Andrade, 1991). There is currently, however, no information regarding a possible role of the serotonergic or adrenergic systems in sADP induction in the olfactory cortex.

As mentioned previously, the data of Constanti & Bagetta (1991) suggested that the cortical sADP was a result of the deactivation and subsequent slow reactivation of a background potassium conductance present in responding neurones. However, on exposure to traditional blockers of potassium channels such as barium or caesium ions, tubocurarine or glibenclamide (\( I_{K(ATP)} \) channel blockers), no inhibition of the sADP was found, although tetrethylammonium (TEA) or tetrabutylammonium (TBA) ions did block the sADP (Constanti et al., 1993); (a later report stated that TEA was not effective in blocking the mAChR-induced sADP in immature rat olfactory neurones; Sciancalepore & Constanti, 1995). These findings do not rule out the possibility that \( I_{\text{ADP}} \) is a potassium-mediated tail current, but they do imply that if \( I_{\text{ADP}} \) does involve such a potassium conductance, it has a different pharmacology from the to more common potassium conductances.
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The calcium dependence of the $I_{\text{ADP}}$ was confirmed in olfactory neurones by lowering extracellular calcium concentration, or replacement of extracellular calcium with Cd$^{2+}$. Further to this, experiments were carried out using the novel anticonvulsant agent felbamate, which was shown to reduce (but not abolish) the mAChR and mGluR-induced sADP (at 100 - 500 μM). The same concentration of felbamate also reduced the duration of calcium spikes revealed by intracellular loading with Cs$^+$ ions (Libri et al., 1996), suggesting an interference with voltage sensitive calcium entry (Galvan et al., 1985). Both of these factors together suggest that voltage-gated calcium channels are involved in the generation of the sADP. It is yet unclear which particular voltage-gated calcium channels are involved in $I_{\text{ADP}}$ induction in the olfactory cortex, although the use of dihydropyridine L-type calcium channel antagonists such as nifedipine or nimodipine have been shown to reduce afterdepolarizations in supraoptic nucleus neurones (Li & Hatton, 1997) and midbrain dopaminergic neurones (Mercuri et al., 1994). On the other hand, Ω-conotoxin (an N-type channel blocker) did not inhibit the ADP induced in either septal cholinergic neurones (Gorelova & Reiner, 1996) or trigeminal motoneurones (Kobayashi et al., 1997). It thus seems likely that of the high threshold voltage gated calcium channels, the L-type calcium channel is commonly involved in generation of depolarizing afterpotentials, whereas the N-type channel has little or no obvious involvement. There is currently no available information on the possible importance of the low threshold T- or P-type voltage gated calcium channels in generation of DAPs.

### 1.6. Calcium-induced calcium release - a possible role in sADP generation?

Entry of calcium into responding neurones during a prolonged depolarizing stimulus is an essential process contributing towards revealing the $I_{\text{ADP}}$ tail current. It is
possible that the slow time course of the $I_{\text{ADP}}$ tail is a result of slow de- and subsequent reactivation of the proposed novel potassium conductance resulting from the intrinsic properties of the channels involved in this process. It might also reflect a slow extrusion of calcium from the cell after a long depolarizing stimulus. A third possibility, however, is that the process of calcium-induced calcium release (CICR), or release of calcium from intracellular stores is somehow involved in the slow nature of the current tail. If calcium release from intracellular stores is involved in sADP generation, then the mechanism of this phenomenon becomes even more complicated.

1.6.1. Intracellular calcium release.

Release of calcium from intracellular stores has been observed in a variety of different cell types, being most well characterised in both cardiac and non-cardiac muscle cells, where CICR from the sarcoplasmic reticulum has been implicated in a variety of cellular processes (e.g. excitation-contraction coupling in skeletal muscle cells; for review see Endo, 1977). Intracellular calcium stores have also been described in various neurone types, particularly ganglionic neurones (Nohmi et al., 1992) and central neurones from different areas, including the hippocampus and the cortex (Henzi & MacDermott, 1992; Kuba, 1994). Intracellular calcium release has been implicated in important neuronal functions such as neurotransmitter release (Smith & Cunnane, 1996) and generation of hyperpolarizing or depolarizing afterpotentials (Torres et al., 1996; Li & Hatton, 1997).

Two types of intracellular calcium store releasing mechanism have been described in neurones, one of which is driven by the ‘ryanodine receptor’ (named because of its sensitivity to the alkaloid ryanodine; also termed ‘calcium release channel’), which displays similar single channel properties to the ryanodine receptor in cardiac muscle (the
The other calcium stores are the ‘IP$_3$-sensitive stores’ which are non-mitochondrial and are sensitive to intracellular concentrations of inositol trisphosphate (IP$_3$; Ferris et al., 1989). These stores appear to be functionally distinct from the ryanodine sensitive stores, located primarily in the dendritic endoplasmic reticulum networks (Takei et al., 1992). Releasable ryanodine-sensitive calcium stores in neurones are generally found in the endoplasmic reticulum networks distributed throughout somatic regions (Seymour-Laurent & Barish, 1995) where the endoplasmic reticulum membrane is in close proximity to the plasma membrane (areas known as subsurface cisternae). The IP$_3$ receptors responsible for calcium release also show some somatic distribution, although they are more prominent in the dendritic networks. There is, however, some evidence that ryanodine receptors can also be found in dendritic portions of the endoplasmic reticulum (Berridge, 1998). Furthermore, there appears to be a large storage capacity for calcium in the mitochondria, although this store does not appear to contribute greatly towards CICR (Carafoli, 1987); in fact, it appears to act as an intracellular calcium buffer, rapidly taking up excess calcium, and then slowly releasing it when cytosolic calcium concentration falls (Taylor & Broad, 1998).

Neuronal CICR was first discovered when the methylxanthine caffeine was applied to bullfrog sympathetic ganglion cells in an attempt to study its effects on transmitter release. Caffeine was found to induce a series of rhythmic membrane hyperpolarizations, which lasted tens of seconds, occurring at frequent intervals (Kuba et al., 1972). In further experiments where cells were loaded with the calcium-sensitive dye fura-2, the changes in fluorescence, in response to caffeine, corresponded to the changes in membrane potential seen under similar conditions (Nohmi et al., 1992). The mechanism underlying the spontaneous membrane hyperpolarizations was attributed to a CICR
process, similar to that seen in skeletal muscle (Endo et al., 1970), whereby calcium released from intracellular stores was subsequently activating one or more calcium-dependent potassium conductances, similar to those described in other systems (Constanti & Sim, 1987). The long time course of the hyperpolarizations was thought to reflect changes in the concentration of calcium within the cells, rather than any gating characteristics of the potassium channels themselves (Magleby & Pallotta, 1983; Lang & Ritchie, 1987). Decay of the hyperpolarizations appeared to correspond to re-uptake of calcium into intracellular calcium-storing organelles, as shown by a corresponding decrease in the intracellular free fura-2 fluorescence (Kuba, 1980). Experiments in which the extracellular calcium concentration was lowered or raised showed an increase or reduction of the hyperpolarization intervals respectively, confirming that entry of calcium into the cells was necessary (Kuba & Nishi, 1976; Kuba, 1980), along with calcium binding to the ryanodine receptor to allow release of calcium from the stores (Kuba, 1994).

The actual mechanism proposed for CICR in neurones was used to construct two separate models. Firstly the single compartment model assumed that the calcium-storing organelle consisted of one compartment in which both calcium release and calcium uptake took place. Entry of calcium into the cell resulted in an initial small rise in intracellular calcium concentration, followed by binding to the calcium release channels on the storage organelle. When the concentration of intracellular calcium reached a certain predetermined level, the release of calcium from the internal stores took place, activating the calcium-dependent potassium conductances; subsequent re-uptake of calcium into the storage organelles then reduced calcium concentration, resulting in a slow deactivation of the potassium channels (Fig. 1.6 A; Kuba, 1994). The second model assumed that there
were two compartments involved in the whole process, one for calcium release and the other for calcium uptake, with a calcium transport mechanism operating between them, similar to that seen in the sarcoplasmic reticulum (Fig. 1.6 B; Winegrade, 1968). The only real functional difference between the two models was that the two compartment model tended to have a shorter CICR phase, leading to a faster decay time of spontaneous membrane hyperpolarizations. The nature of the observed neuronal oscillations in caffeine indicated that the two compartment model was more likely, because the modelled oscillations followed a similar time course (Kuba, 1994). Furthermore the oscillations appeared to result from a decrease in the threshold of activation of the calcium release channels, whereby basal calcium levels in the cell became capable of activating the release process (Nohmi et al., 1992). There was also a dependence on the concentration of calcium within the stores themselves; thus, the CICR process relied on binding of calcium to the calcium release channels on both the inside and the outside, the binding site for calcium most likely being located in the aqueous pore of the ryanodine receptor (Ma et al., 1988).

![Diagram of calcium-induced calcium release (CICR) phenomenon](image)

Fig. 1.6 Schematic representation of the calcium-induced calcium release (CICR) phenomenon. A indicates the single compartment model. Here calcium uptake and release take place from the same compartment,
release occurring when a rise in intracellular calcium activates the calcium release channel. B represents the
two compartment model where calcium uptake and release take place in different compartments, joined by a
calcium transport mechanism. The two compartment model has a shorter time-scale of release and uptake
than the single compartment model (Kuba, 1994).

Re-uptake of calcium into the intracellular stores is mediated by a Ca$^{2+}$-ATPase
(Tsien & Tsien, 1990), similar to that found in sarcoplasmic reticulum (Sah et al., 1994; Taylor & Broad, 1998). This process certainly recharges the intracellular stores ready for
the next signal for calcium release, but the process may also regulate the deactivation of
the CICR response and contribute towards calcium buffering within the cell (Henzi &
MacDermott, 1992). The released calcium is sequestrated back into the intracellular
stores, but additional calcium for uptake into stores can be provided by calcium entry

The IP$_3$-sensitive stores release calcium in response to the binding of inositol
1,4,5-trisphosphate (IP$_3$) to receptors in the membranes of the endoplasmic reticulum
storage vesicles (Ferris et al., 1989). These stores do not appear to release calcium in
response to a rise in intracellular calcium alone, although they do require the presence of
calcium as a cofactor for calcium release (Finch et al., 1991). IP$_3$ can be generated in
neurones by activation of G-protein linked receptors, including muscarinic acetylcholine
receptors (Shimizu et al., 1993) and metabotropic glutamate receptors (Abdul-Ghani et al.,
1996). Following ligand binding, these receptors activate G-proteins which in turn results
in the hydrolysis of the membrane-bound phospholipid phosphatidylinositol 4,5-
bisphosphate (PIP$_2$) by a phosphodiesterase, leading to the formation of diacylglycerol
(DAG) and IP$_3$ (Berridge & Irvine, 1984; Berridge, 1987). The DAG then activates
protein kinase C in and around the plasma membrane, while the IP$_3$ diffuses into the cytoplasm and subsequently activates IP$_3$ receptors in the endoplasmic reticulum, leading to release of intracellular calcium (Berridge & Irvine, 1989). It is possible that the IP$_3$-sensitive stores may contribute towards the maintenance of calcium release from the calcium-sensitive stores, although this is only likely to take place when these stores are in close proximity to the IP$_3$-sensitive sites (Henzi & MacDermott, 1992) (Fig. 1.7).

Fig. 1.7 Activation of calcium release channels and IP$_3$ receptors in neurones leads to release of intracellular calcium. G-protein linked receptors in the plasma membrane activate G-proteins which triggers a cascade in which PLC hydrolyses membrane bound PIP$_2$ to generate IP$_3$, which then diffuses to the IP$_3$ receptors on the IP$_3$-sensitive calcium stores. In addition, calcium enters the cell through voltage- or ligand-gated calcium channels, activating the calcium release channels, or acting as a cofactor at the IP$_3$ receptor. There may be an interaction between the IP$_3$-sensitive stores and the calcium-sensitive stores, providing that the two are in close proximity (Henzi & MacDermott, 1992).
1.6.2. Intracellular calcium release and generation of afterpotentials

As previously mentioned, calcium entry is strongly implicated in the generation of depolarizing and hyperpolarizing afterpotentials in a variety of neurone types. It is possible therefore that CICR may act to amplify the calcium signal inside the cell after calcium entry through voltage-gated calcium channels, then acting on potassium channels to change their conducting characteristics in order to reveal the afterpotentials. Blockade of CICR has been shown to suppress calcium-dependent slow AHPs in vagal neurones (Sah & McLachlan, 1991), otic ganglion neurones (Yoshizaki et al., 1995) and hippocampal neurones (Torres et al., 1996). A comprehensive study has also been carried out on the possible involvement of CICR in the generation of DAPs in supraoptic neurones (Li & Hatton, 1997). In this system, blockade of CICR using ryanodine or dantrolene reduced both the amplitude and time course of the DAPs, as did thapsigargin or cyclopiazonic acid, which are inhibitors of calcium re-uptake into the intracellular stores; caffeine was found to increase the amplitude and time course of these responses. This indicated that CICR was involved in the mechanism of DAP generation in these neurones. In contrast, blockade of IP$_3$-sensitive release by intracellular application of heparin increased, rather than decreased the amplitude of the DAPs, suggesting that IP$_3$-sensitive release was not necessary.

On the basis of this information, it seems likely that CICR could be involved in generation of the sADP seen in responding olfactory cortical neurones, with intracellular calcium release acting to amplify of the rise in intracellular calcium induced by a prolonged train of action potentials. This would account for the slow nature of the tail current, reflecting the slow nature of reactivation of the proposed sADP potassium conductance, as calcium was slowly taken back into the calcium stores from the
cytoplasm. The proposed mechanism of sADP and $I_{ADP}$ induction would thus become extended, as summarised in Fig. 1.8.

When considering CICR mechanisms, it is very important to consider a possible role of the IP$_3$ receptor-mediated release of calcium from internal calcium stores. The most effective method of activating release from the IP$_3$-sensitive stores is to provide both IP$_3$ and Ca$^{2+}$ at the receptors, this dual action having some interesting implications for neuronal function. Firstly, it is surmised that a small elevations of IP$_3$ within

![Diagram](image)

Fig. 1.8. Proposed mechanism of sADP and $I_{ADP}$ induction incorporating the putative involvement of a calcium-induced calcium release (CICR) mechanism. The potassium channels proposed to underlie $I_{ADP}$ are initially sensitized (possibly phosphorylated) by the addition of an mAChR or mGluR agonist. A prolonged depolarizing stimulus allows calcium to enter the cell, which then activates the calcium release channels on the calcium-sensitive calcium stores, and calcium is thus released from these stores, amplifying the calcium concentration increase produced by the depolarization. This increased concentration of intracellular calcium
then causes the deactivation of the $I_{ADP}$ potassium current, leading ultimately to the afterdepolarization. Subsequent slow reactivation of the $I_{ADP}$ conductance takes place as calcium is taken back into the stores, and extruded from the cell (modified from proposed scheme of Constanti et al., 1993).

the cells, which itself is incapable of stimulating release, can enhance the sensitivity of the receptor to calcium (Berridge, 1998) (rather like the facilitatory effect of caffeine acting on the ryanodine receptors; Kuba, 1994), while intracellular calcium loading of cells in the absence of changes in IP$_3$ production is unable to produce any calcium release (this activity only apparently appearing upon subsequent increase of intracellular IP$_3$ levels by receptor activation (e.g. mGluR; Lorenzon et al., 1995).

With this in mind, there is a possible role for IP$_3$ receptor-mediated stores in the generation of the sADP in the olfactory cortex. Muscarinic or metabotropic glutamate receptor activation, as well as activating or ‘priming’ the proposed $I_{ADP}$ potassium conductance to intracellular calcium (see earlier), may also result in stimulation of IP$_3$ production (Shimizu et al., 1993; Abdul-Ghani et al., 1996), which would then sensitize the IP$_3$-sensitive stores to intracellular calcium. The sudden rise in calcium resulting from a large depolarizing stimulus could then act as a trigger for calcium release from these stores, resulting in a similar amplification in intracellular calcium concentration as seen in the case of CICR acting via the ryanodine receptors (Fig. 1.9). It is therefore conceivable that the IP$_3$-sensitive stores can function alongside the ryanodine-sensitive stores in amplifying and prolonging the calcium signal within the cell.

It is also interesting to note that a build-up of calcium in the lumen of the endoplasmic reticulum can result in a positive feedback action, of unknown mechanism, by increasing the calcium sensitivity of both ryanodine and IP$_3$ receptors (Berridge, 1998).
Chapter 1: General Introduction

Fig. 1.9. Possible involvement of IP$_3$-sensitive calcium stores in the generation of the sADP and $I_{ADP}$. Firstly, activation of the mAChR or mGluR results in priming of the sADP potassium channel, and also the production of IP$_3$, which sensitizes the calcium stores to the presence of intracellular calcium. Entry of calcium into the cell during the action potentials produced by a prolonged depolarizing stimulus results in release of calcium from the IP$_3$-sensitive stores, amplifying the intracellular calcium signal, resulting in the inhibition of the $I_{ADP}$ channel (c.f. the proposed mechanism of CICR-induced inhibition of the $I_{ADP}$ in Fig. 1.7) (modified from Constanti et al., 1993).

1.7 Developmental aspects of afterpotential generation.

The brain as a whole undergoes very profound developmental changes both in the embryonic stage and in early postnatal life. Electrophysiological experiments performed on postnatal animals indicate that there are changes in neuronal excitability in a number of areas, such as the hippocampus (Barnes, 1994; Papatheodoropoulos & Kostopoulos, 1996) and cerebellum (Muller & Yool, 1998), reflecting possible alterations in synaptic activity.
(Papatheodoropoulos & Kostopoulos, 1998), expression of ion channels (Muller & Yool, 1998) and function of intracellular machinery such as phosphoinositide metabolism (Tandon et al., 1991). These changes in neuronal excitability may have functional implications in the processes of learning and formation of memories, and also in pathological conditions such as epilepsy.

1.7.1 Postnatal development of the olfactory cortex

Studies of the postnatal development of the olfactory cortex remain few and far between. The time period of interest for the current work was P14-P22 day old animals (consistent recordings from younger rat brains proved too difficult to obtain). A comprehensive study of the formation and migration of different olfactory cell types has been carried out in rats, providing an insight into the developmental process. According to this study, by the 8th day of postnatal life, the laminar arrangement of the olfactory cortex is clearly defined, and the pyramidal cell arrangements of layer II and layer III have attained more or less adult configurations (Valverde & Santacana, 1994) (as have the projections to the olfactory cortex from the olfactory bulbs) despite further growth of the cortical areas and a further synaptogenesis after this point (Schwob & Price, 1978). The developmental profile of the non-pyramidal cells in the deep layer III, however, is unclear (Valverde & Santacana, 1994), which, along with the further development of synaptic connections within the olfactory cortical regions, may have implications for neuronal excitability.
1.7.2. Development of different receptor types and functions during postnatal development

During early postnatal life, there are large changes in the distributions and thus functionality of a number of different receptor types, which has very important implications for neuronal excitability and synaptic plasticity throughout postnatal development. In several regions of the brain, it has been observed that muscarinic receptors develop in number and connectivity to secondary effector systems over the first few weeks of life. In particular, immunocytochemical analyses have shown that numbers of muscarinic receptors increase over the first two weeks in the parietal cortex (Buwalda et al., 1995), but in contrast, the frontal cortex appears to display a level of acetylcholine receptor density which commences at a relatively high level, and then gradually declines towards adulthood (Araki et al., 1996). This same study indicated that levels of cholinergic receptors in the hippocampus were fully developed at a very early age, a finding which was echoed by Reece & Schwartzkroin (1991), who discovered that electrophysiological responses to acetylcholine and carbachol (i.e. membrane depolarization and input conductance decrease measured in neurones from hippocampal brain slices) of rats of as little as two days of age were almost identical to those recorded in neurones of adult animals. This is not surprising considering the proposed involvement of the cholinergic system in learning and formation of memories, processes which are traditionally connected with the cortex and hippocampus.

Neuronal excitability in general is heavily influenced by the GABAergic system, which traditionally has an inhibitory action on cells. There are quite profound changes in GABA receptor density and function during the initial stages of development, in marked contrast to the cholinergic system. Again according to Araki et al. (1996), receptor
autoradiography indicated a wide distribution of GABA_A receptors in various areas of the cortex and hippocampus around the third week of postnatal life, steadily declining towards adulthood. In the hippocampus, however, a paradoxical excitatory action of GABA_A occurs in localised neurones during the first 8 postnatal days, resulting in giant GABA-ergic depolarizing synaptic potentials, which (as well as spontaneous activity in these neurones) can be completely blocked by application of bicuculline (for review see Cherubini et al., 1991). This is in contrast to the adult situation where bicuculline actually enhances the EPSP and causes spontaneous inter-ictal activity (Forti et al., 1997).

In the hippocampus, there is (in contrast to the development of the GABA_A receptor system) a definite postnatal alteration in the GABA_B receptor system. The first indication of this was the discovery that in immature rats, the depolarizing EPSP was rather larger than that seen in adult neurones, and the late slow IPSP was completely absent in the young animal slices. This was accompanied by a depolarizing response to the compound 4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol (THIP) in immature neurones, which in adult cells, traditionally resulted in a membrane hyperpolarization (in retrospect, almost certainly by acting on GABA_B receptors, although this was not inferred in the report; Mueller et al., 1984; c.f. the action of the GABA_B agonist baclofen; Newberry & Nicoll, 1984). Later experiments showed that paired pulse depression (a measure of presynaptic autoreceptor activity) is absent in neonatal animals, indicating that the presynaptic autoreceptor activity of GABA_B was missing (Gaiarsa et al., 1995 (possibly due to an early postnatal lack of GABAergic synaptic density; Rozenberg et al., 1989). There is also a developmental change in GABA_B receptor-mediated inhibition in cortical neurones, specifically studied in the somatosensory cortex of the rat. It was found that around postnatal day 7, there was no difference in paired pulse depression compared
with adult animals, indicating that presynaptic GABA<sub>B</sub> receptor activity was in place at this age. However, it was not until after postnatal day 22 that GABA<sub>B</sub>-mediated slow IPSPs were observed (Fukuda et al., 1993). It also appears that the GABA<sub>B</sub>-mediated slow IPSP was reduced in recordings taken from immature (between P14-P22) olfactory cortical cells (see later; Postlethwaite et al., 1998).

Other receptor systems also undergo developmental changes, but are unlikely to contribute as greatly towards synaptic plasticity and learning behaviour in the young animals. The synaptic NMDA receptors in rat hippocampus undergo modification, probably resulting from a change in NMDA receptor subunit composition, over the first 5 weeks of life, demonstrated by EPSPs being larger in amplitude over this period (Kirson & Yaari, 1996). As in the hippocampus, there may also be developmental changes in cortical NMDA receptor function. With this in mind, it appears that changes are not as profound as with the GABAergic system, since neocortical brain slice neurones exposed to a reduced extracellular magnesium concentration display a similar pattern of oscillatory activity to that seen in adult neurones, with only minor developmental modifications (Flint et al., 1997). The electrophysiological responses to trans-ACPD in hippocampal neurones also apparently do not change during the first weeks after birth, indicating that the metabotropic glutamate receptors responsible for excitation in postsynaptic cells do not alter in their function over this period; however, there is a change in metabotropic glutamate receptor stimulated PI turnover over this same period (Boss et al., 1992). Responses to serotonin also develop over the first three weeks of life in rat hippocampal neurones, adult-type responses being achieved during the third week (Segal, 1990). The adrenergic system, certainly in the hippocampus, appears to be functional very soon after birth, electrophysiological effects of agonists seemingly attaining adult type characteristics
by the 7th postnatal day (Moudy & Schwartzkroin, 1992). There is apparently a large increase in dopamine receptor density and function in the rat frontal cortex between postnatal weeks 3 and 4, then slowly increasing to adult levels after this time period (Noisin & Thomas, 1988). It is not yet known how changes in all these latter receptor systems in the olfactory cortex may contribute towards hyperexcitability or epileptic conditions.

1.7.3. Postnatal development of other electrophysiological characteristics

As well as receptor responses, intrinsic electrophysiological properties undergo developmental regulation in most neuronal systems. There was a profound change in the spiking properties and input resistance of neurones during the initial stages of postnatal life, recorded in hippocampal cells. In rabbit CA1 neurones, input resistance was larger and spike durations longer than in adult neurones, reaching adult characteristics by the second or third week, although calcium and sodium spikes were recorded as early as the first postnatal day. The changes in spiking behaviour were accompanied by an enhanced spike accommodation during the early postnatal days (Shwartzkroin, 1982). This activity is in agreement with recordings from intracerebellar nuclear neurones, where action potentials were prolonged (attributed to sodium and/or potassium channel properties), becoming similar to adult recordings by the end of the first postnatal week (activity of calcium conductances was apparently unchanged throughout this development; Gardette et al., 1985). These reports both also confirmed that a slow IPSP was absent from the evoked compound synaptic potential. The developmental changes in firing patterns could potentially be attributed to progressive development of potassium channel activity (particularly of calcium-activated potassium channels) as found in cerebellar purkinje
neurones (Muller & Yool, 1998), which may also involve regulation by the developmental changes in high voltage activated calcium channel activity (as seen in the hippocampus; Kortekaas & Wadman, 1997). There are relatively few studies of changes in the basic electrophysiological characteristics of cortical cells. It is reported that pyramidal neurones in layer V of the neocortex underwent similar changes to those in the hippocampus, i.e. the action potentials became shorter in duration, and also became larger in amplitude from P1 to P30 (accompanied by a decrease in input resistance), and (in contrast to hippocampal neurones) showed a gradual increase in the amplitudes of calcium-dependent action potentials (N.B. it should be noted that these calcium-dependent action potentials were recorded from P1; McCormick & Prince, 1987). Changes in firing patterns and general neuronal excitability in early life may contribute towards the properties of other electrophysiological phenomena such as the generation of afterpotentials.

1.7.4. Developmental aspects of afterpotential generation.

Afterpotentials often involve calcium-dependent changes in conductance, for example the post-stimulus AHP results from activation of calcium-activated potassium channels (e.g. Lancaster & Adams, 1986; Sah & McLachlan, 1991), and the sADP in the olfactory cortex is thought to be a result of calcium-dependent deactivation of a background potassium current (Bagetta & Constanti, 1991; Constanti et al., 1993). There is thus a possibility that the mechanisms underlying afterpotential generation may undergo developmental regulation. In the hippocampus, the fast post-spike afterdepolarizing potential developed over the first two weeks of life, replacing a post-spike afterhyperpolarization seen in the very young animal neurones (Schwartzkroin, 1982). The afterpotentials were later seen to follow a very specific developmental profile,
achieving peak amplitudes around day 15, and then declining again towards adulthood (Costa et al., 1991), a factor which would be expected to affect firing properties at the different ages. The slow post-stimulus AHP recorded in layer V pyramidal neurones of the sensorimotor cortex was seen to decline in duration over the period of immaturity in slices taken from rats of postnatal age 1-36 days (McCormick & Prince, 1987). In contrast, the muscarinic receptor-induced slow ADP (and its underlying current $I_{ADP}$) recorded in immature olfactory cortical neurones under patch clamp was significantly smaller in younger animals (10-12 day old) than older animals (15 days), indicating a possible developmental profile in this system (Sciancalepore & Constanti, 1995). Developmental characteristics of such afterpotentials may contribute towards neuronal excitability, and certainly have an effect on firing patterns of immature neurones, a factor which may have an impact on behaviours such as formation of memories in the intact animal. This was an area therefore considered worthy of further study in the olfactory cortical system.
1.8 Aims and objectives of present work.

The original aim of this thesis project was to investigate the development of the mAChR/mGluR agonist-induced post stimulus sADP using 'sharp' intracellular recordings taken from olfactory cortical neurones of rats of different ages, ranging from ~14-15 day old up to adulthood (3-4 months old). Further to this, a possible involvement of calcium-induced calcium release in the generation of this afterpotential was to be studied in olfactory neurones taken from adult guinea-pigs, where the sADP phenomenon was originally described. [It should be noted that rats were to be used for the developmental work due to the advanced stage of development attained by guinea-pigs at birth.]

In light of the surprising discovery that application of the muscarinic agonist OXO-M to slices prepared from the neonatal rats (P14-P22) resulted in induction of epileptiform bursting activity in the recorded neurones, the direction of the research changed in order to characterise as far as possible the novel oscillatory behaviour in these cells, and to compare their intrinsic properties with those recorded in adult rats. A parallel investigation was also carried out to examine the effects of various agents interfering with intracellular calcium movements in an attempt to establish whether intracellular calcium mechanisms were involved in the generation of the sADP.

The in vitro slice preparation of guinea-pig or rat olfactory cortex was used in these studies because:-

1. Stable intracellular recordings lasting several hours can be made from individual cells (Galvan et al., 1982; Bagetta & Constanti, 1991).
2. Drugs can be applied to the slices at known concentrations, with relatively fast application/washout times.

3. Cells of the olfactory cortex display a characteristic mAChR/mGluR agonist-induced slow post-stimulus afterdepolarizing potential, and underlying tail current $I_{ADP}$, which is robust, i.e. will not decay or undergo 'desensitization' over the time course of an experiment (Constanti et al., 1993).

4. Intrinsic association fibre networks, which are implicated in models of learning and memory in the intact animal, are still largely intact in the slice preparation, allowing the effects of various pharmacological manipulations upon evoked synaptic activity to be readily studied in a single cell (Hasselmo & Bower, 1992).
CHAPTER 2

MATERIALS AND METHODS
2.1. Brain Slice Preparation

Rostrocaudal brain slices, incorporating the olfactory cortex, were prepared from adult rats (>P40), adult guinea-pigs and immature (P14-P21) rats (either sex) according to the methods of Constanti et al. (1993).

Animals were anaesthetised with halothane in a desiccation chamber and then decapitated using a pair of sharpened secateurs. Immediately after decapitation, the skull was quickly removed from the lambda to the bregma to expose the whole brain, and the meninges were carefully dissected away. The olfactory bulbs were separated from the rest of the brain by means of a scalpel cut and the brain then removed from the skull with the help of a spatula (the optic nerves being cut during this procedure).

The whole brain was cut into two halves down the midline, and one half stored in oxygenated ice-cold (4°C) modified Kreb's solution. A rectangular block was cut from the other half, containing the lateral olfactory tract (LOT) and surrounding olfactory cortex, and stuck with cyanoacrylate glue to the teflon stage of a Campden Vibroslice/M tissue cutter. Transverse slices (~450 μm thick, sectioned perpendicularly to the pial surface, and along the axis of the LOT) were cut in bubbled ice-cold (4°C) Kreb's solution and then stored in oxygenated Kreb's at 32°C for at least 30 minutes to 1 hour recovery period before transferring to the recording chamber. The modified Kreb's solution had the following composition (in mM): NaCl 118; KCl 3; CaCl₂ 1.5; NaHCO₃ 25; MgCl₂.6H₂O 1 and D-glucose 11 (bubbled with 95% O₂: 5% CO₂, pH 7.4). Slices were then placed in a perspex recording chamber (Fig 2.1) and superfused with warmed (29 ± 1°C), oxygenated Kreb's solution at ~ 5 ml/min.
Fig 2.1. Schematic diagram of the brain slice recording chamber.

(1) - Brain slice; (2) - nylon meshes holding brain slice completely submerged; (3) - recording microelectrode; (4) - external Nichrome stimulating electrode; (5) - bath reference (ground) silver/silver chloride pellet electrode; (6) - temperature-sensing probe; (7) - heater; (8) - collecting chamber for Kreb’s solution.
2.2. Brain slice maintenance

The technique used for maintaining brain slices in this study was based on the methods described by Constanti & Sim (1987), Constanti et al. (1993) and Libri et al. (1994).

After pre-incubation, the brain slice was placed on the lower nylon mesh of the perspex recording chamber (Fig 2.1), and held in place with a further nylon mesh. The recording chamber was superfused continuously with Kreb's solution warmed to 29 ±1 °C by a heater, the temperature of the bathing solution being constantly monitored by a temperature-sensing probe. Prior to superfusion, the Kreb's solution was maintained at room temperature in an external reservoir, and constantly bubbled with a 95% O₂ / 5% CO₂ gas mixture. Fluid was circulated using a Watson-Marlow 503s peristaltic pump, maintaining a flow rate of ~ 5ml/min. From the reservoir, the oxygenated Kreb’s solution was carried through fine polythene tubing to a bubble trap, which also served to prevent the fluid level in the bath from changing as a result of the pumping action, and from there was carried, via the heater, to the recording chamber. Fluid was removed from the collecting chamber, again by the action of the peristaltic pump, and passed to waste or recirculated to the original reservoir. The fluid exchange rate in the recording chamber was less than 5 minutes. Intracellular electrophysiological recordings were attempted immediately upon placing a slice within the recording chamber.
2.3 Electrophysiological recording

Intracellular recordings were made using glass microelectrodes prepared from standard-walled, filamented glass capillaries (1.0mm outside diameter, 0.58mm inside diameter; GC100F-15, Clark Electromedical Instruments). Electrodes were produced using a horizontal Livingstone-type electrode puller, and then filled with either 4M potassium acetate (for adult neurones, initial tip resistance 40-60 MΩ) or 2M potassium acetate (for immature neurones, initial tip resistance 50-70 MΩ). Electrodes were coupled to a current-voltage preamplifier (Axoclamp 2A), which allowed recordings to be taken in ‘bridge’, discontinuous current clamp (DCC) or discontinuous single electrode voltage clamp (SEVC) modes.

During ‘bridge’ mode recordings, the operational amplifier arrangement was based on the Wheatstone bridge circuit, allowing microelectrode voltages to be continuously monitored as well as continuous currents to be injected into the cell. The bridge balance control of the Axoclamp was used to balance the voltage drop across the electrode, caused by the product of current flow and electrode resistance, resulting in a recording of membrane potential only. A balanced bridge recording was achieved by altering the bridge balance control until the fast voltage steps at the start and finish of an externally triggered current pulse were just eliminated. A residual voltage transient remained at the start and finish of the injected current step due to the finite response speed of the electrode.

Impalements were routinely made in ‘bridge’ mode by slowly advancing the electrode tip down through the slice until a cell was encountered (indicated by a positive deflection of the recorded potential). A brief oscillation was then applied to the electrode tip using either a short manual switch of the 'positive clear' function of the Axoclamp or a
rapid manual switch from current clamp to voltage clamp mode (with full feedback gain applied). This allowed the electrode tip to enter the soma of the cell, at which point negative holding current was applied to facilitate membrane sealing. Cells with a resting potential in the region -80 to -85 mV (after removal of the holding current) were considered acceptable for recording.

Electrotonic properties of the cells were recorded using current pulses of amplitude 0.5-1 nA in both the positive and negative directions. Negative electrotonic pulses allowed the input resistance of the cell to be calculated and also indicated if any fast or slow inward rectification was present. Positive electrotonic potentials resulted in firing of the cell, the spike amplitude obtained allowing confirmation of the membrane potential (spike amplitude corresponded to an overshoot of ~20 mV above 0 mV when compared with membrane potential).

As well as bridge mode, some recordings were taken using discontinuous single electrode current clamp. This minimised rectification of the electrode during depolarizing or hyperpolarizing current steps of high amplitude. A schematic diagram of the circuitry used for discontinuous current and voltage clamp is shown in Fig. 2.2. Sampling frequency and capacitance neutralization were routinely altered to give a smoothly charging electrotonic potential upon injection of a brief (160 ms) negative or positive current pulse (sampling frequency was typically between 2-3 kHz at 30% duty cycle).

Membrane currents $I_{AHP}$ and $I_{ADP}$ were recorded under discontinuous single electrode voltage clamp mode using the 'hybrid clamp' technique (Pennefather et al., 1985, Lancaster & Adams, 1986, Constanti et al., 1993), in which spike trains were first elicited in the cell by application of a large depolarizing stimulus of ~ +2 nA amplitude.
Fig 2.2. The discontinuous single electrode voltage/current clamp circuitry.

$V_m$ - membrane potential; $V_{pipette}$ - electrode potential; $V_{pipette \text{ sampled}}$ - potential sampled and held by the sample and hold circuit; $V_{cmd}$ - command voltage; $I_{cmd}$ - command current; $I_{m \text{ recorded}}$ - recorded membrane current.

This circuitry allows the tasks of both current injection and voltage recording to be undertaken by the same microelectrode, operating on a time-sharing basis. After penetration of the cell, $V_{pipette}$ is fed into a sample and hold circuit. This circuit feeds a voltage, sampled at a given point in the cycle, into a differential amplifier, which is then compared to a command voltage ($V_{cmd}$). When switch 1 is in the current passing position, the output of the amplifier becomes the input of a controlled current source (CCS), which in turn injects a current (directly proportional to the input voltage of the controlled current source) into the cell. When switch 1 is in the voltage recording position $V_{pipette}$ passively decays towards $V_m$, until it reaches a value within 1 mV of $V_m$ at which point a sample of the $V_{pipette}$ is recorded. After this point, switch 1 returns to the current passing position and the cycle begins again. Membrane current ($I_m$) recordings are taken from a sample and hold circuit connected to the controlled current source. The recorded current (like the input current to the cell) is proportional to the voltage input of the controlled current source. The switching circuit ensures that switch 1 opens and closes at the correct time during the duty cycle, and also that $V_m$ and $I_m$ are sampled at the appropriate times during the cycle. When switch 2 is in the current clamp position, the input to the CCS is a direct command current ($I_{cmd}$) (modified from Finkel & Redman, 1984).
Sample and Hold Circuit for Current

Controlled Current Source

Switching Control

Sample and Hold Circuit for Voltage

Microelectrode

Input Current

Switch 1

Voltage Recording

Current passing

Switch 2

Voltage Clamp

Current Clamp

$V_{\text{pipette}}$

$V_{\text{cmd}}$

$V_{m}$

$I_{m}$

$I_{\text{cmd}}$

$V_{m}$ recorded

$V_{\text{pipette}}$ sampled

$V_{\text{cmd}}$
and 1.6s duration in switched current clamp mode, followed by a manual switch to single
electrode voltage clamp mode, clamping the cell at -70mV (slightly more negative if the
cell persistently fired at this potential); this allowed the ensuing slow current activation (or
deactivation), as a result of the depolarizing stimulus, to be revealed. Clamp feedback
gain of the amplifier was maintained relatively low (~0.3 nA/mV) to prevent amplifier
oscillation and cell death. Sampling rates during single electrode voltage clamp were
between 2-3 kHz, and the duty cycle was 30%. Membrane voltage and current recordings
were filtered at 10 kHz and 10 Hz (low pass) respectively and recorded using a storage
oscilloscope and a Gould RS 3200 chart recorder (frequency response: d.c. to 50 Hz; rise
time ~5 ms for square wave input). In later experiments, membrane voltage and current
recordings were also fed to a Digidata 1200 analog-to-digital interface (Axon instruments,
Foster City, CA) coupled to a PC computer (4DX2-66 Viglen Ltd., UK) to acquire data
and perform off-line analyses with pCLAMP 6.0.1 software (Axon Instruments). Hard
copies of data were obtained using a Hewlett Packard Laserjet 5M printer (Hewlett
Packard Ltd, UK).

2.4 Recording of evoked synaptic events

Evoked synaptic transmission could also be recorded in this system
(typically under bridge mode) using an external bipolar nichrome stimulating electrode
(50 μm diameter, insulated except at the tip) placed within the cell layers II-III, close to
the recording electrode (the aim being to predominantly stimulate local intrinsic fibre
connections, Hasselmo & Bower, 1992). The stimuli were elicited using an external
Digitimer DS2 isolated stimulator (Digitimer LTD., Welwyn Garden, UK), delivering a
stimulus of 5-20 volts amplitude (depending on distance of recording microelectrode from
stimulating electrode) and 0.8 ms duration. Synaptic potentials (EPSP/IPSP complexes) were routinely recorded on an oscilloscope and chart recorder at higher gain. In later experiments, synaptic events were also fed to a Digidata 1200 analog-to-digital interface (Axon instruments, Foster City, CA) coupled to a PC computer (4DX2-66 Viglen Ltd., UK) to acquire data and perform off-line analyses with pCLAMP 6.0.1 software (Axon Instruments).

2.5. Analysis of data and statistical methods

Data were recorded and analysed using both computer and chart recorder traces. During experiments in which bursting was recorded in the immature neurones, the ictal activity consisted of large paroxysmal depolarizing shifts (PDSs) which were characterised by their large amplitude (~15 mV) and long duration (~10 s), and the appearance of repetitive spikes upon the peak of the depolarization. Measurements of PDS mean amplitudes, durations and frequencies (1/ interburst interval) were taken over a standard epoch of time of 3 minutes, the values obtained then being used to calculate the global averages over the total number of recorded cells (values expressed as mean ± SE). Durations of PDSs were measured from the time that the membrane potential depolarized above an arbitrary threshold for burst detection (in this case twice the peak level of membrane noise), until the membrane potential returned to the pre-burst value. Interburst intervals were measured as the times between onset of individual bursts, and the burst amplitudes were taken as the difference between baseline and the peak of the depolarization. The synchronicity of the spontaneous depolarizing events was determined by using the percentage of coefficient of variation (defined as the ratio between the standard deviation and mean value of the measured parameter, expressed as
a percentage; Bracci et al., 1997) calculated for burst duration (CVₜ) and interburst interval (CVᵢ); the values obtained were then used to calculate global averages (mean ±SE).

Measurements of the sADP and Iₐdp in the presence of OXO-M or 1S,3R-ACPD in either young or adult animals was taken either as the depolarizing deflection from the artificial baseline used for the study (-70 mV membrane potential, corrected before each measurement) to the peak of the depolarization in the case of the sADP, or from the artificial baseline (again -70 mV) immediately before application of the prolonged depolarizing stimulus to the peak of the inward tail current trace (in the case of Iₐdp recordings). Global averages of sADP and Iₐdp amplitudes were expressed as mean ± SE. Where applicable, the duration of Iₐdp was taken from the point of switching to voltage clamp mode until the point at which the tail current returned to its resting baseline value.

All recordings were taken before and during (and where appropriate, immediately after) applications of drugs during this study. Statistical significance was generally determined by use of a standard one-tailed Student's paired t-test, however where data were presented as percentage change versus control, statistical differences were assessed using a nonparametric Wilcoxon signed-rank test.
2.6. Drugs

The following drugs were used during this study:

**Oxotremorine-M iodide (OXO-M)** (Semat Ltd, UK), a non-selective muscarinic agonist.

**1S,3R-Aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD)** (Tocris Cookson Ltd, Bristol, UK), a non-selective metabotropic glutamate receptor agonist.

**DL-Amino-5-phosphonovaleric acid (DL-APV)** (Tocris Cookson Ltd, Bristol, UK), an NMDA glutamate receptor antagonist.

**6-Cyano-7-nitroquinoxaline (CNQX)** (Tocris Cookson Ltd, Bristol, UK), a non-NMDA glutamate receptor antagonist.

**Tetrodotoxin (TTX)** (Sigma Ltd, UK), a Na\(^+\) channel blocker.

**Atropine sulphate** (Sigma Ltd, UK), a non-selective muscarinic antagonist.

**(-)-Bicuculline methiodide** (Sigma Ltd, UK), a GABA\(_A\) receptor antagonist.

**Pentobarbitone Na** (Sigma Ltd, UK), an anaesthetic/sedative barbiturate and GABA\(_A\) receptor modulator.

**CGP 52432** ( ), a GABA\(_B\) receptor antagonist.

**(-) Baclofen** (Sigma Ltd, UK), a GABA\(_B\) receptor agonist.

**Caffeine** (Sigma Ltd, UK), an antagonist of the ryanodine receptor.

**3-Isobutyl-1-methylxanthine (IBMX)** (Sigma Ltd, UK), a phosphodiesterase inhibitor.

**Nifedipine** (Sigma Ltd, UK), an L-type Ca\(^{2+}\) channel blocker.

**Ryanodine** (Sigma Ltd, UK), a blocker of intracellular Ca\(^{2+}\) release.

**Dantrolene** (Sigma Ltd, UK), a blocker of intracellular Ca\(^{2+}\) release.

**Thapsigargin** (Sigma Ltd, UK), an intracellular Ca\(^{2+}\) store depletor.

**Cyclopiazonic acid** (Sigma Ltd, UK), an intracellular Ca\(^{2+}\) store depletor.
Chapter 2 : Materials and Methods

All drugs used were freshly dissolved (from stock solutions) in Kreb's solution and superfused at ~10 ml/min (30 second bath exchange time) onto the tissue. 1S,3R-ACPD and DL-APV were pre-dissolved in 1:1 equivalent of 100 mM sodium hydroxide (NaOH) solution whereas CNQX, IBMX, thapsigargin, dantrolene and nifedipine were pre-dissolved in dimethylsulphoxide (DMSO). Nifedipine and dantrolene were weighed and dissolved in semi-darkness, due to their light sensitive nature, and subsequently stored in containers wrapped in silver foil to exclude light. Precautions were also taken to minimise their exposure to light during applications of these agents to cells. They were frozen in 10 mM aliquots and subsequently diluted in Krebs' solution immediately prior to use. Final bath concentrations of DMSO (up to 0.5%) or NaOH (up to 0.1 %) had no deleterious effects on neuronal membrane properties or muscarinic/metabotropic responsiveness. Caffeine stock solutions (30 mM) were routinely prepared on the day of the experiment by dissolving directly into oxygenated Krebs solution. All other drugs were previously prepared as stock solutions in distilled water (pentobarbitone stock at 100 mM; OXO-M, bicuculline and ryanodine stock at 10 mM; TTX and atropine stock at 1 mM).

2.6. Intracellular staining of recorded cells

In some immature animal experiments, intracellular neurobiotin staining techniques were used to reveal the morphology of cells from which recordings were taken. Recording electrodes were filled with 2M potassium acetate with 2% neurobiotin tracer dissolved (Vector Laboratories, CA). Upon impalement, neurobiotin was injected into the cells either by passing depolarizing current pulses (1-2 nA, 300 ms, 3.3 Hz) for 10 minutes or recording normally from the cell for 1-2 hours.
After recording, injected slices were removed from the recording chamber and fixed in 4% paraformaldehyde (in 0.15 M phosphate buffered saline [PBS], pH 7.4) overnight at 4°C. Slices were then rinsed several times in PBS followed by incubation for 30 minutes in 0.3% hydrogen peroxide (diluted with 50% methanol and 50% water) to remove any endogenous peroxidase activity. After incubation, slices were rinsed several times with PBS and then treated with Triton X-100 (0.5% in PBS)(Sigma Ltd, UK) for 2 hours to solubilise the neuronal membrane. After several further washes with PBS, the slices were incubated overnight in a Vectastain ABC reagent (Vector Laboratories, UK) in PBS.

To visualise injected neurones, slices were first rinsed several times with PBS, and then reacted with diaminobenzidine (DAB) (0.05%) and hydrogen peroxide (0.003%) from a DAB peroxidase substrate kit (Vector Laboratories, UK) for 5-10 minutes, followed finally by rinsing with PBS to reduce background staining. The slices were then dehydrated by using sequential ethanol concentrations (50%, 70% & 98%, 5-10 minutes per step) and then mounted on a glass slide and coverslipped for permanent storage. The resulting slides were viewed under a Nikon microscope. Histological procedures were previously described in detail by Libri et al., 1994.
CHAPTER 3

SPONTANEOUS MUSCARINIC AGONIST-INDUCED OSCILLATORY ACTIVITY RECORDED IN IMMATURE RAT OLFACTORY CORTICAL NEURONES.
3.1 Introduction

It has previously been reported that mammalian olfactory cortex \textit{in vitro} is very prone to generation of epileptiform seizure-like activity in response to a variety of chemoconvulsant agents such as 4-aminopyridine (4-AP) (Galvan \textit{et al.}, 1982) or enhancement of N-methyl-D-aspartate (NMDA)-dependent excitatory postsynaptic potentials through reducing extracellular magnesium concentration (Libri \textit{et al.}, 1996; Flint \textit{et al.}, 1997). This brain area is also extremely susceptible to discharges induced by electrical stimulation of afferent fibre inputs (Pelletier & Carlen, 1996) and kindling-induced epileptogenesis \textit{in vivo} (for review see Löscher & Ebert, 1996). Electrical events, known as paroxysmal depolarizing shifts (PDSs) (closely resembling \textit{in vivo} epileptiform events induced by kindling), can be seen in cells of the olfactory cortex slice during the above-mentioned pharmacological and ionic manipulation (Galvan \textit{et al.}, 1982; Hoffman & Haberley, 1989; Libri \textit{et al.}, 1996). In addition, chronic baclofen exposure-induced down-regulation, or direct antagonism of GABA\(_B\) receptors (leading to disinhibition of the olfactory cortex), coupled with activation of muscarinic cholinergic receptors, has been shown to induce epileptiform activity recorded intracellularly from brain slices incorporating this region (Libri \textit{et al.}, 1996; 1998). It has also been suggested that muscarinic acetylcholine receptors (mAChRs) in the olfactory cortex may be involved in epileptogenesis \textit{in vivo}, since bilateral epileptic seizures occurred following unilateral microinjection of the muscarinic agonist carbachol into the piriform cortex, that were abolished by local application of the muscarinic antagonist atropine (Piredda & Gale, 1985).

It is known that substantial age-related changes in muscarinic cholinergic receptor density occur from immaturity to adulthood in various regions of the brain,
particularly the frontal cortex, where there is generally a rapid increase in muscarinic receptor density over the first few weeks of life, followed by a gradual decline towards old age (Araki et al., 1996, Tice et al., 1996). There is also a developmental change in muscarinic-stimulated phosphoinositide (PI) metabolism in cortical neurones (Tan & Costa, 1995) and PI turnover in hippocampal cells (Tandon et al., 1991). It is thus possible that such developmental alterations may have functional consequences on the overall degree of neuronal excitability, in view of the well documented susceptibility of the immature CNS to hyperexcitability and epileptogenesis; for refs see Johnston, 1996).

In the adult rodent olfactory cortical brain slice preparation, mAChR activation has been previously demonstrated to have postsynaptic excitatory effects such as a slow sustained membrane depolarization, an intense neuronal discharge and induction of a slow post-stimulus afterdepolarizing potential (sADP; Constanti et al.; 1993, Libri et al., 1994), as well as causing an inhibition of evoked synaptic transmission (Williams & Constanti, 1988). In the present study, the pre- and postsynaptic muscarinic responsiveness of olfactory cortical brain slices obtained from both immature and adult rats was investigated using a conventional intracellular recording technique. In addition, since metabotropic glutamate receptor (mGluR) activation has been shown to have muscarinic-like effects in several systems (including the olfactory cortex and hippocampus) such as postsynaptic excitation, sADP induction and inhibition of synaptic transmission (Baskys, 1992, Constanti & Libri, 1992, Constanti et al., 1993, Schoepp & Conn, 1993, Libri et al., 1997), the cellular responses of immature and adult rat olfactory neurones to the mGluR agonist 1S,3R ACPD were also investigated for comparison.
3.2 Results

3.2.1 Intrinsic Membrane Properties

This study was based upon intracellular recordings obtained from a total of 63 olfactory neurones of both adult (>P40, n=9) and immature (P14-P22, n=54) rats. Adult cells recorded during this study showed similar electrophysiological properties and firing behaviour to those previously reported in the olfactory cortex (Constanti et al., 1993; Libri et al., 1994) and in other cortical areas (Conners & Gutnick, 1990). A comparison of immature and adult neurones (Fig. 1A,B) showed no statistically significant difference in membrane potential (-81.8 ± 0.4 mV vs -82.1 ± 0.6 mV), input resistance (35 ± 1.8 MΩ vs 28 ± 1.5 MΩ) or evoked action potential amplitude (112 ± 0.8 mV vs 111 ± 1.3 mV), although in some immature neurones a slow I_h-like inward rectification was seen during a brief hyperpolarizing step (-2 nA, 160 ms, n=7), which was not seen in any recordings from adult neurones (c.f. Sciancalepore & Constanti, 1995; 1998) (Fig. 1C). A prolonged depolarizing stimulus (+2 nA, 1.6 s) was followed by a slow afterhyperpolarization (sAHP, routinely measured at -70 mV membrane potential) which was of similar amplitude in both immature (10.6 ± 0.7 mV) and adult cells (11.0 ± 0.6 mV).
Fig 3.1 Comparison of intrinsic properties of adult and immature olfactory neurones.

Comparison of membrane properties of immature and adult olfactory neurones. (A) Shows both positive and negative electrotonic potentials, elicited by current pulses of 160 ms length and 0.5 nA amplitude steps, recorded in an immature (P 19) neurone. Note the activation of a fast inward rectifier with increasing negative current pulses. (B) Shows a typical adult neurone, with very similar spiking properties to the immature cells, and presence of a fast inward rectifier. (C) Shows an immature (P 20) neurone in which a slow inward rectifier ($I_h$) can be seen on hyperpolarization. Note the slow rebound afterdepolarization upon return to resting potential, characteristic of this type of inward rectification. Scale bars apply to all traces.
Table 3.1 Comparison of postsynaptic muscarinic and metabotropic-glutamate response properties of immature and adult rat olfactory cortical neurones. Data shown here are mean ± SE. Depolarizations to OXO-M (10 μM, 2 min) or 1S,3R-ACPD (50 μM, 2 min) were routinely measured from -70 mV membrane potential. Changes in input resistance and sADP amplitude were measured after correction of the agonist-induced membrane depolarization (back to -70 mV). The sADP was induced in all cases by a standard stimulus (+2 nA, 1.6 s). Values in parenthesis refer to the numbers of neurones used; n.m. = not measurable due to bursting activity (see text).


3.2.2 Postsynaptic effects of mAChR/mGIuR activation

Bath-application of a standard dose of the muscarinic agonist oxotremorine-M (OXO-M; 10 μM; 2 min) or the selective mGIuR agonist 1-aminocyclopentane-1S-3R-dicarboxylic acid (1S-3R-ACPD; 50 μM; 2 min) resulted in persistent postsynaptic excitatory effects in both immature and adult olfactory neurones (c.f. Constanti et al., 1993, Libri et al., 1994, 1997). These consisted of a prolonged membrane depolarization, a small increase in input resistance, an intense neuronal discharge (~10 Hz) and appearance of a prominent post-stimulus slow afterdepolarization (sADP), which replaced the sAHP seen in control solution. There was, however, no apparent age-dependent differences in the depolarization amplitude or input resistance change in response to muscarinic or metabotropic-glutamate receptor activation, or appearance of the sADP in response to mGIuR activation (P>0.5; t-test; table 3.1).

3.2.3 Burst discharges induced by muscarinic but not metabotropic activation in immature neurones

During superfusion of the slices with control Kreb’s solution, no burst discharges were ever observed in immature or adult neurones, either spontaneously or in response to depolarizing stimuli. Superfusion of 10 μM OXO-M onto immature slices induced a membrane depolarization within the first 2-3 minutes (see earlier), which was routinely offset by application of maintained hyperpolarizing current. Within 4 minutes of drug application, ~43% of neurones tested (23 out of 54) progressively developed a pronounced pattern of spontaneous synchronous epileptiform behaviour, consisting of rhythmic, large amplitude (mean = 13.5 ± 0.7 mV) bursts of action potentials and oscillations (ictal events) lasting for about 10 s (mean = 10.1 ± 1.0 s) (Fig. 3.2 A).
These events comprised of trains of depolarizing synaptic potentials, with superimposed spikes at their peaks (Fig. 3.2 B,C), generally followed by a prolonged membrane afterhyperpolarization. Ictal bursts occurred at a frequency of between 0.5-7 bursts/min (mean interburst interval = 24.8 ± 3.0 s). Calculation of the coefficient of variation ([SD/mean] * 100 %) for both burst duration (CV_d) and interburst interval (CV_i) revealed that the bursts were of irregular occurrence and duration (CV_d = 30 ± 6 % and CV_i = 33 ± 5 %). The ictal discharges were generally followed by a period during which the cell was either quiescent (n=16), or less frequently (n=7) the cell displayed spontaneous interburst spike discharges, labelled as interictal activity (5-50 mV amplitude, ≤ 2 s duration).

There was no evident relationship between membrane potential and frequency or duration of the OXO-M induced bursting activity (measured between -65 to -105 mV, n=4, in current clamp mode or between -70 to -90 mV, n=3, in voltage clamp mode) (Fig. 3.3). This strongly indicated that the bursting activity did not depend on intrinsic membrane properties of the recorded neurones. Most neurones superfused with OXO-M either displayed an intense neuronal discharge, consisting of repetitive spiking, or an occasional ‘depolarization block’ at the peak of the depolarization. Under these conditions the bursting activity could not be reliably measured, so cells were routinely repolarized back to -70 mV. Bursts were still evident at more depolarized membrane potentials, but they were of reduced amplitude. Due to the unstable nature of the membrane potential during bursting activity, no reliable measurements of the sADP or I_{ADP} could be made while OXO-M was in the bathing solution.

The bursting activity remained relatively stable during continuous superfusion with OXO-M (up to 2 hours) and was readily reversible within 5-10 minutes of
washout. In contrast to this, no bursting activity was ever recorded in neurones of adult rat olfactory cortical slices in the presence of up to 20 μM OXO-M (n=9). In addition, metabotropic glutamate receptor activation produced similar depolarizing effects to OXO-M, but spontaneous epileptiform potentials were never seen during superfusion of either adult (n=4) or immature (n=6) neurones with the metabotropic glutamate receptor agonist 1S,3R-ACPD (10-50 μM), even in immature neurones in which OXO-M induced bursting activity had already been demonstrated; this suggests that mAChR sites were primarily involved in burst generation.

As previously reported by Libri et al. (1994), the cells of the olfactory cortex most responsive to muscarinic and metabotropic activation are those found in the deep cortical layers II-III. Of the immature neurones recorded, only cells electrophysiologically identified as being located in the deep layers II-III were capable of generating a prolonged excitatory response to mAChR activation (i.e. sustained membrane depolarization and repetitive firing); of these cells, 23 out of 27 developed the above described pattern of epileptiform activity. In the remaining 4 cells, rhythmic bursts induced by OXO-M appeared as either low amplitude (8.3 ± 0.9 mV), long duration (66.9 ± 6.1 s) ictal-like spike discharges (frequency ~0.5 bursts/min, n=3) or as a periodic increase in baseline noise (n=1). In contrast, cells electrophysiologically identified as superficial neurones (showing characteristic spike fractionation during large depolarizing current pulses; n=13; Constanti et al., 1993; Libri et al., 1994) showed very little or no sensitivity to the muscarinic agonist, and generated a much weaker pattern of spontaneous bursting activity, consisting of low amplitude (mean = 5.6 ± 0.6 mV), long duration (mean = 27.5 ± 6.5 s) and slow frequency (0.3-1 bursts/min), with isolated spike discharges during the inactive period between bursts.
Fig 3.2 Epileptiform bursting activity induced by OXO-M.

Muscarinic agonist-induced burst firing recorded in a single immature (P 21) rat olfactory cortical neurone maintained at -74 mV membrane potential (closer to firing threshold of approximately -65 mV) by injection of depolarizing current. (A) Continuous bath-application of 10 μM OXO-M to the cell resulted in an initial slow depolarization, followed by repetitive firing upon reaching threshold; (note spikes were truncated by the chart recorder). After readjusting the membrane potential back to the initial level by negative current injection (open arrow), spontaneous rhythmic bursts appeared, which were maintained for the duration of the recording. Initial downward deflections are electrotonic potentials evoked at 0.5 Hz frequency by negative current pulses (-0.5 nA, 160 ms). (*) indicates an expanded portion of a typical ictal burst seen in (B). (C) Shows a trace taken during (B) on a further expanded timescale, indicating more clearly the nature of the bursting phenomenon, consisting of a series of spontaneous synaptic potentials with superimposed spikes.
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Fig 3.3 Voltage dependence of frequency and duration of ictal bursts.

Plots illustrating the voltage dependency of the 10 μM OXO-M-induced bursting frequency (A) or burst duration (B) averaged over 4 experiments. Data points represent means ± SE of parameters measured over 3 minute epochs of time at each level of membrane potential.
3.2.4 Pharmacology of spontaneous bursting in OXO-M

The pattern of bursting activity induced by continuous application of 10 μM OXO-M generally stabilised very quickly, within 5-10 minutes of drug application. The threshold dose of OXO-M required to induce this behaviour was found to be 2.5 μM (n=3). Neurones were routinely recorded for 15-20 minutes before further drug application to ensure the pattern of bursting was consistent with previous data. Some immature neurones, however, failed to generate strong excitatory events and any sort of bursting activity as described above, and these were regularly discarded during the study of the pharmacology of this phenomenon (n=17).

Application of the voltage gated sodium channel blocker tetrodotoxin (TTX, 1 μM) resulted in complete and irreversible inhibition of the spontaneous bursts, as well as a suppression of evoked synaptic activity (n=3, Fig. 3.4 A). Raising the concentration of magnesium in the external bathing solution to 5 mM (5 times normal; n=3, Fig. 3.4 B) also suppressed the bursting activity, reversible within 10 minutes of returning to normal bathing solution with 10 μM OXO-M. Both of these results suggest that the bursting was synaptically mediated. Addition of the general muscarinic antagonist atropine (1 μM, n=3) or the muscarinic M₁-receptor specific antagonist pirenzepine (100-300 nM; n=3) (Williams & Constanti, 1988) also completely and irreversibly inhibited the bursting activity, confirming that muscarinic receptor activation (possibly of the M₁ subtype) was responsible for induction of the epileptiform activity (Fig. 3.5).

The N-methyl-D-aspartate (NMDA) glutamate receptor antagonist DL-APV (100 μM) was effective in abolishing the ictal bursts (n=3), however they were rapidly replaced (within 3 minutes) by a series of spontaneous interictal discharges whose
frequency progressively increased. The mean frequency of interictal activity was 54 ± 9 spikes/min (Fig. 3.6). Ictal activity returned after ~ 5 minutes of washout of the DL-APV (in the presence of OXO-M). Ictal activity was also blocked by application of the non-NMDA glutamate receptor antagonist CNQX (5 μM; n=2) at a concentration known to inhibit non-NMDA responses, without interfering with NMDA activity (Collingridge & Lester, 1989) (Fig. 3.6). As with the DL-APV, a pattern of interictal discharges was observed, which reverted back to ictal type activity after 30-40 minutes of washout of CNQX. 20 μM CNQX (a concentration affecting both NMDA and non-NMDA responses) abolished both ictal and interictal activity, returning after 30-40 minutes washout in the presence of OXO-M (n=3) (Fig. 3.6).
Fig 3.4 Bursting activity in immature neurones is blocked by addition of TTX or raising external magnesium in the bathing solution.

Bursting induced by 10 μM OXO-M in immature neurones is sustained in the continued presence of the muscarinic agonist. (A) Application of 1 μM TTX to a P19 neurone (-70 mV membrane potential) in which muscarinic bursting was evident resulted in a complete and irreversible inhibition of the bursting activity. (B) Increasing the concentration of magnesium in the bathing solution to 5 times normal (5 mM, P22 neurone recorded at -85 mV membrane potential) reversibly blocked the bursting, recovery occurring ~ 10 minutes after return to normal bathing medium containing 10 μM OXO-M. Both of these effects indicate a polysynaptic mode of generation of the oscillatory activity.
A

-70 mV

+ 1 μM TTX

B

-85 mV

5 mM Mg^{2+}
Fig 3.5  OXO-M bursting is blocked by muscarinic antagonists.

Bursting behaviour induced by a sustained application of 10 μM OXO-M is completely and irreversibly blocked by the non-selective muscarinic receptor antagonist atropine (1 μM; P21 neurone) (A), confirming that muscarinic receptors are involved in generation of this phenomenon. Superfusion of the specific M₁ muscarinic antagonist pirenzepine (300 nM; P20 neurone) (B) also inhibited the bursting, suggesting that the M₁ subtype of muscarinic receptor had a functional role in induction of the activity.
A

-80

+ 1 μM Atropine

B

-85

+ 300 nM Pirenzepine
Fig 3.6 NMDA and non-NMDA receptors are important in bursting.

(A) Muscarinic-induced epileptiform activity requires activation of NMDA receptors. Application of 100 μM DL-APV during 10 μM OXO-M induced epileptiform activity (P 22 neurone) abolished the ictal activity, replacing it with a series of spontaneous interictal discharges, reverting back to the previous ictal pattern upon washout of the DL-APV; (* indicates a period where the chart speed was doubled).

(B) Non-NMDA receptor activation is also important for burst generation. CNQX, when applied at a concentration which only blocked non-NMDA receptors (5 μM), resulted in a block of the ictal bursting, being replaced by interictal activity. This reverted back to ictal events after 30-40 minutes washout (P22 neurone).

(C) Both ictal and interictal activity was abolished by 20 μM CNQX (a concentration which blocks both NMDA and non-NMDA receptor activity), and returned within 30-40 minutes washout in the presence of OXO-M (P 21 neurone).

Calibration bars apply to all traces.
3.2.5 GABA_A and GABA_B agonists and antagonists modulate bursting activity.

Pharmacological manipulation of the GABA_A or GABA_B receptor systems strongly affected the bursting activity. Thus, application of the anaesthetic-sedative/ GABA_A receptor modulator pentobarbitone (100 μM; n=3) resulted in a significant reduction in amplitude compared with control values (3.0 ± 1.0 mV; P < 0.01, unpaired t-test), but not duration (13.7 ± 4.1 s) of the ictal bursts; interictal discharges were also reduced in the presence of pentobarbitone. Both ictal and interictal bursting returned within 5 minutes of drug washout (Fig. 3.7 A).

In 4 neurones in which bursting activity was not fully developed, addition of the GABA_A receptor antagonist bicuculline (10 μM) to the bathing solution enhanced the activity, eventually adopting a pattern very similar to the OXO-M induced bursting in other neurones, i.e. large depolarizing ictal events (Fig. 3.7 B, upper panel), and in some cells, interposed interictal activity (not shown). Subsequent exposure of the neurones to atropine (1 μM) resulted in an initial complete abolishment of all bursting, followed (after ~ 4-5 in atropine) by a return of activity, consisting of interspersed interictal-like spiking, consistent with bursting behaviour produced by bicuculline alone (Bracci et al., 1997) (Fig. 3.7 B, lower panel); this confirms that OXO-M can act synergistically with bicuculline to produce full-blown epileptiform activity.

Application of the GABA_B receptor agonist (-) baclofen (10 μM) to slices in which full bursting activity was observed consistently and completely inhibited the bursting (n=3), an effect which was reversible within 5-10 minutes of washout (in the presence of OXO-M) (Fig. 3.8 A).

GABA_B receptor downregulation, in conjunction with muscarinic activation, in the olfactory cortex has been implicated in epileptogenesis in adult rats (Libri et al.,
1996), however it was not known whether this effect of down-regulation could be mimicked by blockade of the GABA_B receptor. The GABA_B receptor antagonist CGP 52432 (1 μM) was therefore used in immature animal slices to mimic the effect of downregulation, and to assess a possible role in epileptogenesis in conjunction with OXO-M. Cells used in this study (n=3) were initially shown to exhibit bursting with 10 μM OXO-M; a threshold dose of 2.5 μM OXO-M was also shown to elicit a very much weaker bursting activity, usually consisting of periodic increases in baseline noise. Superfusion of the slices with 1 μM CGP 52432 alone resulted in an inhibition of the GABA_B mediated slow IPSP, but did not induce any bursting activity. However, after 15 minutes of treatment of slices with 1 μM CGP 52432, co-application of the low dose of OXO-M (2.5 μM) resulted in the appearance of a pattern of bursting activity almost identical to that seen upon prior treatment of the slices with 10 μM OXO-M (Fig 3.8 B) that was completely abolished by atropine (1 μM) or baclofen (10 μM). These results confirm that antagonism of GABA_B receptors, in conjunction with muscarinic receptor activation, is strongly epileptogenic in the immature olfactory cortex.

As previously mentioned, application of up to 50 μM 1S,3R ACPD failed to induce bursting activity in immature neurones. In accordance with this, co-application of 50 μM 1S,3R ACPD to slices pretreated with 1 μM CGP 52432 also failed to generate any spontaneous discharges, indicating that metabotropic glutamate receptor activation is not implicated in this behaviour.
Fig 3.7 Modulation of GABA\(_A\) receptor activity affects OXO-M induced epileptogenesis.

(A) The anaesthetic-sedative agent pentobarbitone (100 \(\mu M\)) reduced the amplitude of ictal bursting activity; (note discharges were not completely inhibited: see text) without significantly affecting their frequency (P 22 neurone). There was still evidence of interictal activity in the presence of pentobarbitone. The blocking action was reversed within 5-10 minutes of washout (in the presence of OXO-M). (B) (Upper panel): blockade of GABA\(_A\) receptors by 10 \(\mu M\) bicuculline in conjunction with muscarinic activation revealed a full blown pattern of epileptiform activity, not seen in the presence of 10 \(\mu M\) OXO-M alone (P 20 neurone). Bursts induced by the synergistic action of bicuculline and OXO-M were abolished by co-application of atropine, confirming that muscarinic activation is necessary for appearance of the full-blown ictal events (same neurone; lower panel). After \(~5\) minutes of atropine, a pattern of interictal type spike discharges was seen, which is consistent with application of bicuculline alone, and does not resemble the activity produced by bicuculline with OXO-M (or 10 \(\mu M\) OXO-M alone in responding cells).
A

-86

+ 100 μM Pentobarbitone

B

-72

+ 10 μM Bicuculline

+ Bicuculline

+ 1 μM Atropine
Fig 3.8  Activation or blockade of GABA_B receptors affects OXO-M induced epileptogenesis.

(A) OXO-M-induced bursting activity (10 μM; P 19 neurone) was reversibly blocked by co-application of the GABA_B receptor agonist (-) baclofen (10 μM). Recovery took place within 5-10 minutes of washout (in the presence of 10 μM OXO-M). (B) A lower concentration of OXO-M (2.5 μM; same neurone as above) failed to induce any bursting activity already seen in the presence of 10 μM OXO-M, however blockade of GABAB receptors by the compound CGP 52432 (B_2) in the same neurone, while having no intrinsic effect, was able to act synergistically with the lower dose of OXO-M, together inducing a ‘full blown’ bursting activity.
A

-85

10 μM Baclofen

5 min

B

\[ +2.5 \mu M \text{OXO-M} \]

B_1

-85

2.5 μM OXO-M

20 s

B_2

-87

1 μM CGP 52432

+ 2.5 μM OXO-M

20 s

50 mV
3.2.6 Evoked synaptic potentials in immature and adult neurones.

Synaptic potentials could be elicited in both adult and immature slices using an external bipolar stimulating electrode. In adult slices, focal stimulation (10-30 V; 0.2 ms) of local association fibre terminals (layer III of the olfactory cortex) during superfusion with control solution resulted in the appearance of a characteristic EPSP/IPSP complex consisting of an initial short latency EPSP (glutamate-mediated), with a threshold for triggering an action potential of around -65 mV, followed by an initial fast IPSP (mediated by GABA_A receptors) and a slow (GABA_B mediated) IPSP (Malcangio et al., 1995; Libri et al., 1996a) (see Table 3.2). In adult neurones, no repetitive spike discharges were ever recorded superimposed upon the EPSP, even following stimuli of supramaximal intensity (30 V) or depolarizing the membrane potential up to -60 mV (n=5). Neurones in immature animal slices displayed a fast EPSP of significantly smaller peak amplitude (mean = 11.9 ± 0.8 mV; P<0.01, t-test), but longer (although variable) duration (mean 180.7 ± 18.2 ms; P<0.01, t-test) compared to adult neurones. The EPSP was again followed by the fast and slow IPSP complex (Fig. 3.9). The GABA_B-mediated slow IPSP was significantly reduced in amplitude in the immature cells, compared with adult cells (3.9 ± 0.3 mV, n=30, compared to 5.4 ± 0.6 mV, n=9; P<0.05 t-test). Occasionally (10 out of 34 neurones) there were several spikes superimposed upon the fast EPSP as the membrane potential was depolarized, or the stimulus intensity was increased above 10-30 V.
### Table 3.2
Comparison of peak EPSP amplitude and duration of immature and adult olfactory neurones measured in control solution. Values are mean ± SE. Values in parenthesis refer to the number of neurones used.

<table>
<thead>
<tr>
<th></th>
<th>Immature (P14-P22)</th>
<th>Adult (≥P40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPSP amplitude in control solution</td>
<td>$11.9 \pm 0.8$ mV (n= 34)</td>
<td>$18.0 \pm 1.8$ mV (n=9)</td>
</tr>
<tr>
<td>EPSP duration in control solution</td>
<td>$180.7 \pm 18.2$ ms (n= 34)</td>
<td>$98.8 \pm 9.6$ ms (n=9)</td>
</tr>
</tbody>
</table>

### 3.2.7 Evoked postsynaptic potentials are affected by muscarinic/metabotropic activation

Modulation of evoked synaptic potentials by muscarinic or metabotropic receptor activation was studied in both adult and immature neurones. In adult slices, presynaptic muscarinic activation by 10 μM OXO-M led to a decrease in peak amplitude of the EPSP evoked at -84 mV membrane potential (% inhibition = $70.5 \pm 5.6\%$, P< 0.01; n=6). Activation of presynaptic metabotropic glutamate receptors with 10 μM 1S,3R ACPD also resulted in an inhibition of peak amplitude of the EPSP (42.3 ±11.5 %; n=5; Wilcoxon signed rank tests) (Fig. 3.10, upper panel). Both of these inhibitions were recorded after correction for membrane potential shifts induced by postsynaptic muscarinic or metabotropic activation, and were consistent with previous reports of presynaptic muscarinic/metabotropic action in this preparation (Constanti et al., 1988; Libri et al., 1997). Inhibition caused by either muscarinic or metabotropic...
activation was reversed after 20-30 minutes washout of either agonist, and no desensitisation of the OXO-M or 1S,3R ACPD effects on synaptic transmission could be seen, even after up to 30 minutes exposure to the agonist.

Neurones of immature animals displayed a pronounced difference in their response to muscarinic receptor activation. In contrast to the inhibitory action observed in adult neurones following application of 10 μM OXO-M, this concentration of agonist applied to immature neurones induced a dramatic prolongation of the evoked synaptic potentials (up to 10 s duration). This was accompanied by a recurrent spike discharge superimposed upon the lengthened PSP (Fig. 3.10, middle panel), consistent with the discharge observed in other models of epileptiform behaviour recorded in adult olfactory cortical neurones; e.g. reduced magnesium in the bathing solution or treatment of slices with 4-AP (Libri et al., 1996). These effects were readily reversible upon washout of OXO-M. In some neurones (n=3), hyperpolarization of the membrane (by negative current injection) allowed prolonged PSPs to be recorded without contamination by superimposed spikes, indicating that the lengthening of the PSP was at a presynaptic level, and not due to changes in postsynaptic membrane properties induced by repetitive firing (Fig. 3.10, lower panel). Unlike the response to OXO-M, synaptic potentials evoked in immature neurones, as in the adult slices, were consistently inhibited by the presence of 10 μM 1S,3R ACPD (50.7 ± 7.1 % inhibition; P< 0.01; Wilcoxon test; n=6). This inhibition was not significantly different to the inhibition observed in adult neurones upon exposure to 10 μM 1S,3R ACPD (P> 0.05, t-test). In addition, EPSP-superimposed spike discharges were never observed during metabotropic activation in immature neurones.
Fig 3.9 Comparison of evoked postsynaptic potentials in adult and immature neurones in control solution.

Postsynaptic potentials could be elicited in both adult and immature neurones by subthreshold orthodromic stimuli (5-30 V; 0.2 ms) delivered to local association fibres. Typical recordings of the EPSP/IPSP complex taken from an adult (A) and an immature (P 16, B) neurone. Traces were routinely taken at a membrane potential of -84 mV for comparison. The EPSP/IPSP complex consists of a fast glutamate mediated EPSP, followed by an initial fast GABA_A mediated IPSP and a slow GABA_B mediated IPSP. Note that the EPSP is reduced in amplitude, but increased in duration in the immature neurone.
Fig 3.10 Effects of muscarinic/metabotropic activation on evoked synaptic transmission

Recordings taken from an adult neurone (upper panel) indicate that either muscarinic (10 μM OXO-M) or metabotropic (10 μM 1S,3R-ACPD) receptor activation inhibits the peak amplitude of the EPSP. The middle panel shows the effects of applying 10 μM OXO-M to a neurone of an immature rat (P 19); the evoked PSP is clearly prolonged with superimposed repetitive spike discharges. In contrast, 10 μM 1S,3R ACPD strongly depressed the PSP, recorded in the same neurone. When the membrane potential was deliberately maintained at -90 mV by negative current injection (set from -84 mV to prevent orthodromic action potentials; bottom panel), a similar prolongation of the PSP was once again observed upon treatment with 10 μM OXO-M, this time without contamination by superimposed spikes. In this case, there is a clear and characteristic irregular oscillation of the membrane potential during the PSP. All agonists were applied for 2 min; a 30 minute washout period was allowed between each drug application.
Adult

Control

+ OXO-M

+ 1S,3R-ACPD

Immature

-84 mV

50 mV

100 ms
3.2.8 Comparison of OXO-M-induced bursting with 4-AP-induced effects

The convulsant potassium channel blocker 4-aminopyridine (4-AP) has been shown to induce epileptiform-like activity in adult guinea-pig olfactory cortical neurones in vitro (Galvan et al., 1982; Libri et al., 1996). In the present study, exposure of immature neurones to 4-AP (100 μM, n=2; 200 μM, n=4) resulted in a similar pattern of seizure-like potentials, with interspersed interictal spiking; this was accompanied by a dramatic prolongation of evoked synaptic potentials, with superimposed spike discharges (Fig. 3.11). These effects were indistinguishable from those induced in the immature neurones by superfusion with 10 μM OXO-M. However, co-application of up to 5 μM atropine during the 4-AP-induced bursting activity did not reduce either the spontaneous or the stimulus-evoked bursts (n=3), indicating that muscarinic receptor activation was not responsible for induction of this behaviour.

The threshold dose required for 4-AP to induce a pattern of spontaneous activity was 10 μM (n=3). In some cells in which 10 μM OXO-M did not produce a full pattern of bursting activity, co-application of 10 μM 4-AP (n=2) resulted in an augmentation of the epileptiform activity, increasing the burst frequency. Co-application of atropine to these cells resulted in an inhibition of the OXO-M induced component of bursting activity, leaving the 4-AP effects intact. This suggests a synergistic action of low doses of 4-AP and OXO-M in inducing epileptiform activity in this preparation.
Fig 3.11 Bursting in immature neurones induced by 4-AP

Exposure of an immature neurone to the convulsant 4-AP (100 μM; P 22 neurone) results in induction of a pattern of spontaneous seizure-like activity similar to that induced by OXO-M in other immature cells (A). This activity consisted of depolarizing ictal events, with interposed interictal spiking activity. The bursting was, however, unaffected by a high dose (5 μM) of atropine. Evoked synaptic potentials (B) in the same neurone displayed a dramatic prolongation upon exposure to 4-AP (100 μM), with accompanied repetitive spike discharges, comparable to those seen in neurones exposed to 10 μM OXO-M.
A

-84
+ 100 μM 4-AP

20 min

+ 5 μM Atropine

50 mV
20 s

B

-84
control

+ 4-AP

50 mV
100 ms
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3.2.9 Intracellular staining of recorded neurones

Some recorded immature neurones were also stained using intracellular neurobiotin in an attempt to determine whether significant dye-coupling existed between cells, and to compare their morphology with those of previously reported adult neurones; such electrical connectivity between cells could possibly explain their enhanced susceptibility towards propagation of epileptiform behaviour (Strata et al., 1997). However, four neurones successfully labelled in this manner indicated that the neurobiotin injected into single recorded cells did not leak into adjacent neurones (Fig. 3.12). Moreover, there appeared to be no obvious difference between the morphology of 'deep pyramidal' neurones recorded in immature slices in this study and adult neurones previously reported (c.f. Libri et al., 1994).
Fig 3.12 Recorded immature neurones stained with neurobiotin

Photomicrographs of individual immature olfactory cortical neurones stained following intracellular injection of neurobiotin. The morphologies of these neurones were similar to the characteristic morphologies of pyramidal neurones located in the cortical layer III, consisting of a pyramidal-shaped cell body, a prominent apical dendrite, and extensive basal dendritic tree. The neurones showed a strong excitatory response to OXO-M with superimposed spontaneous bursting behaviour. It should be noted that the neurobiotin stain remained within individual recorded neurones, indicating that cells of the immature olfactory cortex were not directly coupled through gap junctions. The morphology of recorded bursting neurones was very similar to that of neurones recorded and stained in adult guinea-pig olfactory cortical slices (Libri et al., 1994).
3.3 Discussion

3.3.1 Age-dependent changes in muscarinic but not metabotropic responsiveness

It is very clear from the results obtained in this study that although the resting membrane properties (i.e. resting membrane potential, input resistance and action potential amplitude) of immature rat olfactory neurones (P14-P22) were indistinguishable from those of adult neurones (> P40), the response profile of immature neurones to muscarinic receptor activation differs substantially from that observed in neurones from adult animals. Thus, a progressive pattern of both spontaneous and stimulus-evoked epileptiform activity was consistently observed in the immature neurones which was not seen in adult neurones after treatment with up to 20 μM OXO-M. By contrast, the responses of the immature neurones to metabotropic glutamate receptor activation by up to 50 μM 1S,3R-ACPD were similar in both immature and adult neurones, suggesting that metabotropic glutamate receptor development in this system reaches adult levels much earlier in life than that of muscarinic receptors. It has been reported that electrophysiological responses to metabotropic activation show no apparent difference between immature and adult neurones in the hippocampus (Boss et al., 1992), or in the spinal cord (Ishida et al., 1993), although there does appear to be a clear developmental profile of PI hydrolysis in response to metabotropic activation in the hippocampus (Boss et al., 1992) and hypothalamus (Sortino et al., 1991). In both adult and immature neurones, exposure to 10 μM OXO-M generally (in ‘type I’ responding neurones; Libri et al., 1994) led to a depolarization of the cell, leading to repetitive spiking activity (~10 Hz) which was almost identical in character in the two sets of neurones. It is therefore possible that postsynaptic excitatory effects of
muscarinic receptor activation as recorded in the adult neurones, and reported previously in guinea-pig olfactory neurones (i.e. muscarinic depolarization and induction of a post stimulus sADP; Constanti et al., 1993; Libri et al., 1997), are present in the immature neurones, but are heavily masked by superimposed bursting activity. It is in fact conceivable that such postsynaptic excitatory effects as sADP induction may contribute to the epileptiform behaviour; for example, a brief spontaneous depolarizing potential may induce an sADP, which then appears as an ictal-like event. Close examination of the ictal bursting, however, indicated that burst generation and spiking activity arose from trains of spontaneous depolarizing synaptic potentials, each with an action potential superimposed. In addition, the sudden hyperpolarizations observed at the termination of the ictal bursts were not consistent with persistent excitation arising from a sADP (Constanti et al., 1993). It should also be noted that bursting was completely independent of membrane potential, recorded either in current clamp or voltage clamp modes, indicating that burst generation was most likely to originate outside the recorded cell soma. Considering that the sADP in this system is strongly voltage dependent, disappearing at membrane potentials greater than -100 mV (Constanti et al., 1993), if the sADP was a strong contributory factor to bursting behaviour, a reduction in bursting activity would be expected upon membrane hyperpolarization beyond -100 mV, which was not seen.
3.3.2 Pharmacology of epileptiform activity generated by OXO-M

The bursting activity was consistently blocked by addition of the muscarinic antagonist atropine, confirming that muscarinic receptor activation was essential for the seizure-like activity. Moreover, superfusion of bursting neurones with the muscarinic M₁-selective antagonist pirenzepine (at nM concentrations) also resulted in an inhibition of the bursts, suggesting an involvement of this receptor subtype in burst generation; a similar effect of pirenzepine was also seen towards cholinergic-induced seizures induced in the zona-incerta and surrounding structures of the basal diencephalon (Cruickshank et al., 1994). A full investigation of the pre- or postsynaptic muscarinic receptor subtypes responsible for the induction of epileptiform behaviour in the immature olfactory cortex was, however, outside the scope of the present study.

Bursts were also significantly shortened by superfusion with the selective glutamate NMDA receptor antagonist DL-APV or the selective non-NMDA antagonist CNQX, indicating that activation of both sub-classes of ionotropic glutamate receptor are necessary for ictal burst generation. Indeed, a high dose of CNQX (affecting both NMDA and non-NMDA receptors) completely abolished the activity; this suggested that release of glutamate from presynaptic terminals was taking place during ictal bursts.

Adding TTX to the bathing medium completely and irreversibly inhibited both ictal and interictal activity, indicating that the bursting behaviour was being initiated outside the recorded soma, i.e. presynaptic in origin, and most probably driven by a polysynaptic network. Raising the concentration of magnesium in the bathing solution (to reduce transmitter release, and prevent influx of calcium into cells through ionotropic glutamate receptors; e.g. Flint et al., 1997) also inhibited the behaviour, again suggesting a polysynaptic mode of burst generation.
3.3.3 Modulation of GABA$_A$ or GABA$_B$ receptors can influence bursting activity

OXO-M-induced epileptiform activity could be reversibly inhibited by use of the anaesthetic-sedative pentobarbitone, most likely by augmenting GABA$_A$ receptor-mediated inhibition in this system, although at the concentration used (100 µM) it is possible that this agent was also suppressing glutamate-mediated excitation (Schulz & MacDonald, 1981; Cullen & Martin, 1984). Use of the selective GABA$_A$ receptor antagonist (-) bicuculline had a facilitatory effect on the OXO-M induced bursting, either increasing the amplitude of ictal events in cells previously demonstrating low amplitude bursts, or leading to full amplitude bursting in cells previously demonstrating only small amplitude increases in baseline noise in response to OXO-M application. This suggested that blockade of GABA$_A$ inhibition, while not in itself causing full blown epileptiform activity at the concentration of bicuculline used (small interictal type events were usually evident, coupled with a small increase in evoked synaptic activity) could act synergistically with the excitatory action of OXO-M to produce bursting activity. A similar pattern of bursting has been reported in immature hippocampal neurones, where application of muscarinic agonists along with bicuculline induced spontaneous synchronous activity (Psarropoulou & Dallaire, 1998; Psarropoulou et al., 1998); this was attributed to the release of inhibitory control of GABA-ergic polysynaptic excitatory circuits functioning alongside the excitation produced by muscarinic agonists.

Epileptiform activity was also reversibly reduced by co-application of the GABA$_B$ receptor agonist (-) baclofen, similar to the anticonvulsant action previously reported in hippocampal slices (Ault & Nadler, 1983). Activation of presynaptic GABA$_B$ receptors in the CNS has been shown to reduce evoked synaptic potentials
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(Allerton et al., 1989; Potier & Dutar, 1993) and release of neurotransmitters from presynaptic terminals (Bowery et al., 1980), as well as causing a hyperpolarization and decrease in input resistance postsynaptically (Lacey et al., 1988; Brooks et al., 1992). Considering the lack of voltage-dependence of the muscarinic bursting in immature olfactory neurones, it is unlikely that any baclofen-induced hyperpolarization per se suppressed the spontaneous discharges. It is more likely that baclofen blocked bursting by reducing or preventing release of transmitters from presynaptic terminals, coupled with a general increase in postsynaptic potassium conductance (Lacey et al., 1988; Osmanović et al., 1988).

Blockade of GABA_B receptors with the selective GABA_B receptor antagonist CGP 52432 (1 μM) completely abolished the slow GABA_B-mediated IPSP, however this reduction in inhibitory activity was not a sufficient drive towards excitation to induce synchronous activity. OXO-M, at a dose (2.5 μM) insufficient to cause spontaneous activity on its own (in a cell previously demonstrating muscarinic bursting at a higher concentration of OXO-M), was able to provide the further excitation needed to induce the spontaneous activity (blockable by atropine or pirenzepine). It is interesting to note that 10 μM (-) baclofen was still able to block the bursting in the presence of both CGP 52432 and OXO-M. The CGP 52432 should, at this concentration, behave as a potent antagonist of GABA_B receptors, so baclofen would be expected to have no further effect in this system. The disappearance of the bursting in baclofen, however, suggests that the mechanisms controlling bursting are more complicated than originally thought. Neurochemical data (Bonanno et al., 1997) indicates that another GABA_B antagonist CGP 35348 may selectively block presynaptic GABA_B heteroreceptors (mediating inhibition of glutamate release) without inhibiting
GABA<sub>B</sub> autoreceptors located on GABAergic terminals. Conversely, experiments in the rat spinal cord suggest that the GABA<sub>B</sub> antagonist CGP 56999A antagonises the baclofen-induced inhibition of GABA (but not glutamate release) (Theo et al., 1996). It is thus possible that CGP 52432 in the olfactory cortex was preferentially blocking only one of these presynaptic GABA<sub>B</sub> receptor subtypes, promoting the bursting, while leaving the other receptor subtypes open to activation by applied baclofen, which then inhibited the bursting activity. Further experimentation will be required in order to clarify this issue.

3.3.4 Evoked synaptic potentials in immature neurones differ from those of adult neurones

When measured in control solution, it was found that both immature and adult neurones displayed an initial EPSP, followed by a fast IPSP and a slow IPSP. The EPSPs recorded in immature cells were of significantly lower amplitude than those in adult neurones, but they were of significantly longer (although variable) duration (~200 ms, as opposed to ~100 ms). There have been reports of similar activity in neonatal animals in other areas such as the primary somatosensory cortex (Kim et al., 1995). In addition, it was found that the slow GABA<sub>B</sub>-mediated IPSP was of significantly smaller amplitude in immature neurones. This suggests that there may be a delayed maturation of GABA release from presynaptic terminals, or a deficiency of either postsynaptic GABA<sub>B</sub> receptors or GABA<sub>B</sub> receptor-effector coupling. Age-dependent changes in postsynaptic GABA<sub>B</sub>-mediated responses and GABA<sub>B</sub> inhibitory synaptic transmission has been noted in the hippocampus (Gaiarsa et al., 1994; Gaiarsa et al., 1995). This age-related difference in inhibition may have functional significance in formation of
excitatory synaptic inputs during development, but could also lead to an increased susceptibility for generation of seizure-like activity during early postnatal life.

### 3.3.5 Age-related changes in presynaptic muscarinic/metabotropic activation

The present results show that there is no significant difference in the depression of synaptic activity caused by metabotropic activation in rat olfactory cortex slices studied between 14 days and adulthood; this suggests that there is no functional difference in the presynaptic metabotropic glutamate receptor system in this brain area during this period.

On the other hand (in contrast to the postsynaptic neuronal excitatory effects of muscarinic activation already apparent at an early age), it is suggested that the inhibitory effects of *presynaptic* mAChR activation may not be present, or at least may be greatly reduced during the first few weeks of postnatal life. It has previously been reported that mAChRs generally increase in number in the brain from birth to about postnatal day 30, when they reach adult density (Tice *et al.*, 1996), and also there appears to be a delayed development of cholinergic depression of the evoked field EPSP potential in the hippocampus (Milburn & Prince, 1993), indicating a clear developmental profile of presynaptic muscarinic receptors. In the immature olfactory cortex, evoked EPSPs underwent a pronounced prolongation, with a superimposed repetitive spike discharge following mAChR activation. This prolongation was not apparently dependent on postsynaptic voltage-dependent conductances because in some experiments, the effect on the EPSP was apparent without the presence of the superimposed spikes. It is also unlikely that the prolongation of the EPSP was a result of an increase in membrane input resistance (~20% in 10 μM OXO-M) as this was also present in adult neurones, in
which EPSP prolongation was never seen during muscarinic activation. Further experiments in the future, using pre- and postsynaptic muscarinic receptor subtype-specific agonists/antagonists would be useful to investigate the mechanism underlying this novel synaptic phenomenon.

Previous work by Williams and Constanti (1988) indicated that the muscarinic inhibition of evoked synaptic transmission was most likely mediated by a phosphoinositide (PI)-coupled M\textsubscript{1} mAChR, while the mGluR-mediated inhibition is thought to result from activation of two distinct mGluRs (group II and III; negatively linked to cAMP formation) (Libri et al., 1997). It is thus conceivable that the lack of inhibition of evoked synaptic transmission in the immature neurones is simply the result of a lack of presynaptic PI-coupled muscarinic receptors (i.e. reduced receptor density), previously reported in mouse cerebral cortex (Tan & Costa, 1995).

3.3.6 Comparison of OXO-M induced activity with 4-AP induced bursting

The bursting activity induced by application of OXO-M was very similar to the epileptiform behaviour induced in these slices by the widely known convulsant agent 4-AP, and was comparable to the bursting previously reported in adult guinea-pig olfactory cortical neurones with 4-AP (Galvan et al., 1982; Libri et al., 1996). The prolongation of the evoked EPSP in the presence of OXO-M was also very similar to that seen in the presence of 4-AP. This points towards a similar mechanism of spontaneous burst generation by both agents. In an atrial muscle preparation, it was discovered that there was an interaction between the effects of muscarinic agonists (carbachol and acetylcholine) and the effects of 4-AP, with carbachol antagonising its physiological effects, and 4-AP also acting as a direct antagonist of cardiac muscarinic
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receptors (Urquhart & Broadley, 1991). In our system, the reverse appeared to be true, with low doses of OXO-M actually *promoting* the bursting activity induced by 4-AP. A similar effect was reported in hippocampal neurones, where it was found that a blockade of potassium conductances by 4-AP increased the probability of neurones to display rhythmical activity in response to application of carbachol (Bianchi & Wong, 1994). Furthermore, in an attempt to investigate a possible direct interaction of 4-AP with the muscarinic receptors in our system, it was found that atropine (1 µM) completely abolished the muscarinic agonist-induced bursting and evoked EPSP prolongation, while very high concentrations of atropine (up to 5 µM) consistently failed to inhibit the epileptogenic effects of 4-AP, confirming that 4-AP was not inducing the bursting in this system by activating muscarinic receptors; (c.f. the antagonism of electrophysiological effects of 4-AP by atropine seen in feline atrial myocytes; Navarro-Polanco & Sanchez-Chápula, 1997). Given that 4-AP is believed to induce bursting by a blockade of presynaptic potassium channels (Galvan *et al.*, 1982), and that muscarinic agonists have been shown to inhibit postsynaptic potassium channel activity in a wide variety of systems (Benson *et al.*, 1988; Womble & Moises, 1992; Washburn & Moises, 1992), it is conceivable that OXO-M was also able to inhibit presynaptic potassium channel activity in the immature slices, leading to induction of seizure-like events and prolongation of evoked synaptic potentials.
3.3.7 Mechanism underlying spontaneous OXO-M-induced bursting activity

The observations made during this series of experiments demonstrate that the bursting activity requires activation of muscarinic cholinergic receptors (most likely of the M₁ subtype), as well as the activation of both NMDA and non-NMDA glutamate receptors, suggesting that the phenomenon is multifactorial. The precise point of origin and mechanism of propagation of the bursts, however, is presently unclear, but almost certainly involves excitatory actions of muscarinic agonists upon a distinct population of ‘responding’ neurones located in the deep olfactory cortical layers (Constanti et al., 1993; Libri et al., 1994). Such excitation is most likely propagated to surrounding neurones through a complicated network of local intrinsic association fibres (Haberly & Bower, 1984; Hasselmo & Bower, 1993) similar to the synchronous activity seen through local circuitry in hippocampal slices (MacVicar & Tse, 1989). It is also possible that epileptiform activity was synaptically driven by cells of the subjacent endopiriform nucleus (proposed deep layer IV of the piriform cortex), known to display spontaneous bursting properties (Hoffman & Haberly, 1991; Hoffman & Haberley, 1996; Pelletier & Carlen, 1996).

It is noteworthy that only neurones from the deep cortical layer III were capable of generating fully developed bursts, whereas neurones electrophysiologically identified as superficial pyramidal neurones (Constanti et al., 1993; Libri et al., 1994) generated a much weaker pattern of bursting activity. This may reflect a relatively stronger network of synaptic connectivity through intrinsic fibres on the deep neurones compared with those of the superficial neurones (Forti et al., 1997).

It was also interesting to note that some cells (4 out of 27), identified as deep cells, did not display full bursting activity in response to muscarinic activation. While
this may reflect a variation in the timescale of development of some of the immature animals (i.e. in a few cases, the muscarinic system may be fully developed by the age of recording), it may also reflect a disruption in the association fibre network (implicated in the propagation of the bursts) during the process of slicing. Patch clamp recordings taken from thin olfactory cortical slices (200 μm) of immature animals within the appropriate age range (P10-P22) displayed no bursting in response to muscarinic activation (Sciancalepore & Constanti, 1996; 1998); this implies that the more intact architecture of the fibre network, as would be found in the thicker slices used in this study (450 μm), is vital for observing epileptiform activity.

It has been suggested that in the brains of immature animals, neurones may form electrical gap junctions between each other, which can be identified by dye-coupling between cells, and may contribute towards oscillatory activity, particularly in the hippocampus (Strata et al., 1997). In the present study the neurobiotin dye, injected into the cell soma of immature cells, remained within the recorded neurones, confirming that there was no obvious dye coupling between adjacent cells; this indicated that propagation of oscillatory activity was unlikely to be taking place through gap-junctions in this preparation.

A comparison of the muscarinic-induced epileptiform activity in the immature olfactory neurones with bursting activity observed in other neuronal systems of both adult and immature animals may provide further insight into the cellular mechanisms involved in its generation. One of the most common types of similar bursting is that induced by 4-AP (as described above), most likely resulting from the block of presynaptic potassium channels (Galvan et al., 1982; Voskuyl & Albus, 1985; Perreault & Avoli, 1991). Another popular in vitro model of epileptic events is induced by
reducing the concentration of magnesium ions in the bathing solution (Flint et al., 1997), which is thought to remove the background inhibition of NMDA receptor channel activity, thereby enhancing NMDA conductance (Traub et al., 1994). This type of bursting activity is similar in its manifestation to the muscarinic bursting, having similar frequency and duration and displaying a similar prolongation of evoked synaptic potentials (Flint et al., 1997). The results of our study show that NMDA receptor activity is paramount for the muscarinic bursting activity to take place, although it seems more likely that this is occurring as a result of a greater release of excitatory transmitters resulting from a developmental lack in presynaptic muscarinic inhibition, rather than any change in the intrinsic activity of the NMDA receptors themselves, particularly since the bursting could be reduced by blockade of non-NMDA receptors.

A further common method of inducing epileptiform activity within the piriform cortex in vitro is by repeated electrical stimulation, in the form of kindling (Hoffman & Haberly, 1996) or repeated tetanic stimulation (Pelletier & Carlen, 1996). Both of these types of activity are thought to originate in the underlying endopiriform nucleus, which is considered a crucial epileptogenic site within the piriform cortex. It is highly likely that the slices used within the present study contained cells of the endopiriform nucleus, which may have enhanced the susceptibility of the piriform cortex to epileptogenesis by muscarinic agonists; further studies would be needed to confirm this. It should be stressed that the actual manifestation of bursting activity in the latter-mentioned models was different to that seen in the present muscarinic model; for example the evoked epileptiform EPSPs in both cases of electrically-induced activity took place within a 50 ms timescale, whereas the EPSPs in the immature rats were prolonged by several seconds in the presence of OXO-M. Also, the epileptic events induced by electrical
stimulation appeared not to be as dependent on NMDA-receptor activation as the present muscarinic-induced bursting (Hoffinan & Haberly, 1996; Pelletier & Carlen, 1996).

Compounds believed to block sodium channels have been shown to reduce the appearance of tonic-clonic and partial epileptic seizures both in clinical and experimental situations (Macdonald & Kelly, 1993). Furthermore, it has been found that treatment of hippocampal slices with veratridine (a compound which causes persistent activation of sodium channels at normal membrane potentials) leads to a cellular model for epileptiform activity (Otoom et al., 1998). This mechanism of burst generation, however, can be discounted in consideration of the muscarinic bursting, since there was no evidence that fast sodium channel activity was being affected in our system; moreover, synaptic transmission (in contrast to the muscarinic-induced activity) was completely inhibited by the veratridine, presumably due to a persistent presynaptic depolarization.

The most likely mechanism of induction of spontaneous and evoked epileptiform activity in the immature slices appears to involve an imbalance between inhibitory and excitatory processes within the neuronal networks. As mentioned previously, the most similar in vitro model of epileptiform activity found was that recently described by Psarropoulou & Dallaire (1998) in immature hippocampal slices, where the cholinergic agonist carbachol was administered in the presence of the GABA<sub>A</sub> receptor antagonist bicuculline. It is possible that in the immature olfactory cortex there is a reduced level of GABAergic inhibition, and/or an over-expression of muscarinic-induced excitatory activity (not intrinsically epileptogenic) which results in
induction of the epileptiform behaviour in the presence of muscarinic receptor activation.

There is obviously a profound difference in the muscarinic receptor response profiles between immature and adult neurones in slices of olfactory cortex, adult neurones never displaying bursting activity or EPSP prolongation in response to muscarinic activation. There is evidence that in humans, as well as in other species, neonates and infants are considerably more prone to epileptic seizures than adults (Johnston, 1996). There have also been reports of specific periods during development in which there is a heightened tendency for epileptiform activity (Sutor et al., 1994), reflecting a general increase in neuronal excitability in immature neurones. The in vitro findings of this study indicate that mAChR development in the olfactory cortex, and possibly other systems, may play a very important role in epileptogenesis in neonates. This finding is also consistent with the reported occurrence of absence seizures in epileptic children following olfactory stimulation (Komárek, 1994). It is proposed that a delayed maturation or change in presynaptic muscarinic receptor density or function during the first few weeks of life could contribute towards this heightened susceptibility for seizure generation in immature cortical neurones.
3.4. Summary

1) The effects of muscarinic receptor activation were studied in slices of immature (P14-P21) and adult (> P40) rat olfactory cortex using standard intracellular current and voltage clamp recording techniques.

2) Application of the muscarinic agonist OXO-M (10 μM) to slices prepared from immature animals resulted in the induction of a spontaneous epileptiform behaviour, not seen in cells of the adult olfactory cortex.

3) Epileptiform bursts were inhibited by atropine (1 μM) or pirenzepine (300 nM), confirming the involvement of muscarinic receptors in their generation.

4) Bursting behaviour was also blocked by application of 1 μM TTX or raising the concentration of magnesium in the bathing solution to 5 times normal (5 mM), indicating a polysynaptic mode of generation.

5) The NMDA antagonist DL-APV (100 μM) or the non-NMDA receptor antagonist CNQX (5 μM) suppressed bursting, indicating that both glutamate receptor types were necessary for the propagation of the epileptiform activity, which was most likely of presynaptic origin.

6) GABA_A or GABA_B receptor modulators/agonists (pentobarbitone, 100 μM, or baclofen, 10 μM, respectively) suppressed bursting, while GABA_A/GABA_B antagonists (bicuculline, 10 μM, or CGP52432 1 μM, respectively) promoted the activity, working synergistically with a lower dose of OXO-M (2.5 μM), which itself did not cause oscillatory activity.

7) Muscarinic bursting strongly resembled that induced by the known convulsant 4-AP (100 μM), and indeed in cells which did not burst fully in OXO-M (10 μM), a low dose of 4-AP (10 μM) acted synergistically to induce this oscillatory behaviour.
8) Evoked synaptic potentials were dramatically prolonged by 10 μM OXO-M in the immature neurones, rather than being depressed, as seen in adult slices. 50 μM 1S,3R-ACPD, however, depressed evoked EPSPs in both adult and immature neurones.

9) In conclusion, epileptiform behaviour was consistent with a lack of presynaptic inhibitory muscarinic receptor activity, possibly resulting from a delayed maturation of inhibitory presynaptic muscarinic receptors.
CHAPTER 4

THE EFFECTS OF CAFFEINE ON THE MUSCARINIC AGONIST-INDUCED POST-STIMULUS AFTERDEPOLARIZATION (sADP) AND ITS UNDERLYING CURRENT $I_{ADP}$. 
4.1 Introduction

It has previously been shown that exposure of adult mammalian olfactory cortex in vitro to muscarinic agonists results in an inhibition of synaptic transmission (Williams & Constanti, 1988; Bagetta & Constanti, 1990), and postsynaptic excitatory effects such as a depolarization, repetitive spike discharge, and appearance of a slow post-stimulus afterdepolarizing potential (sADP), believed to involve a calcium-dependent deactivation of a novel potassium conductance (Constanti & Bagetta, 1991; Constanti et al., 1993; Libri et al., 1994, 1996). Similar effects were seen in these neurones following metabotropic glutamate receptor activation (Constanti & Libri, 1992; Libri et al., 1997). The dependence of the sADP on calcium entry into the recorded cells has been confirmed by other workers on cortical neurones (Schwindt et al., 1988; Andrade, 1991), as well as other systems such as dorsal root ganglia (White et al., 1989) and supraoptic nucleus neurones (Li et al., 1995). Furthermore it has been reported that L-type voltage-gated calcium channels and release of calcium from intracellular stores play an important role in generation of depolarizing afterpotentials in these latter neurones (Li & Hatton, 1997).

Methylxanthines such as caffeine and theophylline have a wide range of pharmacological actions in neurones of the central nervous system (e.g. direct interaction with voltage gated calcium channels, inhibition of phosphodiesterases and direct antagonism of adenosine receptors). However, one of the most well known actions of these compounds is the mobilisation of calcium from intracellular stores through interaction with the ryanodine receptor (for reviews see Nehlig et al., 1992; Sawynok & Yaksh, 1993). Considering the effects of compounds promoting or inhibiting calcium release on DAPs recorded in supraoptic nucleus neurones (Li &
Hatton, 1997), and also taking into account the previously reported role of neuronal calcium entry and release from intracellular calcium stores in the generation of the sAHP in guinea-pig vagal neurones (Sah & McLachlan, 1991) or rabbit otic ganglion cells (Yoshizaki et al., 1995), we were interested in examining the possible role of calcium release from intracellular stores in olfactory cortical sADP generation. The effects of caffeine, and some other compounds which modulate calcium entry/release from intracellular stores, upon induction of the slow afterdepolarizing potential and its underlying inward tail current were therefore investigated under the present conditions.
4.2 Results

4.2.1. Effects of caffeine on neurones of the olfactory cortex.

Caffeine was superfused onto neurones of the olfactory cortex both in the absence and presence of OXO-M or 1S,3R-ACPD. The dose of caffeine generally used in other studies to potentiate CICR was 10 mM (e.g. Marrion & Adams, 1992; Nohmi et al., 1992); this concentration was therefore used initially during the experiments performed on olfactory cortical neurones. This dose, however, appeared to consistently damage the neurones (causing loss of membrane potential and intrinsic membrane properties of the cells) and was not used further. Alternatively, 3 mM caffeine was generally employed, a concentration which appeared to be non-toxic, and also had no effect on membrane potential throughout superfusion.

4.2.2. Intrinsic neuronal membrane properties.

The present series of experiments were based on stable intracellular recordings of 1-5 hours duration obtained from a total of 46 neurones in tissue taken from adult (>P40) guinea-pigs. The predominant cell type (~80% of recordings) corresponded electrophysiologically to the 'deep' cell type of olfactory cortical layer III (Fig. 4.1). In control solution, neurones had a mean resting membrane potential of -84.2 ± 0.3 mV (after sealing), a mean first-spike amplitude (evoked by an injected current pulse) of 115 ± 0.8 mV, and a mean resting input resistance (calculated from <20 mV hyperpolarizing electrotonic potentials) of 40.1 ± 1.4 MΩ. The other main cell type encountered was the 'type III non-responding' neurones, which displayed significantly lower resting membrane potential (mean = 77.3 ± 1.9 mV), smaller spike amplitude (mean = 106 ± 2
Chapter 4: Caffeine blocks the sADP and $I_{ADP}$

mV) and reduced resting input resistance (mean = 16.6 ± 1.3 MΩ). These cells tended to display typical spike fractionation with increasing depolarizing electrotonic current pulses, and strong accommodation of spiking activity in response to long depolarizing currents. The latter cell type did not respond to muscarinic or metabotropic agonists (e.g. Libri et al., 1994), so was regularly discarded.

When 3 mM caffeine was applied to the type I cells in control solution (n=5), or in the presence of 10 μM OXO-M (n=3) or 10 μM 1S,3R-ACPD (n=3), there was no significant effect on the intrinsic membrane properties of recorded neurones (p>0.5, t-tests).

4.2.3 Muscarinic or metabotropic glutamate receptor activation in guinea-pig neurones

A prolonged application of either 10 μM OXO-M (n=36) or 10 μM 1S,3R-ACPD (n=3) to the olfactory neurones resulted in a slow membrane depolarization (mean = 8.3 ± 0.4 mV in OXO-M or 5.0 ± 1.5 mV in 1S,3R-ACPD) with superimposed repetitive spike discharge at around 10 Hz, coupled with a decrease in membrane input resistance (8.5 ± 3.6 % in OXO-M or 22.4 ± 7.6 % in 1S,3R-ACPD, measured from -70 mV membrane potential). These neurones also displayed a post-stimulus afterdepolarization (sADP) lasting several seconds (Fig. 4.2). 3 mM caffeine consistently failed to affect the depolarization, and subsequent repetitive firing, induced by either OXO-M (n=3; Fig. 4.3) or 1S,3R-ACPD (n=3; not shown) in responding neurones.
Fig 4.1. Resting membrane properties of a typical ‘deep’ neurone of the guinea-pig olfactory cortex.

(A) shows a typical current/voltage relationship recorded from a ‘deep’ guinea-pig olfactory neurone in which the spiking activity was regular, and there was no spike fractionation with larger depolarizing stimuli (stimulus width = 160 ms; 0.5 nA negative, + 0.5 and 1.5 nA positive steps). This cell had a relatively large input resistance, and there was a clear fast inward rectifier revealed with increasing hyperpolarizing stimuli. (B) shows that such a ‘deep’-type neurone (same cell as in A) showed little or no spike accommodation during a spike train elicited by a prolonged depolarizing stimulus (+ 2 nA, 1.6 s), and there was also a small post-stimulus sAHP present in this neurone type.
Fig 4.2. Effects of OXO-M on a responding 'type I' neurone.

(A) shows the sAHP (upper panel) and its underlying outward tail current $I_{AHP}$ (lower panel, revealed under hybrid voltage clamp) following a prolonged depolarizing stimulus in control solution, measured at -70 mV membrane potential (stimulus parameters were +2 nA, 1.6 s). (B) shows the corresponding sADP (upper panel) and its underlying inward tail current $I_{ADP}$ (lower panel) induced by a similar long depolarizing stimulus (+2 nA, 1.6 s) in the presence of 10 μM OXO-M. (All recordings were from the same neurone throughout).
Fig 4.3. Effects of 3 mM caffeine on the sAHP, $I_{AHP}$, muscarinic depolarization, sADP and $I_{ADP}$.

(A) A prolonged depolarizing stimulus (1.6s, + 2 nA) leads to induction of a post-stimulus sAHP in the control solution. Middle panel shows the sAHP recorded under current clamp, bottom panel shows the corresponding hybrid voltage clamp trace of the current underlying the sAHP represented as a slowly decaying outward current deflection. (B) Upper panel shows effects of applying 10 μM OXO-M (commencing at solid line) leading to depolarization of the cell and eventual superimposed repetitive firing (downward deflections represent induced negative electrotonic potentials, evoked by injection of -0.5 nA at ~ 0.3 Hz). Second panel shows the induction of the post-stimulus sADP with superimposed repetitive firing. In both cases, a small direct hyperpolarizing current was applied at the open arrow leading to a gradual hyperpolarization of the cell to the original potential. The lower panel shows the corresponding $I_{APD}$ tail current measured under hybrid voltage clamp conditions, represented as a slowly decaying inward current. (C) Effect of 3 mM caffeine on the sAHP recorded in control. Caffeine reduced the sAHP amplitude recorded in current clamp (upper trace) and the corresponding outward tail current recorded in voltage clamp (lower trace). (D) When OXO-M was re-applied in the presence of caffeine, the depolarizing response to muscarinic activation was unaffected, whereas a clear reduction was observed in sADP amplitude in the presence of OXO-M under current clamp, and the $I_{ADP}$ under hybrid voltage clamp.
4.2.4 The sADP and its underlying tail current $I_{ADP}$ in responding neurones.

Guinea-pig olfactory neurones, when challenged with a prolonged depolarizing stimulus (2.0 nA, 1.6 s) in control solution, showed a mean post-stimulus slow afterhyperpolarization (sAHP) amplitude of $11.0 \pm 0.4$ mV (n=34) (routinely measured close to threshold membrane potential; ~-70 mV). Under ‘hybrid clamp’ conditions, the sAHP was found to have a mean underlying outward tail current ($I_{AHP}$) amplitude of $0.22 \pm 0.01$ nA at -70 mV holding potential (n=34) (Fig. 4.2 A). When these cells were exposed to either 10 μM OXO-M or 10 μM 1S,3R-ACPD, the sAHP disappeared and was replaced with a post-stimulus sADP, with a mean amplitude (at -70 mV) of $8.7 \pm 0.4$ mV in OXO-M (n=34) or $7.0 \pm 1.5$ mV in the presence of 1S,3R-ACPD (n=3). The underlying inward tail current ($I_{ADP}$) revealed under hybrid clamp, at -70 mV holding potential, had a mean amplitude of $0.35 \pm 0.02$ nA and mean duration $35.4 \pm 1.4$ s in OXO-M (n=33) (Fig. 4.2 B) and $0.28 \pm 0.01$ nA amplitude, $27.3 \pm 3.2$ s duration in 1S,3R-ACPD (n=3). Induction of the sADP and $I_{ADP}$ were readily reversible upon washout of either OXO-M or 1S,3R-ACPD from the bathing solution.

4.2.5. Effects of caffeine on the sADP and $I_{ADP}$.

The most notable effect of caffeine on this system was a dramatic reduction in the amplitude of the OXO-M-induced sADP and its underlying current $I_{ADP}$ (Fig. 4.3). This reduction was concentration dependent, being reduced by $31.6 \pm 1.4$ % and $30.2 \pm 3.0$ % in 0.5 mM (n=3), $26.7 \pm 14.5$ % and $51.5 \pm 15.5$ % in 1 mM (n=3), $60.8 \pm 5.8$ % and $74.5 \pm 9.1$ % in 2 mM (n=3) and $64.1 \pm 15.0$ % and $74.2 \pm 8.1$ % in 3 mM caffeine respectively (n=8; P<0.05) (Figs. 4.4; 4.5). 3 mM caffeine reduced the 10 μM 1S,3R-ACPD induced sADP and $I_{ADP}$ by $77.8 \pm 13.8$ % and $73.3 \pm 15.0$ % respectively (n=3,
P<0.05). The effects of 3 mM caffeine are summarised in Table 4.1; all effects were readily reversible after 15-20 minutes of caffeine washout.

Theophylline (another methylxanthine) also significantly inhibited $I_{ADP}$ in a concentration-dependent reversible manner, with a similar potency to caffeine; inhibition was $41.1 \pm 8.4 \%$ in 0.5 mM, $68.9 \pm 9.3 \%$ in 1 mM, $85.5 \pm 0.4 \%$ in 2 mM and $96.2 \pm 3.9 \%$ in 3 mM theophylline ($p<0.05$, n=3).

4.2.6. Effects of caffeine on evoked synaptic potentials.

As in the experiments involving the immature and adult rat neurones (chapter 3), in the present series of experiments, synaptic potentials could be elicited using an external stimulating electrode placed in layer II-III of the cortical slice. Focal stimulation (10-30 V; 0.2 ms) of the local association fibres again resulted in the appearance of the characteristic EPSP/IPSP complex as described in the previous chapter. In the guinea-pig neurones, there were no spike discharges superimposed on the EPSP, even following stimuli of maximal intensity (30 V) or depolarizing the membrane potential (up to -60 mV, n=3). The threshold for appearance of an action potential was around -65 mV. The mean amplitude of evoked EPSPs in control solution was $14.6 \pm 0.7$ (n=28) (Table 4.1). In the presence of OXO-M, the mean EPSP amplitude was reduced by $58 \pm 3 \%$ (n=28), and in 1S,3R-ACPD the amplitude was reduced by $80 \pm 10 \%$ (n=3). In contrast, in 3 mM caffeine, there was a clear augmentation of evoked synaptic transmission, the EPSP being enhanced by $93 \pm 33 \%$ (n=5, $P<0.05$ in all cases) (Table 4.1).
Fig 4.4. The $I_{ADP}$ tail current is reduced by increasing concentrations of caffeine.

Left panel shows the $I_{ADP}$ tail current (in the absence of caffeine) induced by a prolonged depolarizing stimulus during superfusion of the neurone with 10 μM OXO-M. Right panel shows superimposed $I_{ADP}$s in the same neurone in the presence of 0.5, 1 or 3 mM caffeine. Stimulus parameters for $I_{ADP}$ induction were consistent with those used previously (+2nA, 1.6 s).
10 μM OXO-M

10 μM OXO-M + caffeine

- 0.5 mM
- 1 mM
- 3 mM
- 0.5 mM

0.1 nA

5 s
Fig 4.5. The inhibition of $I_{\text{ADP}}$ amplitude by caffeine or theophylline was concentration-dependent.

(A) shows the concentration-dependent percentage decrease in $I_{\text{ADP}}$ amplitude in the presence of 0.5 mM, 1 mM, 2 mM (n=3) or 3 mM (n=8) caffeine respectively. (B) shows the inhibition of $I_{\text{ADP}}$ amplitude in a concentration-dependent manner by 0.5 mM, 1 mM, 2 mM or 3 mM theophylline (n=3). Data are presented as mean percentage decreases, with error bars referring to S.E.M.
A

% Inhibition of $I_{ADP}$

Concentration of caffeine (mM)

B

% Inhibition of $I_{ADP}$

Concentration of theophylline (mM)
Chapter 4 : Caffeine blocks the sADP and $I_{\text{ADP}}$

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<tr>
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<th>Control</th>
<th>$+3$ mM Caffeine</th>
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<tbody>
<tr>
<td><strong>EPSP Amplitude (mV)</strong></td>
<td>$15.6 \pm 1.0$ (n=5)</td>
<td>$30.0 \pm 5.4$ (n=5)</td>
</tr>
<tr>
<td><strong>sADP Amplitude (mV) in</strong></td>
<td><strong>OXO-M</strong></td>
<td></td>
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<tr>
<td></td>
<td>$7.7 \pm 0.8$ (n=9)</td>
<td>$2.9 \pm 1.1$ (n=9)</td>
</tr>
<tr>
<td><strong>$I_{\text{ADP}}$ Amplitude in OXO-M (nA)</strong></td>
<td>$0.40 \pm 0.06$ (n=8)</td>
<td>$0.11 \pm 0.07$ (n=8)</td>
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</table>

Table 4.1. Effects of superfusing 3 mM caffeine onto responding neurones of the guinea-pig olfactory cortex. EPSP amplitudes were measured routinely at -84 mV membrane potential. sADP amplitudes were measured close to threshold at -70 mV membrane potential, and $I_{\text{ADP}}$ amplitudes were measured under 'hybrid' clamp at -70 mV holding potential. Values in parenthesis refer to the numbers of neurones used for each value. All changes were significant (P<0.05, t-tests)

4.2.7. Requirement of calcium entry for sADP generation.

Calcium entry into the cell was previously found to be necessary for induction of the sADP (Constanti et al., 1993), but there was no indication of the type(s) of calcium channel involved. Blockade of L-type voltage-gated calcium channels has been shown to inhibit the slow afterhyperpolarization in the hippocampus (Tanabe et al., 1998), so the possible contribution of these channels towards sADP generation was investigated by application of the L-type calcium channel blocker nifedipine (10 µM) in the absence and presence of 10 µM OXO-M. Figure 4.6 shows that the amplitude and time course of the sADP and underlying $I_{\text{ADP}}$ were significantly reduced by $41.7 \pm 4.8$ % and $46.6 \pm$
Chapter 4: Caffeine blocks the sADP and $I_{ADP}$

1.7 % respectively (n=4) by 10 μM nifedipine in the presence of 10 μM OXO-M (p<0.05, paired t-tests). As with caffeine, however, the nifedipine did not interfere with the muscarinic depolarization response (n=3). The effects of nifedipine were reversed after 40 minutes of washout; (due to the light sensitivity of nifedipine, light was excluded from the test solution, and experiments were performed in near darkness).

4.2.8. Effects of ryanodine receptor blockers and calcium store uptake inhibitors.

The possible role of CICR in generating the olfactory cortical sADP and the underlying $I_{ADP}$ was investigated using the membrane-permeable inhibitors of intracellular calcium release, ryanodine and dantrolene (Henzi & MacDermott, 1992). Further to this, the effects of intracellular calcium store depletion were also tested by using the membrane permeable calcium store uptake inhibitors thapsigargin and cyclopiazonic acid (CPA; Markram et al., 1995). Ryanodine (10 μM) reduced $I_{ADP}$ amplitude by a small, but significant, percentage (9.9 ± 4.2%, p<0.05; n=7; Fig. 4.7), while bath-application of dantrolene (n=3) at a concentration of 10 μM for up to 40 minutes failed to significantly reduce the sADP or $I_{ADP}$ amplitude (p>0.5, paired t-test; Table 4.2; Fig. 4.7); (due to the light sensitivity of the dantrolene, recordings were performed in near darkness, and light was excluded from the dantrolene solution).

Thapsigargin and CPA have previously been shown to suppress calcium uptake into intracellular stores in neurones via inhibition of calcium ATPase in the endoplasmic reticulum, making less calcium available for subsequent release (Markram et al., 1995). However, neither thapsigargin (3 μM, n=3) nor CPA (15 μM, n=3) significantly reduced the amplitude of $I_{ADP}$ (p > 0.1 in both cases, paired t-test), even after 30-40 minutes exposure to these agents (Table 4.2; Fig. 4.7).
Fig 4.6. Inhibitory effects of 10 μM nifedipine on the sADP and $I_{ADP}$ measured in a single olfactory cortical neurone.

(A) shows the sADP and $I_{ADP}$ recorded in 10 μM OXO-M, in the absence of nifedipine, recordings being taken at -70 mV membrane potential and -70 mV holding potential respectively. (B) shows sADP and $I_{ADP}$ recorded in the same neurone in the presence of 10 μM OXO-M and 10 μM nifedipine; note the reduction in sADP and $I_{ADP}$ amplitude. Stimulus parameters were identical to those used previously (+2 nA, 1.6 s).
A 10 μM OXO-M

B 10 μM OXO-M + 10 μM nifedipine
Fig 4.7. Effects of intracellular calcium release modulators on $I_{ADP}$ amplitude in the presence of 10 μM OXO-M.

Left panels of each section show the control $I_{ADP}$ evoked in OXO-M and right panels show $I_{ADP}$ in the presence of drug. (A) Superfusion of ryanodine (10 μM) caused a significant overall depression of around 10% (see text), but in all other cases (10 μM dantrolene [B], 3 μM thapsigargin [C] or 15 μM CPA [D]) there was no significant inhibition of $I_{ADP}$. In each experiment, $I_{ADP}$ was recorded under hybrid clamp at -70 mV holding potential, and was elicited using stimulus parameters as described previously (+2 nA, 1.6 s). Scale bars refer to all recordings in this section; (A–D were obtained from different neurones).
A

10 μM OXO-M

-70

B

10 μM OXO-M

+ 10 μM ryanodine

+ 10 μM dantrolene

C

10 μM OXO-M

+ 3 μM thapsigargin

+ 15 μM CPA

D

0.2 nA

5 s
### Table 4.2.
The effect upon $I_{ADP}$ amplitude of ryanodine or dantrolene (which interfere with release of calcium from intracellular stores), or thapsigargin or CPA (which prevent calcium reuptake into the stores, depleting them of calcium). All recordings of $I_{ADP}$ were taken under hybrid clamp, at a holding potential of -70 mV. In all cases, apart from ryanodine, there was no significant difference between the $I_{ADP}$ amplitude before or after administration of the compound ($p>0.5$, paired t-tests). The reduction of $I_{ADP}$ amplitude by ryanodine was significant ($p<0.05$, paired t-test). Values in parenthesis refer to the numbers of neurones used for each investigation.

### 4.2.9. Other possible pharmacological actions of caffeine.

Caffeine is known to have several different sites of action (Nehlig *et al.*, 1992) apart from its classical interaction with intracellular calcium store release channels (Sawynok & Yaksh, 1993; Kuba, 1994). Thus, caffeine also inhibits the enzyme

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<tr>
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<th>Absence of compound</th>
<th>Presence of compound</th>
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<tr>
<td>$I_{ADP}$ amplitude in 10 μM ryanodine (nA)</td>
<td>0.38 ± 0.06 (n=7)</td>
<td>0.35 ± 0.06 (n=7)</td>
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<tr>
<td>$I_{ADP}$ amplitude in 10 μM dantrolene (nA)</td>
<td>0.28 ± 0.04 (n=3)</td>
<td>0.25 ± 0.03 (n=3)</td>
</tr>
<tr>
<td>$I_{ADP}$ amplitude in 3 μM thapsigargin (nA)</td>
<td>0.47 ± 0.13 (n=3)</td>
<td>0.43 ± 0.12 (n=3)</td>
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<tr>
<td>$I_{ADP}$ amplitude in 15 μM CPA (nA)</td>
<td>0.26 ± 0.06 (n=3)</td>
<td>0.23 ± 0.06 (n=3)</td>
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phosphodiesterase, leading to an increase in intracellular cAMP concentration (Sawynok & Yaksh, 1993), as well as being a direct antagonist at adenosine receptors (Daly et al., 1981; Greene et al., 1985).

Experiments were therefore carried out to test the possibility of these systems being implicated in the inhibition of the sADP and $I_{\text{ADP}}$. A low dose of IBMX (100 μM) was used as a phosphodiesterase inhibitor to test if an intracellular buildup of cAMP was responsible for sADP inhibition. Out of the 3 neurones tested with 100 μM IBMX in the presence of 10 μM OXO-M, none showed any reduction of sADP or $I_{\text{ADP}}$ amplitude compared to OXO-M alone, even after up to 30 minutes exposure to IBMX (p>0.5, paired t-test; Fig. 4.8). In addition, 100 μM IBMX failed to alter the amplitude of evoked excitatory synaptic potentials (p>0.5, paired t-test), indicating that phosphodiesterase inhibition does not account for the enhancing effects of caffeine on synaptic transmission in the olfactory cortex.

In light of the previously reported inhibition of the sAHP and its underlying $I_{\text{AHP}}$ by caffeine (Haas & Greene, 1988), and its enhancement by adenosine (Haas & Greene, 1984) in hippocampal CA1 neurones, the possible effects upon $I_{\text{ADP}}$ of caffeine behaving as an antagonist of endogenous adenosine was investigated (c.f. Motley & Collins, 1983). During these experiments, an sADP and $I_{\text{ADP}}$ were initially recorded in the presence of 10 μM OXO-M alone, and then in the presence of 3 mM caffeine, which almost completely abolished the sADP and $I_{\text{ADP}}$ (mean reduction = 86.8 ± 5.6 %, n=3) and enhanced evoked synaptic potentials (mean enhancement = 144.2 ± 66.7 %, n=3) (Fig. 4.9). Following this, 100 μM adenosine was added in an attempt to reverse any possible antagonism of endogenous adenosine receptors produced by caffeine. After exposure of the slices to adenosine for 15 minutes, a definite reversal in the
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enhancement of the EPSP was seen (i.e. EPSP amplitude was reduced by 73.1 ± 14.7 %, n=3; p<0.05, paired t-test), suggesting that adenosine was reversing the effect of caffeine presynaptically. The sADP and $I_{ADP}$, however, did not return during superfusion with 100 µM adenosine in 10 µM OXO-M plus 3 mM caffeine, in fact there was no detectable difference between $I_{ADP}$ in 3 mM caffeine alone, or with 100 µM adenosine added to the bathing solution (p>0.1, paired t-test), suggesting that caffeine blockade of adenosine receptors was not responsible for the observed reduction in the $I_{ADP}$ (Fig. 4.9).
Fig 4.8. IBMX fails to inhibit $I_{\text{ADP}}$.

Superfusion of the phosphodiesterase inhibitor IBMX (100 μM) has no significant effect on the amplitude of $I_{\text{ADP}}$ evoked at -70 mV in the presence of OXO-M (10 μM). Scale bars apply to both traces.
10 µM OXO-M

10 µM OXO-M + 100 µM IBMX
**Fig 4.9. Attempts to reverse the effects of caffeine with adenosine.**

(A) Shows the $I_{AHP}$ tail current (top) and the evoked synaptic potential (bottom) recorded in control solution. (B) shows the $I_{ADP}$ and evoked synaptic potential in the presence of 10 μM OXO-M alone. (C) shows inhibition of the $I_{ADP}$ and *enhancement* of the EPSP produced by adding 3 mM caffeine. (D) shows that in the presence of 100 μM adenosine, $I_{ADP}$ was still inhibited by caffeine, while the EPSP was reduced. Top scale bars refer to all top traces and bottom scale bars refer to all bottom traces. $I_{AHP}$ and $I_{ADP}$ were elicited under hybrid clamp at -70 mV holding potential using previously described stimulus parameters (+2 nA, 1.6 s). Synaptic potentials were all evoked at -84 mV membrane potential, using identical stimulus parameters throughout (2.0 V, 0.8 ms).
A: Control

B: 10 μM OXO-M

C: 10 μM OXO-M + 3 mM caffeine

D: 10 μM OXO-M + 3 mM caffeine + 100 μM adenosine

-70 mV

5 s

0.2 mA

20 mV

100 ms
4.2.10. A possible direct blockade of calcium channels by caffeine

There are several reports showing a direct interaction of caffeine with calcium currents in cell membranes of muscle cells (Zholos et al., 1991; Varro et al., 1993; Yoshino et al., 1996), indicating an overall decrease in conductance through these channels. To test whether caffeine was inhibiting $I_{\text{ADP}}$ by blocking calcium currents in the olfactory cortical neurones, cells were recorded with 2 M caesium chloride-filled electrodes. It has previously been shown that intracellular caesium blocks voltage-gated potassium channels, thereby delaying repolarization after an action potential. This reveals a characteristic plateau potential resulting from activation of voltage-gated calcium channels (Galvan et al., 1985; Libri et al., 1996), very similar to the prolonged plateau seen on the repolarization phase of the cardiac action potential.

Cells impaled with caesium chloride electrodes were routinely left for at least 15 minutes to allow the caesium to diffuse into the cell interior. Subsequent blockade of voltage-gated potassium channels resulted in the calcium plateau developing over this time, and the action potential duration was lengthened accordingly (Fig. 4.10) (the spike half-width, i.e. the width of the action potential at half peak amplitude from baseline, was taken as a standard measure during the caesium electrode experiments). Superfusion of the neurones with 10 μM nifedipine reduced the half-width of the initial action potential from $41.7 \pm 2.6 \text{ ms}$ to $24.9 \pm 6.2 \text{ ms (n=3)}$, a significant reduction ($40.0 \pm 15.0 \%$, $p<0.05$, paired t-test), indicating that the plateau resulted, in part, from the activity of L-type voltage-gated calcium channels (Fig. 4.10). Likewise, superfusion of cells with 3 mM caffeine resulted in reduction of the spike half-width to $33.0 \pm 10.8 \text{ ms (n=3)}$, also a significant reduction ($45.0 \pm 15.0 \%$, $p<0.05$, paired t-test). This strongly indicated that caffeine was partially blocking voltage-gated calcium conductances in the
olfactory neurones (Fig. 4.10); (care was taken to ensure that all recordings made with caesium chloride-filled electrodes were taken at the same membrane potential, as action potentials recorded under these conditions were highly sensitive to changes in membrane potential.
Fig 4.10. Action potentials recorded with caesium chloride-filled electrodes, revealing calcium plateaus.

Top panel shows a depolarizing electrotonic potential evoked in control solution, with a clear calcium plateau present on the first and subsequent action potentials. The middle panel shows a clear reduction of half-width of the initial spike in the presence of 10 μM nifedipine. Lower panel shows a similar reduction in initial spike half-width resulting from superfusion with 3 mM caffeine. Scale bars refer to all traces. Stimulus parameters were identical for each trace (+ 0.5 nA, 160 ms). (A) and (B) were obtained from different neurones.
A

control

-80

+ 10 μM nifedipine

B

control

+ 3 mM caffeine

20 mV

50 ms
4.3. Discussion

4.3.1. Basic membrane properties of neurones.

The basic membrane properties, i.e. membrane potential, input resistance and spike amplitude, of guinea-pig olfactory neurones recorded during this study were similar to those previously reported in this system (Constanti et al., 1993; Libri et al., 1994). Neurones displaying the characteristic properties of 'non-responding' cells of the superficial pyramidal cell layer (e.g. lower membrane potential, spike fractionation and strong accommodation of spikes during spike trains) were discarded due to their inability to generate an sADP or $I_{ADP}$ (Constanti et al., 1993). Cells which did not display spike fractionation but did display an sADP were studied as a single group, regardless of whether they were type I (deep pyramidal) or type II (non-pyramidal) neurones (Libri et al., 1994).

4.3.2. mAChR or mGluR activation in olfactory neurones.

Application of OXO-M or 1S,3R-ACPD to responding neurones within the olfactory cortical slices resulted in characteristic effects as previously reported (see also chapter 3); i.e. a slow membrane depolarization, thought to be largely a result of suppression of a variety of background membrane conductances (e.g. Adams et al., 1982; Constanti & Sim, 1987; McCormick & Prince, 1986; for further refs., see Introduction) or activation of nonspecific cation conductances (Haj-Dahmane & Andrade, 1996) leading to the depolarization, and the induction of a post-stimulus sADP with self-perpetuating repetitive firing (Constanti et al., 1993). This was in conjunction with a reduction in evoked EPSP amplitude expected upon administration of OXO-M or
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1S,3R-ACPD (e.g. Williams & Constanti, 1988; Libri et al., 1997), indicating that both the pre- and postsynaptic muscarinic/metabotropic receptor systems were functioning normally in this system during recordings.

4.3.3. The sADP and $I_{\text{ADP}}$ in responding neurones.

The sADP and its underlying tail current $I_{\text{ADP}}$ could be readily revealed by application of mAChR or mGluR agonists in all neurones from layer III of the olfactory cortex. The sADPs and $I_{\text{ADP}}$s had similar amplitudes and time courses to those recorded previously in the olfactory cortex (Constanti & Bagetta, 1991; Constanti et al., 1993; Libri et al., 1997). The appearance of the sADP was unlikely to be merely a result of a muscarinic block of the sAHP, as it has been previously reported that the sAHP can be blocked by application of catecholamines, without the induction of any afterdepolarizing response (Constanti & Sim, 1987). The mechanism of induction of the sADP was originally proposed as being a calcium-dependent inactivation of a novel ongoing potassium conductance, with a subsequent reactivation of this current over a relatively long time course (several seconds), to give the sADP its long-lasting deflection (Constanti & Bagetta, 1991; Constanti et al., 1993). The important questions for this series of experiments were: the nature of the calcium channels involved in calcium entry during the generation of the afterdepolarizing potential, and the possible involvement of intracellular calcium movements in this process. To this end, a series of compounds were tested, which traditionally interfere with neuronal calcium entry, or augment/inhibit release of calcium from intracellular stores.
4.3.4. Caffeine inhibition of the sADP and $I_{ADP}$.

Caffeine is now widely acknowledged as an agent which can augment the release of calcium from intracellular stores (for refs. see introduction, section 1.6.1), having a profound effect on the activity of neurones in which this process is essential for normal function. Early experiments on intracellular calcium release used a relatively high dose of caffeine (10 mM; Marrion & Adams, 1992; Nohmi et al., 1992); however, in several experiments where this concentration was used on the olfactory neurones, it was found to be very toxic, consistently causing rapid cell death. On this basis, the concentration of caffeine used was lowered, although at the lower concentrations it became less likely that caffeine was directly interfering with intracellular calcium release. 3 mM caffeine was consistently able to inhibit $I_{ADP}$ (and the concomitant sADP) induced by either OXO-M or 1S,3R-ACPD; thus caffeine was not acting directly on either receptor system to cause the inhibition, an observation which was further confirmed by the apparent lack of inhibition of the muscarinic or metabotropic depolarizations by caffeine during these recordings (note that caffeine has been previously found to directly antagonise the effects of carbachol and the binding of $^3$H-N-methylscopolamine in rat pancreatic acini; Grosfils et al., 1996). Also, the inhibition of the OXO-M induced $I_{ADP}$ was concentration-dependent over the range of caffeine doses used (0.5 mM - 3 mM). The inhibition of $I_{ADP}$ by caffeine was mimicked by theophylline, another member of the methylxanthine group. This inhibition was similar to that of caffeine, and was dose-dependent over a similar concentration range (i.e. 0.5 - 3 mM); thus the block of $I_{ADP}$ by caffeine was not specific to this methylxanthine. Considering that both caffeine and theophylline act to augment release of calcium from intracellular stores (e.g. Smith et al., 1983; Endo, 1977; Kuba, 1994), and taking into
account the proposed mechanism of induction of $I_{\text{ADP}}$ in the olfactory cortex (i.e. raised intracellular calcium leading to a decrease in a novel potassium conductance; Constanti et al., 1993), it seems very unlikely that either caffeine or theophylline was further raising the intracellular calcium level during a long depolarizing stimulus, as augmenting release of calcium from intracellular stores would be expected to increase the amplitude of $I_{\text{ADP}}$, as seen in the case of the depolarizing afterpotentials recorded in supraoptic nucleus neurones (Li & Hatton, 1997). Caffeine must be therefore be acting on other systems to cause inhibition of $I_{\text{ADP}}$. If the olfactory cortical $I_{\text{ADP}}$ had some other underlying mechanism such as the proposed increase in nonspecific cationic conductance seen in the neocortex (Haj-Dahmane & Andrade, 1998), then one would still expect an increase in $I_{\text{ADP}}$ amplitude in caffeine, as this other afterdepolarization was also dependent on intracellular calcium concentration.

4.3.5. Intracellular calcium stores - a role in $I_{\text{ADP}}$ generation?

The importance of intracellular calcium concentration in the generation of the sADP and $I_{\text{ADP}}$ allows speculation about the possibility of a functional role of calcium stores in this process. It was impossible to study the involvement of the IP$_3$-sensitive calcium stores during this study, as there are currently no membrane permeant agents available which affect these stores. Other authors have used agents (the most common being heparin) in recording pipettes, which are allowed to enter the cell and inhibit release from the IP$_3$-sensitive stores (e.g. Abdul-Ghani et al., 1996; Li & Hatton, 1996); however, it was impractical to perform recordings in the olfactory neurones with such agents in the pipette solution for intracellular perfusion because of the inconsistency of the sADP response: i.e. it was impossible to guarantee that recordings were being taken
from responding neurones in the first instance, and it was also impossible to gain control readings of the sADP response in the presence of OXO-M, due to the continual presence of heparin within the intracellular environment.

The possible involvement of release of calcium from intracellular stores was studied using a range of compounds routinely used to interfere with this process in other systems. Thus, application of the calcium release channel blocker ryanodine caused a small, yet significant, decrease in amplitude of $I_{ADP}$, which implies that there are calcium stores present within the olfactory neurones, possibly contributing towards generation of the slow afterpotential responses. Nevertheless, it seems that these putative stores have only a very small functional role in this type of cellular activity, unlike the generation of DAPs in the supraoptic nucleus (Li & Hatton, 1997) or the AHP in hippocampal neurones (Torres et al., 1996). Furthermore, there was no significant reduction of $I_{ADP}$ amplitude in the presence of the CICR blocker dantrolene, or the intracellular calcium store depletors thapsigargin or cyclopiazonic acid, again indicating that there is no great involvement of these stores in cortical $I_{ADP}$ generation, in contrast to supraoptic nucleus (Li & Hatton, 1997) or hippocampal (Torres et al., 1996) neurones where these agents all reduced afterpotential amplitudes.

A further possibility is that ryanodine somehow exerts a direct blockade of calcium currents in the plasma membrane, preventing entry of calcium, and thus reducing the $I_{ADP}$ current. However, there is currently no evidence that ryanodine can directly block any high or low voltage-activated calcium channels in neuronal membranes (e.g. Kawai & Watanabe, 1989). There is, however, a report suggesting that ryanodine may interfere with potassium ion fluxes in the plasma membrane. Vais et al. (1996) found that several ryanoid compounds altered the ion selectivity of plasma
membrane-bound potassium channels in the locust skeletal muscle, which altered the reversal potential of these channels from close to \( E_K \), more towards that of \( E_{Na} \). The authors also noted that the compounds reduced the reversal potential of a high conductance \( \text{Ca}^{2+} \)-activated \( K^+ \) channel in mouse muscle. Considering once more the proposed mechanism of induction of \( I_{ADP} \) in the olfactory cortex, it is possible that ryanodine was exerting a small block of the putative potassium conductance underlying the sADP, leading to a smaller background \( 'I_{ADP}' \) conductance to be inactivated by intracellular calcium, thus leading to a smaller \( I_{ADP} \) tail current being recorded. If this was the case, then one would expect to see a small depolarization during superfusion of ryanodine onto the preparation; however, since the observed reduction of \( I_{ADP} \) was very small (~10%), any effects of altering conductance through this type of channel would probably be lost among other background currents present such as leak conductances, making such a depolarization difficult to detect.

4.3.6. Other pharmacological actions of caffeine.

Caffeine is a rather nonspecific drug, in that it has a number of pharmacological actions in addition to augmenting release of calcium from the ryanodine-sensitive intracellular stores. Firstly, caffeine behaves as an inhibitor of the enzyme phosphodiesterase, the enzyme responsible for the breakdown of intracellular cyclic adenosine monophosphate (cAMP), thus leading to a buildup of cAMP concentration within treated cells (for reviews see Nehlig et al., 1992; Sawynok & Yaksh, 1993). In order to test whether this action of caffeine was responsible for the inhibition of \( I_{ADP} \), a more selective inhibitor of phosphodiesterase, IBMX, was added to the bathing solution in the presence of OXO-M, this compound having little or no effect on intracellular
calcium release at the concentrations used (normally observed at millimolar concentrations of IBMX; Usachev & Verkhratsky, 1995). In the olfactory cortical neurones, co-application of IBMX failed to significantly reduce the amplitude of \( I_{\text{ADP}} \), even after 20-30 minutes in the presence of this compound. This would suggest that inhibition of phosphodiesterase (and buildup of intracellular cAMP) is not playing a role in \( I_{\text{ADP}} \) inhibition.

It is interesting to note that IBMX can also act as an adenosine receptor antagonist at similar concentrations to phosphodiesterase inhibition, and certainly at the concentration used in this study (Schwabe et al., 1985; Prestwich et al., 1987). Considering that caffeine also acts as a potent antagonist of adenosine receptors (Greene et al., 1985; Haas & Greene 1988) it seems rather unlikely that pharmacological inhibition of endogenous adenosine activity can inhibit \( I_{\text{ADP}} \). Further to this, in experiments where high concentrations of adenosine were used to try and reverse any possible antagonism of adenosine receptors imposed by the presence of caffeine, there was no effect on the \( I_{\text{ADP}} \) depression.

In the presence of OXO-M, EPSP amplitudes were consistently reduced while \( I_{\text{ADP}} \) was evident. In the presence of caffeine, EPSP amplitudes were increased (most likely as a result of inhibiting endogenous adenosine activity on presynaptic adenosine receptors; c.f. Haas & Greene, 1988), while \( I_{\text{ADP}} \) amplitude was reduced. Co-application of adenosine with OXO-M and caffeine resulted in a reduction of EPSP amplitude in the olfactory cortical neurones, yet there was no evidence of any reversal of the inhibition of \( I_{\text{ADP}} \), so it is very unlikely that inhibition of endogenous adenosine activity by caffeine contributes towards block of \( I_{\text{ADP}} \).
4.3.7. Caffeine affects calcium entry into neurones during generation of $I_{ADP}$

The original experiments of Constanti et al. (1993) clearly showed that calcium entry into neurones was essential for the generation of $I_{ADP}$, insofar as removal of calcium from the external bathing medium abolished the afterdepolarizing response. There is evidence that caffeine directly interferes with inward calcium currents in muscle preparations (Zholos et al., 1991; Varro et al., 1993; Yoshino et al., 1996), and so it is conceivable that caffeine was blocking calcium entry through voltage-sensitive Ca$^{2+}$ channels, leading to an inhibition of $I_{ADP}$. Intracellular loading with caesium ions results in blockade of voltage-gated potassium conductances, resulting in an inability of a cell to easily return to resting potential after firing an action potential. The outcome of this is that the action potential (provided that Ca$^{2+}$ conductances are present) attains a lengthened plateau on the downward phase, resulting from increased activity of voltage-gated calcium channels (Galvan et al., 1985; Libri et al., 1996), rather like in the case of the cardiac action potential. In recordings made in cells loaded with caesium ions, caffeine was able to significantly reduce the half-width of the initial spike in an evoked spike train, indicating that the compound was indeed causing a blockade of voltage-gated calcium currents. For comparison, nifedipine (a potent blocker of L-type calcium channels) produced a similar degree of spike shortening as was seen in the presence of caffeine (40 % inhibition in nifedipine, and 45 % inhibition in caffeine).

Thus, it is likely that inhibition if $I_{ADP}$ by caffeine may partly result from a direct block of voltage-gated calcium conductances similar to that seen in the presence of nifedipine. However, it is worth noting that nifedipine was able to inhibit $I_{ADP}$ amplitude by about 42 % (similar to the reduction spike half-width in the caesium-loading experiments), whereas caffeine was able to reduce $I_{ADP}$ amplitude by around 75
% while only reducing the spike half-widths by 45 %. A further blocking mechanism for caffeine was therefore suggested.

4.3.8. Mechanism of block of $I_{\text{ADP}}$ by caffeine.

If the inhibition of $I_{\text{ADP}}$ by caffeine is not a result of its effect on intracellular calcium mobilisation, inhibition of phosphodiesterase, blockade of endogenous adenosine action on adenosine receptors, or solely due to direct inhibition of voltage-gated calcium channel activity, then what mechanism can be responsible for this action? A further reported effect of caffeine is an interference with the benzodiazepine binding site on the GABA$_A$ receptor (Boulenger et al., 1982; Sawynok & Yaksh, 1993), preventing binding of benzodiazepines, and also possibly interfering with GABA$_A$ receptor-chloride channel coupling (Lopez et al., 1989). This effect, however, is unlikely to explain the inhibition of the $I_{\text{ADP}}$ in the present experiments unless a tonic modulatory effect of an ‘endogenous’ benzodiazepine receptor ligand on $I_{\text{ADP}}$ is proposed.

One further action of caffeine which could explain the residual inhibition of $I_{\text{ADP}}$ is a direct block of the $I_{\text{ADP}}$ potassium conductance. Caffeine is known to block certain types of potassium conductance (Greene et al., 1985), without interfering with intracellular signalling mechanisms (Yamamoto et al., 1995), e.g. the delayed rectifier ($I_{\text{DR}}$) or the transient outward current ($I_A$) in neurones (Reiser et al., 1996), or $I_A$ in smooth muscle cells (Noack et al., 1990). It should be noted that the authors of these studies claimed that these currents were independent of intracellular calcium concentration (which must always be a consideration when dealing with caffeine).
Interestingly, theophylline has also been shown to block a calcium-independent potassium conductance in dissociated cortical neurones (Munakata & Akaike, 1993). It is unclear, however, why in the study of Reiser et al. (1996), IBMX was shown to inhibit the same potassium currents as caffeine, independent of its action on phosphodiesterase. This is in contrast to the present findings, where IBMX had no significant effect on $I_{ADP}$ amplitude. This may be a result of a higher concentration of IBMX used in their study, or a result of IBMX having no blocking action on voltage gated calcium channels in our experiments.

In conclusion, it is clear from the experiments carried out in this series that the $I_{ADP}$ tail current, as originally thought, has a very complicated mechanism of induction. It seems that calcium entry takes place at least in part via L-type voltage-gated calcium channels, and it also appears that agents capable of manipulating intracellular calcium stores have little or no effect on $I_{ADP}$ generation. Similarly, inhibition of phosphodiesterase in the cell, or pharmacological manipulation of adenosine receptors in the slices does not affect $I_{ADP}$. It is likely that in the olfactory cortical neurones, the concentrations of caffeine used may have been simultaneously blocking voltage-gated calcium currents (reducing calcium entry into the neurones), and also causing a direct block of the putative potassium conductance underlying the $I_{ADP}$. Further studies are clearly needed to gain a full understanding of the mechanism(s) underlying this unusual phenomenon.
4.4. Summary.

1) Caffeine (0.5-3 mM) had no effect on intrinsic membrane properties of guinea-pig olfactory cortical neurones; however, evoked excitatory synaptic transmission was enhanced.

2) Caffeine or theophylline (0.5-3 mM) reversibly reduced the sADP and its underlying tail current $I_{ADP}$ induced by 10 μM OXO-M in these neurones, without affecting the depolarization observed during initial superfusion of this compound. 3 mM caffeine also reduced the sADP induced by 10 μM 1S,3R-ACPD.

3) Superfusion of 10 μM nifedipine onto the recorded neurones was also able to inhibit the sADP and $I_{ADP}$, suggesting an involvement of L-type calcium channels in generation of the tail current.

4) Superfusion of the calcium release inhibitor ryanodine (10 μM) produced only a small reduction in $I_{ADP}$ amplitude (~10 %); however, neither 10 μM dantrolene (another release inhibitor) or the calcium re-uptake inhibitors thapsigargin (3 μM) or 15 μM cyclopiazonic acid (CPA) had any significant effect on $I_{ADP}$ amplitude.

5) Inhibition of phosphodiesterase with IBMX (100 μM) had no effect on $I_{ADP}$ amplitude, and the inhibition of $I_{ADP}$ imposed by caffeine was not reversed by co-application of 100 μM adenosine, suggesting that a caffeine blockade of endogenous adenosine activity was not involved.
6) Caffeine (3 mM) was found to inhibit voltage gated calcium channel activity, in a similar fashion to nifedipine (10 μM), as revealed by intracellular loading with caesium ions.

7) In conclusion, it is suggested that the block of the $I_{\text{ADP}}$ by caffeine was due to a combination of voltage-gated calcium channel blockade, and a direct inhibitory action on the putative novel $I_{\text{ADP}}$ potassium conductance.
The original aim of the work presented in this thesis, as mentioned previously, was to investigate the ontogeny of the novel mAChR/mGluR-induced post-stimulus afterdepolarization and its underlying current $I_{ADP}$, and also to attempt to gain a further understanding of the possible involvement of intracellular calcium stores in the generation of this tail current. Throughout the course of these investigations, however, some surprising and unexpected results were seen, namely the epileptiform activity induced by the muscarinic agonist OXO-M in immature animal slices, and also the blockade of $I_{ADP}$ by caffeine, both of which required further careful study.

Investigation of the muscarinic bursting activity revealed that it was firstly very consistent, appearing in the majority of recorded immature neurones, and not appearing in adult neurones; this indicated a profound functional difference in development of aspects of the neuronal muscarinic receptor system in the rat, an area which is still relatively unexplored. The muscarinic bursting could certainly be considered as a useful model for cellular epileptic activity, as it responded to both traditional and novel agents which are widely known to suppress such activity in other in vitro models of epileptogenesis, and may allow further understanding of the events leading towards muscarinic epileptic phenomena, another area in need of further investigation. This model may even contribute towards an understanding of more clinical aspects of juvenile epilepsies, possibly allowing further lines of exploration towards finding ways of relieving these conditions.
The sADP and its underlying tail current $I_{ADP}$, elicited in the presence of OXO-M in olfactory neurones, remains an extremely complicated phenomenon. From the results of the present experiments, it is unlikely that calcium-induced calcium release plays any significant role in generation of this response. Caffeine and theophylline, however, clearly inhibited the tail current (and the accompanying membrane depolarization), which is a rather anomalous methylxanthine action, considering that these compounds generally tend to promote neuronal excitation. This study also provides further evidence that the current underlying the sADP is a potassium current, and moreover indicates that calcium entry through L-type voltage-gated calcium channels is necessary for its appearance; however there are still questions about this conductance which need answering, for example there is recent evidence that the cortical $I_{ADP}$ may be dependent on the concentration of sodium in the extracellular bathing medium. This may be interpreted in two ways: either that the sADP is not the result of a decrease in potassium conductance at all, and that $I_{ADP}$ is indeed a non-specific cation conductance, or that the $K^+$ channel underlying the $I_{ADP}$ conductance somehow requires the binding of external sodium to allow opening. Clearly there is room for continued investigation into the sADP phenomenon and its underlying mechanism of induction.

The olfactory cortex thus remains an area of the brain which can be useful and interesting for the investigation of neuronal activity, and pathological conditions such as epilepsy. It also provides a model for the ongoing processes of learning and memory, and developmental aspects of function of certain receptor systems. The in vitro brain slice preparation continues to be a very useful device for studying these activities.


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Full Papers


Abstracts


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