THE ROLE OF P2 RECEPTORS IN FAST SYNAPTIC TRANSMISSION IN THE RAT MEDIAL HABENULA NUCLEUS

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A thesis submitted for the degree of Doctor of Philosophy
at the University of London

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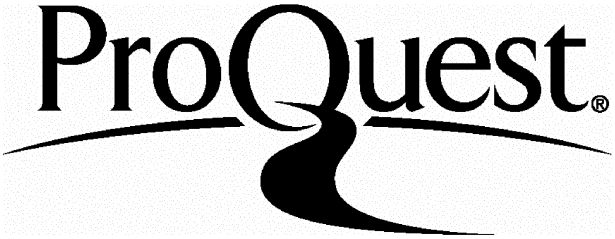
March 2002



Abstract

Fast excitatory glutamatergic and purinergic synaptic transmission was studied in acute slices of the rat medial habenula nucleus using whole-cell patch clamp recordings.

The subunit composition of P2X receptors mediating fast purinergic transmission was investigated using the selective $P2X_1$ and $P2X_2$ antagonist NF279. This inhibited currents in a dose-dependent manner but the block was less than 100%. An additional facilitatory effect was evident at low concentrations.

Glutamate transmission was modulated by the two P2Y receptor agonists UTP and UDP. Both low concentrations of UTP (10 and 30μM) and high concentrations of UDP (200μM) potentiated evoked glutamate currents. Analysis of failures revealed a presynaptic locus for both of these effects. Potentiation was long-lasting and inhibited by the P2 receptor antagonist RB2. Increasing the concentration of UTP inhibited evoked glutamate currents. This was also a presynaptic effect, as indicated by analysis of failures. Inhibition was reversible and not sensitive to RB2.

α,β-methyleneATP, a desensitizing agonist at the $P2X_1$ and $P2X_3$ receptors and an antagonist at the $P2X_4$ receptor, reversibly inhibited glutamate transmission in 50% of cells. Analysis of failures indicated a presynaptic effect. The non-selective P2 antagonist PPADS reversibly reduced the frequency of spontaneous miniature glutamatergic currents.

The data indicates that glutamate afferents in the medial habenula posses at least two distinct presynaptic P2 receptors which facilitate release. A P2Y receptor mediates a novel form of long-term potentiation. A second P2 receptor (probably P2X) mediates brief facilitation.

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Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
UTP	Uridine triphosphate
UDP	Uridine diphosphate
2MeSATP	2-methylthio-Adenosine triphosphate
2MeSADP	2-methylthio-Adenosine diphosphate
PLC	Phospholipase C
PKC	Protein kinase C
AC	Adenylyl cyclase
DRG	Dorsal root ganglion
RB2	Reactive Blue 2
PPADS	Pyridoxalphosphate-6-azophenol-2',4'-disulphonic acid
NF279	8,8'-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis(1,3,5-naphthalenetrisulphonic acid)
SCG	Superior cervical ganglion
VDCC	Voltage-dependent Ca^{++} channel
TS	Triangular Septal nucleus
SFi	Septofimbrial nucleus
ADA	Adenosine deaminase
NMDA	N-methyl-D-aspartate
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
EPSC	Excitatory post-synaptic current

Acknowledgements

I am grateful to the Medical Research Council who provided the studentship which allowed me to pursue this work.

I would like to thank the following people: my supervisor, Frances Edwards, for her support, encouragement and enthusiasm throughout the last three years; Susan Robertson, for much help during my early days in the lab and continued help since; my second supervisor, Jonathan Ashmore, who was always available for advice; Isabel Dean, for many hours of helpful discussion on numerous topics; Steve McGuiness, Michiko Takahashi, Anna di Simoni, Richard Hodkinson and Stephanie Parsley who have all helped me out at various times; and a special thank you to my parents, my grandfather and my brother for their support.

Chapter 1

Introduction

This thesis is concerned with the role of ATP in fast synaptic transmission in the central nervous system (CNS) and, in particular, with its role in modulating synaptic glutamate release in the medial habenula nucleus. This section introduces ATP receptors and their distribution and possible functions in the CNS; the medial habenula and its anatomy and physiology; and LTP, an important form of synaptic plasticity in which extracellular nucleotides have been implicated and are further implicated by the results which are presented here.

1.1 Nucleotide Receptors

To account for the observed effects of extracellular ATP and other adenine nucleotides and nucleosides on a variety of cell types, it was suggested that there are specific cell surface receptors for these ligands, so-called purinoceptors. In 1978, Burnstock (Burnstock, 1978) proposed a subdivision of purinoceptors into P1 and P2: P1 were receptors for adenosine while P2 were ATP receptors. Adenosine receptors are now classified as A1 and A2 receptors with further subdivisions of these classes.

In 1985, Burnstock and Kennedy (Burnstock & Kennedy, 1985) proposed a further subdivision of P2 receptors into P2X and P2Y, based on agonist profiles, desensitization, tissue distribution and physiological effects. Studies on signal transduction mechanisms further supported a subdivision of P2 receptors. In some cells the application of ATP led to the accumulation of intracellular IP_3 and the release of Ca^{++} from intracellular stores in a manner similar to G-protein-coupled receptors (Dubyak, 1991); in other preparations the response to ATP was indicative of the receptor being a ligand-gated ion channel (Benham & Tsien, 1987).

A few studies have produced evidence for a third class of purinoreceptor, responsive to ATP (ie P2-like) but inhibited by methylxanthines (ie P1-like) (Shinozuka *et al.*, 1988;Todorov *et al.*, 1994): this has been called the P3 receptor.

The rapid development of molecular biological techniques during the 1990s led to the cloning of a number of P2 receptors of both the P2X and P2Y subtypes. This, in turn, facilitated the study of their molecular structure, pharmacology and transduction mechanisms. Those receptors now classified as P2X are all ligand-gated cation channels while the P2Y receptors are all G-protein coupled.

1.2 Problems with studying P2 Receptors

Studies on the effects of ATP in different systems have been hampered by a variety of complicating factors such as the current lack of selective pharmacological agents, and the conversion of ATP to biologically active metabolites e.g. ADP and adenosine. Some of these problems have been partly circumvented by cloning receptors and expressing them in appropriate cell types. For example, expressing a particular P2 receptor in a cell which does not express native receptors for ATP or adenosine allows that particular receptor to be studied in isolation from effects possibly mediated by other receptors. However, problems remain. For example, some of the initial experiments on cloned P2Y receptors ascribed agonist activity to nucleotides which later studies showed to be spurious and actually due to a metabolite. Furthermore, the metabolites potentially arise from two sources: interconversion by ectonucleotidases in the preparation or contamination of the original compound. Estimates of the contamination of nucleotide triphosphates with nucleotide diphosphates range from 5% to 40% (Connolly & Duley, 2000); even accepting the lower figure, a solution of 1 μM UTP could contain 50nM UDP, an amount which may significantly activate the sensitive $P2Y_6$ receptor (Nicholas *et al.*, 1997;Filippov *et al.*, 1999). Consequently studies on the agonist profiles of

these receptors now make use of purified compounds and experimental conditions are designed to minimise the build up of metabolites.

A second factor relevant to pharmacological studies is the level of receptor expression in the host cells. An agent acting as a weak agonist when there is a high receptor reserve may be ineffective or even act as an antagonist when there are fewer receptors present. Such is the case for ATP at the $P2Y_1$ receptor (Palmer *et al.*, 1998) and may explain some of the other discrepancies in the literature. Another factor which may lead to apparent discrepancies between studies is the choice of assay used to measure biological activity.

1.3 P2Y Receptors

So far six P2Y receptors have been cloned from mammalian tissue ($P2Y_{1,2,4,6,11,12}$) and three from non-mammalian tissue ($P2Y_3$ from chick and turkey, p2y8 from xenopus and tp2y from turkey). The non-mammalian receptors may all be species homologues of mammalian receptors: the avian $P2Y_3$ receptor is most similar in agonist profile and sequence homology to the $P2Y_6$ receptor (Li *et al.*, 1998); the tp2y (Boyer *et al.*, 2000) and p2y8 (Bogdanov *et al.*, 1997) receptors resemble the rat $P2Y_4$ receptor in their agonist selectivities.

The mysteriously missing numbers in the sequence of P2Y receptors were originally assigned to receptors thought to belong to the family but which were later shown to be erroneous classifications.

1.3.1 P2Y Receptor Structure

The P2Y receptors so far cloned comprise between 328 and 538 amino acids (Von Kügelgen & Wetter, 2000). Analysis of the predicted amino acid sequence revealed that these receptors possess the seven transmembrane spanning domains characteristic of G-protein coupled receptors, an extracellular N-terminus and an intracellular C-terminus containing several potential phosphorylation sites (Webb et al., 1993; Lustig et al., 1993) (fig 1, B). Site-directed mutagenesis implicated several

amino acid residues within the exofacial regions of transmembrane spanning domains three, five, six and seven in the binding of agonists (Jiang et al., 1997; Erb et al., 1995). A comparison of cloned P2Y receptors also revealed highly conserved amino acid residues on transmembrane spanning domains three, six and seven close to the intracellular loops and these may be involved in transduction of the signal to the G-protein (Von Kügelgen & Wetter, 2000).

1.3.2 $P2Y_1$

The first P2 receptor was cloned by Webb et al (Webb *et al.*, 1993) in 1993 from the chick embryo and expressed in xenopus oocytes. Subsequently a $P2Y_1$ receptor has been cloned from several other species (Leon *et al.*, 1996;Henderson *et al.*, 1995;Tokuyama *et al.*, 1995;Filtz *et al.*, 1994). The receptor is selective for adenine nucleotides (uridine nucleotides are inactive) and may be a specific diphosphate nucleotide receptor (Leon *et al.*, 1997); the 2-methylthio derivatives of adenine nucleotides are generally more potent than their non-substituted analogues.

1.3.3 $P2Y_2$

Shortly after the $P2Y_1$ receptor was cloned, Lustig et al (Lustig *et al.*, 1993) cloned a second P2Y receptor from the mouse with a very different pharmacological profile; rat and human homologues have since been cloned (Chen *et al.*, 1996; Parr *et al.*, 1994). This receptor is approximately equisensitive to ATP and UTP with 2MeSATP (the most potent $P2Y_1$ agonist) being much less potent. Being responsive to UTP this receptor was initially equated with the so-called P2U receptor, a UTP-sensitive receptor identified in a number of in situ studies. It later became apparent that there is more than one UTP-sensitive P2Y receptor.

1.3.4 $P2Y_4$

In 1995 two groups cloned a third P2Y receptor subtype from human cDNA libraries (Communi *et al.*, 1995;Nguyen *et al.*, 1995b); the rat

orthologue has also been cloned (Bogdanov *et al.*, 1998). Initial studies reported conflicting effects of ATP at the human receptor; it now appears that ATP is an antagonist (Thier *et al.*, 2000). There is also a clear species difference: the rat receptor is equisensitive to UTP and ATP while the human receptor is UTP selective.

1.3.5 $P2Y_6$

In 1995 a P2Y receptor was cloned from rat aortic smooth muscle with an agonist profile distinct from that of the other known P2Y receptors (Chang *et al.*, 1995): it is now classified as $P2Y_6$. Initially this receptor was thought to be most sensitive to UTP with ADP and ATP being approximately 50 and 500 times less potent but it is now recognized that UDP is the most potent agonist (Nicholas *et al.*, 1997).

1.3.6 $P2Y_{11}$

This receptor was cloned form a human genomic DNA library (Communi *et al.*, 1999). It is an adenine nucleotide specific receptor: ATP is the most potent agonist followed by 2MeSATP; ADP, 2MeSADP, adenosine, UTP and UDP are ineffective. At present there is no known rat homologue and the receptor is unique amongst the P2Y receptors so far cloned in that it couples to both PLC to produce IP_3 and AC to stimulate cAMP formation.

1.3.7 $P2Y_{12}$

This P2Y receptor was very recently cloned from rat and human platelets (Hollopeter *et al.*, 2001) and is thought to be the ADP receptor required for platelet aggregation (variously called $P2Y_{ADP}$, $P2Y_{AC}$, $P2Y_{CYC}$ and $P2T_{AC}$) (Gachet *et al.*, 1992; Gachet *et al.*, 1990); similarly to $P2Y_1$, 2MeSADP is an even more potent agonist. The receptor inhibits adenylyl cyclase to reduce intracellular cAMP levels; whether or not the cloned receptor stimulates PLC does not seem to have been tested.

1.3.8 Signal Transduction Mechanisms

1.3.8.1 Phopholipase C and Adenylyl Cyclase

All the P2Y receptors couple to phospholipase C (PLC) with the subsequent formation of IP_3 and Ca^{++} release from intracellar stores (Ralevic & Burnstock, 1998). Three of the P2Y receptors also couple to adenylyl cyclase either to stimulate or inhibit it. As mentioned above, the $P2Y_{11}$ receptor is unique in stimulating both PLC and adenylyl cyclase (Communi *et al.*, 1999). B10 cells, a clonal line of rat brain endothelial cells, express a single P2Y receptor, identified as $P2Y_1$, which inhibits adenylyl cyclase in a PTX-sensitive manner (Webb *et al.*, 1996). Adenylyl cyclase is also inhibited by the $P2Y_{12}$ receptor. Of the other receptors, the human $P2Y_2$ (Nguyen *et al.*, 1995a) and the rat $P2Y_6$ receptors (Chang *et al.*, 1995) do not couple to adenylyl cyclase, although in the former study there was no positive control. Other studies on the cloned P2Y receptors have not assayed cAMP accumulation or inhibition.

1.3.8.2 Inhibition of N-type Ca^{++} channels and M-type K^+ currents

In a series of studies, Filippov and colleagues expressed three rat P2Y receptors in superior cervical ganglion neurons and investigated their signalling mechanisms (Filippov *et al.*, 1999; Filippov *et al.*, 1998; Filippov *et al.*, 2000). The $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors all inhibit N-type Ca^{++} channels in a voltage-dependent manner. This inhibition is partially sensitive to pertussis toxin indicating the possible involvement of more than one family of G proteins, one of which may be the PTX-sensitive Go. They also show that $P2Y_2$ and $P2Y_6$ receptors inhibit M-type K^+ currents through a PTX-insensitive G protein; this is an interesting finding as it is unusual for a single receptor to couple to two different ion channels through two different G proteins. The physiological significance of these signalling pathways in neurons would most likely be to increase neuronal excitability. However, an inhibition of N-type Ca^{++} channels at the presynaptic terminals would probably lead to the inhibition of transmitter release.

1.4 P2X Receptors

From studies in native tissue, it was clear that there was more than one type of ionotropic P2X receptor but the lack of selective pharmacological agents meant that receptors were classified into broad groups such as α,β-methyleneATP-sensitive and insensitive receptors. Molecular biology has advanced the search for different subtypes and the expression of cloned receptors in appropriate cells has aided in the study of their kinetics, pharmacology and structure.

P2X receptors are ligand-gated, Ca^{++}-permeable cation channels (Brake *et al.*, 1994; Evans *et al.*, 1995; Chen *et al.*, 1995). Seven P2X receptor subunits have so far been cloned (see below), each one between 379 and 595 amino acid residues long (Khakh et al., 2001); the identity between subunits ranges from 26-47% (Khakh et al., 2001). From the amino acid sequence, a subunit is predicted to have two transmembrane spanning domains and a large extracellular loop (Brake et al., 1994; Valera et al., 1994) and the overall structure of the receptor resembles that of the amiloride-sensitive epithelial Na^{+} channel (fig 1, A). The ATP-binding site is thought to be on the extracellular loop, but close to the pore of the channel (Ennion et al., 2000). Various other sites with specific roles have been identified, including glycosylation sites on the extracellular loop, an intraterminal PKC consensus site and several sites involved in determining the time course of the response along the length of the protein involved in determining the time course of the response (for review see Khakh, 2001).

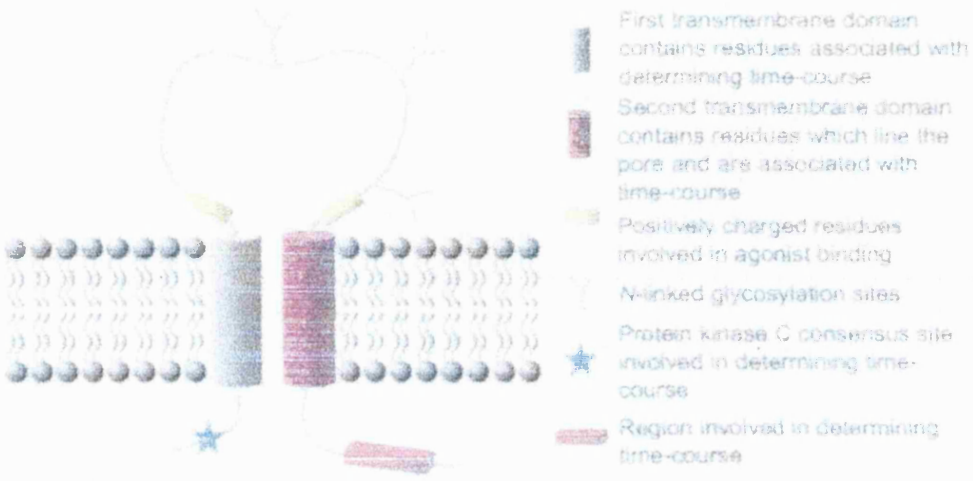
Functional monomeric receptors can form but the fact that their Hill co-efficient is greater than one (Evans et al., 1995) suggests that a receptor comprises more than one subunit. Additional evidence that the P2X receptors comprise more than one subunit comes from studies showing that functional heteromeric receptors can form (Lewis et al., 1995; King et al., 2000; Torres et al., 1998; Le et al., 1998). Several

Fig 1 Structure of P2X and P2Y receptors

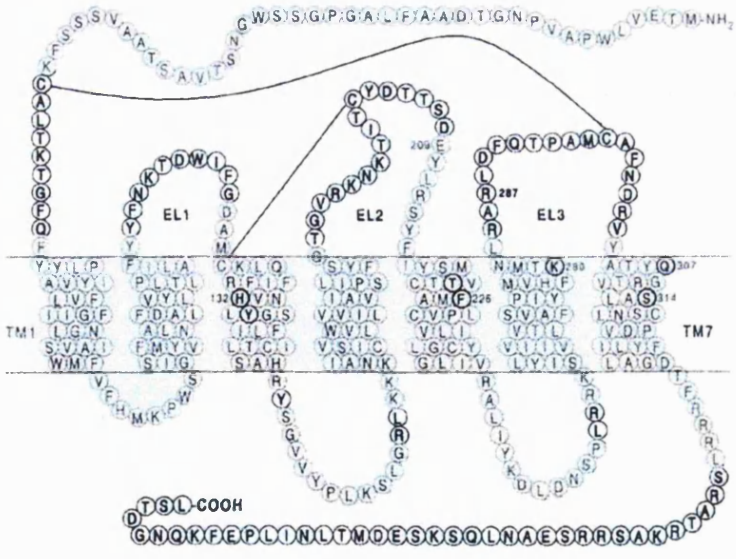
A. Cartoon showing the structure of the P2X receptor. Adapted from Robertson et al., 2001. See references therein.

B. Predicted secondary structure of the human $P2Y_1$ receptor. The highlighted amino acids in transmembrane domains three, five, six and seven are believed to part of the nucleotide binding site. The solid lines represent two predicted disulphide bridges. From Von Kugelgen and Wetter, 2000. See references therein.

A


First transmembrane domain contains residues associated with determining time-course
Second transmembrane domain contains residues which line the pore and are associated with time-course
Positively charged residues involved in agonist binding
N-linked glycosylation sites
Protein kinase C consensus site involved in determining time-course
Region involved in determining time-course

B


EL1
EL2
EL3
TM1
TM7
COOH

different approaches have been taken to ascertain how many subunits combine to form a functional receptor. When Nicke et al (Nicke et al., 1998) subjected $P2X_1$ or $P2X_3$ receptors to polyacrylaminde gel electrophoresis under non-denaturing conditions, the receptors ran as trimers; in the same study, bi-functional derivatives of PPADS cross-linked receptors as trimers. Using a different approach, based on the block by methanethiosulphonate of a $P2X_2$ receptor carrying a cysteine mutation, Stoop and colleagues (Stoop et al., 1999) also concluded that three subunits are likely to form a receptor.

The functional recombinant heteromeric receptors which have been described are combinations of two different subunits, although the ratio in which they combine to form heteromers is not known. Torres and colleagues (Torres et al., 1998) have shown that numerous pairs of subunits can co-immunoprecipitate. Given that three subunits probably form a receptor, heteromers comprising three different subunits are presumably possible, making the potential number of functional heteromers very large. The recombinant heteromers which have so far been studied are combinations of subunits with distinct pharmacological profiles or kinetic properties. It is also possible that homomers with similar properties combine: in such cases the resulting heteromer might have properties which are only subtly different from the parent homomers.

The seven P2X receptor subunits which have been cloned to date can be divided into groups based on their pharmacological profiles and kinetic properties.

1.4.1 $P2X_1$ and $P2X_3$

The $P2X_1$ receptor was originally cloned from the rat vas deferens (Valera *et al.*, 1994) and has since been cloned from human and mouse urinary bladder (Valera et al., 1995); the $P2X_3$ receptor was cloned from the rat DRG (Chen *et al.*, 1995;Lewis *et al.*, 1995). The receptors are characterized by their rapid desensitization (hundreds of milliseconds for

$P2X_1$ and less than 100ms for $P2X_3$) and their sensitivity to α,β-methyleneATP. The greater potency of α,β-methyleneATP at receptors I n mature tissue is thought to result from its relative resistance to hydrolysis by ectonucleotidases compared to ATP.

1.4.2 $P2X_2$ and $P2X_5$

The $P2X_2$ receptor was first cloned from rat pheochromocytoma PC12 cells (Brake *et al.*, 1994); it is insensitive to α,β-methyleneATP and the agonists ATP and 2MeSATP are much less potent than at the $P2X_1$ and $P2X_3$ receptors. The $P2X_5$ receptor was cloned from rat coeliac ganglion (Collo *et al.*, 1996). It has a similar pharmacology to $P2X_2$ in terms of its agonist profile, including its insensitivity to α,β-methyleneATP. Both receptors show little or no desensitization (Collo *et al.*, 1996; Brake *et al.*, 1994).

1.4.3 $P2X_4$ and $P2X_6$

The $P2X_4$ and $P2X_6$ receptors were both originally cloned from rat SCG (Buell *et al.*, 1996; Collo *et al.*, 1996); ATP is the most potent agonist at both receptors, which are α,β-methyleneATP insensitive; at the rat $P2X_4$ receptor, α,β-methyleneATP is reported to be an antagonist (Jones *et al.*, 2000). Currents show very little desensitization. Several studies have found that cloned $P2X_6$ receptors are difficult to express functionally (Collo *et al.*, 1996; Lê *et al.*, 1998a), suggesting that they might be expressed predominantly as heteromers with another subunit (see below).

1.4.4 $P2X_7$

This receptor was cloned from rat macrophages (Surprenant *et al.*, 1996) and is now recognized as the receptor previously known as P2Z, a cytotoxic receptor described in several different cells of the immune system (Chow *et al.*, 1997). Under physiological conditions the receptor is permeable to small cations; however, the receptor has the unusual property that in the prolonged presence of ATP and low divalent cation levels the channel becomes a pore permeable to small molecules, an effect which is

associated with cytotoxicity (Rassendren *et al.*, 1997;Surprenant *et al.*, 1996). The endogenous agonist is probably ATP^{4-} (ATP not chelated to Ca^{++} or Mg^{++}) which constitutes only a small proportion of extracellular ATP with normal extracellular divalent cation concentrations. This probably explains the lower sensitivity of the receptor to ATP (EC_{50} ~ 100μM) than the other P2X subtypes. The most potent agonist is 3'-O-(4-benzoyl)benzoyl ATP (BzATP).

1.4.5 Heteromeric P2X receptors

In addition to the seven homomeric receptors, co-expression of subunits has so far revealed four heteromeric combinations with distinct characteristics: $P2X_{2/3}$ (Lewis *et al.*, 1995), $P2X_{1/5}$ (Torres *et al.*, 1998), $P2X_{2/6}$ (King *et al.*, 2000) and $P2X_{4/6}$ (Le *et al.*, 1998); $P2X_4$ and $P2X_6$ receptors have the widest CNS distribution which largely overlaps (Collo *et al.*, 1996; Lê *et al.*, 1998b), raising the possibility that they predominantly form heteromers in situ. As discussed above, it is likely that there are numerous more such heteromers to be described.

Table 1 summarizes some of the pharmacology of mammalian P2X and P2Y receptors as well as the signal transduction mechanisms associated with P2Y receptors.

1.5 P2 Receptors in the CNS

1.5.1 Detection of P2Y receptors in the CNS

The detection and localization of P2Y receptor in the central nervous system lags behind that of P2X receptors, partly due to a current lack of antibodies for all but the $P2Y_1$ receptor. The reverse transcription polymerase chain reaction (RT-PCR) and Northern blotting have been used to screen for P2Y receptor mRNA, often in homogenized brain tissue which reveals nothing of the cell types involved. Nonetheless, using these techinques the mRNA for both rat (Tokuyama *et al.*, 1995) and human (Leon *et al.*, 1996) $P2Y_1$ receptors has been detected in the brain whilst

neither the rat (Rice *et al.*, 1995) nor the human (Parr *et al.*, 1994) $P2Y_2$ receptor mRNA was detected in the whole brain, despite the fact that this receptor was first cloned from a mouse neuroblastoma / rat glioma cell line (Lustig *et al.*, 1993); it has been detected in astrocytes using single cell RT-PCR (Zhu & Kimelberg, 2001). $P2Y_4$ receptor mRNA was detected by RT-PCR in the whole brain and also in cultured cortical astrocytes (Webb *et al.*, 1998); note that the same group did not detect the mRNA in whole brain using Northern blotting, a reminder that the failure to detect a signal can be due to the sensitivity of the assay rather than a lack of the signal. Rat $P2Y_6$ mRNA was not detected in the brain by Northern Blot (Chang *et al.*, 1995). The $P2Y_{12}$ receptor was detected in the brain by in situ hybridization and appeared to be located in glia but not neurons (Hollopeter *et al.*, 2001).

While the detection of mRNA strongly suggests that a cell expresses the corresponding protein, to be certain requires visualising the protein with either a specific antibody or a selective ligand; in the case of P2Y receptors the antibodies are only just becoming commercially available. Using a polyclonal antibody recognizing the $P2Y_1$ receptor Moore and colleagues (Moore *et al.*, 2000a) visualised this receptor in a number of regions of the human brain where it appeared to be localized exclusively to neuronal cells, with a prominent expression in hippocampal and cortical pyramidal cells. The neuropil of many regions stained, leading them to propose that the receptor might also be located on axons and terminals; however, this awaits confirmation at the electron microscopic level - the light microscope only allowed them to confirm its location on neuronal somata and dendrites. In rat brain, there was prominent labelling of cerebellar Purkinje cells as well as cortical and hippocampal CA1 neurons (Moran-Jimenez & Matute, 2000); astrocytes and oligodendrocytes of the bovine corpus callosum were also labelled.

References from Table 1

1. Leon *et al.*, 1997
2. (Leon *et al.*, 1997;Webb *et al.*, 1996)
3. (Chen *et al.*, 1996)
4. (Charlton *et al.*, 1996)
5. (Bogdanov *et al.*, 1998)
6. (Chang *et al.*, 1995)
7. (Robaye *et al.*, 1997)
8. Communi *et al.*, 1999
9. (Communi *et al.*, 1999)
10. (Hollopeter *et al.*, 2001)
11. (Torres *et al.*, 1998)
12. (Evans *et al.*, 1995)
13. (Liu *et al.*, 2001)
14. (Buell *et al.*, 1996)
15. (Brake *et al.*, 1994)
16. (Liu *et al.*, 2001;Chen *et al.*, 1995)
17. (Garcia-Guzman *et al.*, 1997)
18. (Hollopeter *et al.*, 2001)
19. (Wang *et al.*, 1996)
20. (Buell *et al.*, 1996;Bo *et al.*, 1995)
21. (Wang *et al.*, 1996;Bo *et al.*, 1995)
22. (Torres *et al.*, 1998)
23. (Collo *et al.*, 1996)
24. (Khakh *et al.*, 2001;Ralevic & Burnstock, 1998)
25. (Surprenant *et al.*, 1996)
26. (Khakh *et al.*, 2001)
27. (Suarez-Herta *et al.*, 2001)

Table 1

	Agonists	**RB2**	**PPADS**	**Signal Transduction Mechanims**
$P2Y_1$	ADP[1]	Antagonist[2]	Antagonist[2]	Stimulates PLC Inhibits AC
$P2Y_2$	ATP = UTP[3]	[A]	No effect[4]	Stimulates PLC
$P2Y_4$	ATP =UTP[B,5]	Antagonist[5]	Antagonist[27]	Stimulates PLC
$P2Y_6$	UDP > UTP[6]	Antagonist[6]	Antagonist[7]	Stimulates PLC
$P2Y_{11}$	ATP > ADP[8]	No effect[9]	No effect[9]	Stimulates PLC Stimulates AC
$P2Y_{12}$	ADP>ATP[18]			Inhibits AC

A. In situ responses to UTP which are RB2 insensitive have been reported. These probably represent effects at $P2Y_2$

B. In the rat; the human receptor is UTP selective

	α,βmethyleneATP	**PPADS**	**RB2**
$P2X_1$	Agonist[11]	Antagonist[12]	
$P2X_2$	No effect[13]	Antagonist[14,12]	Antagonist[15]
$P2X_3$	Agonist[16]	Antagonist[17]	
$P2X_4$	Antagonist[19]	No effect[20]	No effect[21]
$P2X_5$	No effect[22]	Antagonist[23]	
$P2X_6$	No effect[23]	No effect[24]	
$P2X_7$	No effect[25]		

A blank square means the compound doesn't appear to have been tested on the cloned receptor. No effect has been entered where concentrations of 100μM or greater have no effect or only a small effect

In a follow up study on post mortem brains of Alzheimer's disease patients (Moore *et al.*, 2000b), Moore's group found $P2Y_1$ receptors associated with a number of structures characteristic of the disease, including neurofibrillary tangles, neuritic plaques and neuropil threads; the significance of this is unclear. Currently there are no similar studies with other P2Y antibodies but presumably it is only a matter of time before the antibodies become available and the data follows.

To summarize the data as it presently stands, it is clear that the $P2Y_1$ receptor is expressed in neurons and glial cells in the mammalian CNS and that the $P2Y_4$ receptor is expressed in some CNS cell types including (and possibly restricted to) glial cells. $P2Y_2$ has been detected in astrocytes as has $P2Y_{12}$. $P2Y_6$ has so far gone undetected in the CNS.

1.5.2 Functional Evidence for P2Y receptors

In addition to the anatomical evidence for P2Y receptors in the CNS, there is functional evidence for their presence on both neuronal and glial cells in various nuclei. Ca^{++} signals initiated by P2Y receptors have been detected in cortical pyramidal neurons in acute slices (Lalo *et al.*, 1998) as well as cerebellar Purkinje cells (Kirischuk *et al.*, 1996), hippocampal and thalamic neurons (Mironov, 1994). Similarly P2Y receptor-dependent Ca^{++} signals have been detected in astrocytes from the dorsal spinal cord (Idestrup & Salter, 1998), hippocampus (Bernstein *et al.*, 1998) and cerebellum (Jimenez *et al.*, 1998). Zhu and Kimelberg (Zhu & Kimelberg, 2001) combined Ca^{++} imaging with single cell RT-PCR, detecting mRNA for $P2Y_1$ and $P2Y_2$ receptors. Note that previously whole brain RT-PCR failed to detect $P2Y_2$, indicating that its expression might not be widespread within the CNS.

The functional significance of astrocyte P2Y (and possibly also P2X) receptors may well be related to the spread of Ca^{++} waves between astrocytes. In some preparations, it is the release of ATP (or another nucleotide) which signals to, and initiates the Ca^{++} rise in neighbouring

astrocytes; ATP is subsequently released to continue propagation of the signal (Guthrie *et al.*, 1998). The mechanism by which astrocytes release ATP is not known but in cultured C6 glioma cells the expression of connexins greatly increases ATP release (Cotrina *et al.*, 2000); it is possible that ATP exits the cells through the pore of the connexin molecules. Whilst it is clear that extracellular ATP is one signalling mechanism by which Ca^{++} waves propagate, they can also spread between networks of coupled astrocytes by diffusion of IP_3 through gap junctions (Finkbeiner, 1992). Furthermore, ATP can stimulate glutamate release from cultured astrocytes (Frech *et al.*, 1989) although there is currently no evidence that glutamate signals between astrocytes to propagate Ca^{++} waves. The relative importance of the different mechanisms is not clear; neither is the functional significance of astrocytic Ca^{++} waves understood.

Neuronal P2Y receptors have been studied in the sympathetic neurons of the superior cervical ganglion mainly in a series of experiments by Stefan Boehm and his colleagues; whilst these are not CNS neurons, they are the only ones which have received much attention. These cells have distinct receptors for UTP and ATP (Connolly, 1994) both of which are capable of triggering noradrenaline release by TTX and Ca^{++}-sensitive mechanisms (Boehm *et al.*, 1995;Connolly, 1995;Boehm, 1994). The ATP effect is probably through a P2X receptor (Connolly, 1994) whilst the UTP receptor is equisensitive to UDP and has a pharmacology resembling $P2Y_6$ (Boehm, 1998). Subsequent experiments have revealed two intracellular signalling cascades activated by UTP: inhibition of the K^+ current I_M (Boehm, 1998) and activation of PKC (Vartian *et al.*, 2001). The first pathway probably involves the following steps: P2Y receptor $\rightarrow$ $Gq_{/11}\rightarrow$ PLC $\rightarrow$ IP_3 $\rightarrow$ Ca^{++} release $\rightarrow$ I_M inhibition (Bofill-Cardona *et al.*, 2000). However, whilst inhibition of I_M is a mechanism of excitating these neurons common to UTP, acetylcholine (Brown *et al.*, 1981) and bradykinin (Jones *et al.*, 1995), it is not responsible for the release of noradrenaline by UTP (Bofill-Cardona *et al.*, 2000); this depends on the

second intracellular pathway involving the activation of PKC (Vartian *et al.*, 2001).

Despite the presence of a uridine nucleotide-sensitive receptor on these neurons, it is currently not known whether either UTP or UDP is released from neurons by exocytosis; therefore it remains to be seen whether UTP or UDP functions as a neurotransmitter in the SCG or elsewhere.

1.5.3 Detection of P2X receptors in the CNS

Various techniques have been used to detect and localize P2X receptors within the CNS including in situ hybridization, Northern Blotting, RT-PCR and immunohistochemistry; because antibodies are available for all seven cloned homomers, there is currently more information available about cellular and subcellular localization of P2X receptors than there is for P2Y receptors. Different techniques do not always agree completely but it usually the case that a more sensitive technique detects a receptor where a less sensitive technique previously failed to detect one.

As the evidence currently stands, the distribution of CNS P2X receptors is as follows: $P2X_4$ and $P2X_6$ have the widest distributions which largely, but not entirely, overlap (Collo *et al.*, 1996;Lê *et al.*, 1998b); $P2X_4$ receptors were exclusively localized to neurons and commonly to the somata and proximal dendrites. $P2X_2$ is more restricted, but still widespread (Collo *et al.*, 1996;Kidd *et al.*, 1995); in the hypothalamus there was clear labelling of neuronal somata, dendrites and axons (Xiang *et al.*, 1998) whilst in DRG it labelled both neuronal and non-neuronal cells (Vulchanova *et al.*, 1997). The signal for $P2X_1$ was either weak or undetectable (Kidd *et al.*, 1995;Vulchanova *et al.*, 1996;Brake *et al.*, 1994) consistent with a highly localized distribution. $P2X_5$ was detected exclusively in the mesencephalic nucleus (Collo *et al.*, 1996); $P2X_3$ was initially detected only in primary afferents of sensory neurons (Collo *et al.*, 1996;Chen *et al.*, 1995) but has since been detected in other CNS nuclei (Kidd *et al.*, 1995;Séguéla *et al.*, 1996). Similarly, $P2X_7$ receptors were initially reported to be absent from neurons in the CNS but present on ependymal cells and macroglia (Collo

et al., 1997); however, they have since been detected on presynaptic terminals of vagal (Deuchars *et al.*, 2001) and hippocampal neurons (Sperlagh *et al.*, 2002).

Two of the in situ hybridization studies looked at numerous CNS nuclei including the medial habenula (Kidd *et al.*, 1995;Collo *et al.*, 1996). Kidd et al only searched for $P2X_1$ and $P2X_2$ which they failed to detect. Collo et al searched for $P2X_{1-6}$ and detected just $P2X_4$ and $P2X_6$ subunits. In addition, whole habenula RT-PCR detected $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_6$ (F.Soto, personal communication). From this limited data, it appears that the two most common subunits in the CNS are the ones most prominently expressed in the medial habenula.

1.5.4 Subcellular Localization: Presynaptic P2 receptors

In the peripheral nervous system it is clear that that as well as postjunctional P2 receptors there are also prejunctional receptors controlling transmitter release (Von Kügelgen *et al.*, 1989; Von Kügelgen *et al.*, 1993; Von Kügelgen *et al.*, 1995) Inhibitory prejunctional receptors are more common (Von Kügelgen *et al.*, 1989;Fuder & Muth, 1993;Goncalves & Queiroz, 1996;Koch *et al.*, 1998) and in some cases have been classified as P2Y on the basis of their sensitivity to PTX (Von Kügelgen *et al.*, 1993); facilitatory prejunctional receptors appear to be less common but a facilitatory P2X receptor has been described (Sperlagh & Vizi, 1996). As ATP is a co-transmitter in the periphery (Burnstock, 1999a;Von Kügelgen & Starke, 1991), these are commonly presynaptic autoreceptors, activated by ATP released from the same terminal (Von Kügelgen *et al.*, 1993). In the CNS there is anatomical (Vulchanova *et al.*, 1996; Vulchanova *et al.*, 1997; Atkinson *et al.*, 2000; Rubio & Soto, 2001; Lê *et al.*, 1998b; Loesch & Burnstock, 1998) and physiological (Gómez-Villafuertes *et al.*, 2001; Gu& MacDermott, 1997; Nakatsuka & Gu, 2001; Khakh & Henderson, 1998; Kato & Shigemoto, 2001) evidence for presynaptic P2 receptors, including P2 receptors on terminals which are not known to release ATP (heteroreceptors) (Gómez-Villafuertes *et al.*, 2001; Kato & Shigemoto, 2001; Deuchars *et al.*, 2001).

At the light microscopic level limited information can be obtained about the subcellular distribution of P2X receptors; nonetheless, some results indicated a presynaptic localization. Following ablation of the nodose ganglion (Vulchanova *et al.*, 1996) and dorsal rhizotomy (Vulchanova *et al.*, 1997), $P2X_2$ immunoreactivity was greatly reduced in the nucleus of the solitary tract and the dorsal horn respectively, suggesting that the receptors are located on primary afferent terminals. In such studies, however, the possibility always remains that there is transneuronal degeneration causing a loss of postsynaptic receptors. Conclusive evidence, therefore, requires a combination of immunolabelling and electron microscopy to unequivocally identify the receptors on terminals. Such evidence is now available.

Electron microscopy confirmed the presence of presynaptic $P2X_2$ receptors in the NTS (Atkinson *et al.*, 2000) and further showed that they were located on vagal afferent and other glutamatergic terminals but not on GABAergic terminals. The receptors were close to the synapse, which is consistent with the functional study of Kato et al (see below). One study looked at the subcellular localization of $P2X_2$, $P2X_4$ and $P2X_6$ subunits in the cerebellum and hippocampus (Rubio & Soto, 2001). In Purkinje cells all three subunits were detected at parallel fibre synapses whilst climbing fibre and basket cell synapses remained unlabelled; likewise in the hippocampus all three subunits were detected at the synapse of Schaffer Collaterals onto CA1 pyramidal cells. Co-immunolabelling with an antibody to the GluR2/3 subunit confirmed the expression of these P2X subunits at excitatory synapses. Although occasional gold particles appeared to be located presynaptically the overwhelming majority of the receptors were on the postsynaptic membrane; furthermore, the receptors were located towards the perimeters of the synapses: this may mean that they are only activated by synaptically released ATP during high frequency bursts of activity; alternatively they may be well positioned to detect ATP release from perisynaptic glial cells. $P2X_4$ receptors have also been clearly shown on axon terminals synapsing on dendrites in the dorsal

horn and in the olfactory bulb (Lê *et al.*, 1998b). Immunoreactive dendrites were observed as well so the data is suggestive of both a pre- and postsynaptic role for P2X receptors in synaptic transmission in these regions. The distribution of the $P2X_1$ receptor has also been studied in the cerebellum and found to be located at a small subpopulation of parallel fibre-Purkinje cell synapses (Loesch & Burnstock, 1998). Curiously, whilst labelling was seen on presynaptic boutons and postsynaptic spines, labelled boutons were never seen contacting labelled spines. In addition, axons with en passant boutons were seen where the axon shaft was labelled but the bouton was not: this distribution of presynaptic receptors may be relevant to the electrophysiological studies (see below) which reveal different mechanisms by which P2X receptors can facilitate glutamate release.

A combined functional and anatomical study immunolabelled the $P2X_3$ subunit on GABAergic synaptosomes from the rat midbrain and demonstrated a Ca^{++} response to applied ATP and α,β-methyleneATP (Gómez-Villafuertes *et al.*, 2001). The pharmacology of this response indicated that the $P2X_3$ receptor might exist as a heteromer with a second P2X receptor, although there was no immunolabelling for either $P2X_1$, $P2X_2$ or $P2X_4$.

Other than the study of Moore et al using the $P2Y_1$ antibody (the only P2Y antibody currently available) there is no anatomical data on the subcellular localization of P2Y receptors.

1.6 Presynaptic P2 receptors on glutamate terminals: electrophysiological and other functional evidence.

Physiologically, there is evidence for presynaptic P2 receptors controlling the release of dopamine (Trendelenburg & Bültmann, 2000), serotonin (Von Kügelgen *et al.*, 1997), noradrenaline (Koch *et al.*, 1997;Von Kügelgen *et al.*, 1994), glycine (Rhee *et al.*, 2000), GABA (Hugel & Schlichter, 2000) and glutamate in the CNS (see below for references).

Some of these studies need to be interpreted cautiously because, although the results point to the involvement of a presynaptic P2 receptor, it is not necessarily located on the afferent terminals or the axons. However, there are studies where the presynaptic receptor is clearly located on the terminals or the axon. One such study is that of Gu and MacDermott (Gu & MacDermott, 1997) on a preparation of cultured dorsal horn neurons in which the somata had been removed. In this study the P2X selective agonist α,β-methyleneATP increased the frequency of spontaneous, TTX-insensitive glutamate currents by two mechanisms: the facilitation was partly action potential dependent presumably indicating that P2X receptors depolarized the axons to firing threshold; the remainder of the facilitation was action potential independent and resulted from Ca^{++} influx through the P2X receptors per se. This work has been extended to acute slices of the spinal cord with similar results, except that the Ca^{++} influx was solely through P2X receptors (Nakatsuka & Gu, 2001). Khakh and Henderson (Khakh & Henderson, 1998) reported presynaptic P2X receptors on terminals of trigeminal neurons. Facilitation of glutamate release was greatly reduced by TTX and abolished by Cd^{++} indicating that, unlike primary afferents in the spinal cord, the Ca^{++} influx depended on voltage-dependent Ca^{++} channels (VDCCs). In contrast, in the nucleus tractus solitarius, where ATP and α,β-methyleneATP reversibly increased the frequency of spontaneous, TTX-insensitive glutamate currents, the effect was completely insensitive to Cd^{++}, indicating that the Ca^{++} influx was entirely through P2X receptors (Kato & Shigetomi, 2001). Similarly, in a preparation of cultured sympathetic ganglion neurites (no somata present) ATP stimulated noradrenaline release was also Cd^{++}-insensitive (Boehm, 1999). It appears, therefore, that P2X receptors can facilitate transmitter release by two distinct mechanism, possibly depending on the position of the receptors relative to the release site. To trigger release independently of VDCCs, the P2X receptors presumably have to be very close to the Ca^{++}-sensing release machinery in order to achieve the local high concentration of Ca^{++} required (Heidelberger *et al.*, 1994). In contrast, distant P2X receptors, perhaps located on the axons, are unlikely to produce the

necessary Ca^{++} signal without the opening of VDCCs. In addition, P2X receptors producing a Ca^{++} signal below the release threshold could still enhance action-potential dependent release. The relative physiological importance of axonal versus terminal P2X receptors is not known, although clearly there is a difference between a receptor which enhances action-potential dependent release and one which induces release independently of cell firing; with respect to the latter effect, it is interesting to note that the application of ATP in the NTS released sufficient glutamate from presynaptic terminals to fire the postsynaptic cell (Kato, F, unpublished observation). Furthermore, axonal and terminal receptors might be exposed to different and independent sources of ATP.

One noteworthy study is that of Deuchars and colleagues (Deuchars *et al.*, 2001) in which they combined immunohistochemistry and electrophysiology to show functional presynaptic $P2X_7$ receptors on vagal afferent terminals; immunopositive terminals were clearly visible under the electron microscope and the application of the $P2X_7$ agonist BzATP depolarized the postsynaptic cell, an affect completely blocked by glutamate antagonists; this was interpreted as due to a release of glutamate following activation of the $P2X_7$ on presynaptic terminals. The electron microscope also identified presynaptic $P2X_7$ receptors at the neuromuscular junction and these were shown to be functional by destaining FM1-43 loaded terminals with BzATP. This identification of presynaptic $P2X_7$ receptors was unexpected not only because they had previously gone undetected in CNS neurons (Collo *et al.*, 1997) but also because the $P2X_7$ receptor can form a wide pore permeable to large molecules and associated with cell lysis (Rassendren *et al.*, 1997;Surprenant *et al.*, 1996). However, the presynaptic $P2X_7$ receptor appeared not to be forming such a pore, as there was no loading of terminals by either YO-PRO or 6carboxyfluorescein. Very recently, the $P2X_7$ receptor has also been detected on glutamate terminals in the hippocampal CA1 and CA3 regions (Sperlagh *et al.*, 2002) raising the possibility that presynaptic $P2X_7$ receptors are widespread.

The Ca^{++} permeability of the P2X receptors predicts that they would facilitate transmitter release at terminals where they are expressed, exactly in the manner described for glutamate in the spinal cord and the NTS. In contrast, inhibitory presynaptic P2X receptors have yet to be described. There is evidence for release-inhibiting G-protein coupled receptors on glutamate terminals; however, the evidence seems less clear cut than that for P2X receptors, perhaps unavoidably so because the data is complicated by concomitant, and well documented, inhibitory effects of adenosine (Dolphin & Prestwich, 1985;Prestwich *et al.*, 1987;Lupica *et al.*, 1992;Fredholm & Dunwiddie, 1988). For example, at the Schaffer collateral-commissural fibre synapses onto hippocampal CA1 neurons, Mendoza-Fernandez and colleagues found evidence for ATP-mediated presynaptic inhibition of glutamate release (Mendoza-Fernandez *et al.*, 2000); while some of the data were consistent with the inhibition being due to breakdown to adenosine (eg lack of effect of P2 antagonists but competitive antagonism by adenosine antagonists), other data was inconsistent with this interpretation (eg ATP was a more potent inhibitor than adenosine, and its effects were not blocked by adenosine deaminase). Evidence that the ATP effect was via a G-protein coupled receptor came from its sensitivity to pertussis toxin. The authors concluded that there were inhibitory effects of ATP per se, acting at a P2Y receptor sensitive to theophylline-derivatives (possibly the P3 receptor proposed by others (Shinozuka *et al.*, 1988;Todorov *et al.*, 1994)). In contrast, in a similar study by Cunha et al (Cunha *et al.*, 1998), the inhibitory effects of ATP were reduced by adenosine deaminase and ectonucleotidase inhibitors and potentiated by adenosine uptake inhibitors, leading them to conclude that the effects were subsequent to breakdown to adenosine. (In the same study, they were able to inhibit the effects of γ-substituted ATP analogs with ectonucleotidase inhibitors whilst being unable to detect any hydrolysis of these compounds; consequently they proposed that there is highly localized catabolism of ATP at the synapse, "channelling" the adenosine so produced directly towards the adenosine receptors. They further proposed that this could explain the higher potency of ATP compared to adenosine -

as reported by Mendoza-Fernandez – because the ATP, unlike adenosine, could slip past the adenosine uptake sites to deliver adenosine to the receptors.) It is interesting to note that the Mendoza-Fernandez study reported both P2X receptors facilitating release and P2Y receptors inhibiting release at the same glutamate synapses. Why might ATP have bidirectional effects on the same terminals? As the P2 receptors have different sensitivities to ATP, one hypothesis is that the effect of extracellular ATP is concentration dependent. A second possibility is again the idea that the receptors might be spatially separated on the terminals and therefore selectively activated by ATP from different sources; application of exogenous ATP would be unable to make this distinction.

In summary, there is good evidence for presynaptic facilitatory P2X receptors on glutamate terminals in the CNS. There is also evidence for P2X receptor-mediated presynaptic facilitation of glycine (Rhee *et al.*, 2000), GABA (Hugel & Schlichter, 2000;Gómez-Villafuertes *et al.*, 2001) and noradrenaline (Boehm, 1999) release in the CNS, suggesting that this mechanism of facilitation may be widespread. In the cases of noradrenaline and GABA in the spinal cord, these are examples of positive feedback at an ATP autoreceptor.

1.7 Fast Purinergic Transmission in the CNS

Neuronal responses to exogenously applied ATP were reported in the early 1980s (Jahr & Jessell, 1983;Salt & Hill, 1983) raising the question of whether ATP might be a fast transmitter between neurons. It was not until the early 1990s that fast purinergic transmission between neurons was described, in the ceoliac ganglion (Evans *et al.*, 1992;Silinsky *et al.*, 1992); this was shortly followed by the first description of fast purinergic transmission in the CNS, in the medial habenula nucleus (Edwards *et al.*, 1992). Since then, fast purinergic currents have been discovered in the hippocampal CA1 (Pankratov *et al.*, 1998) and CA3 neurons (Mori *et al.*, 2000), the locus coeruleus (Nieber *et al.*, 1997), the hypothalamus (Jo & Role, 2000) and the spinal cord (Jo & Schlichter, 1999). However, it is by no means obvious what the physiological role of these synapses might be.

In hippocampal CA1 neurons the residual epsc after block of AMPA receptors was approximately 25% of the current prior to the block; furthermore, this residual current was only detected when stimulating at very low frequencies. A similar proportion of the epsp remained after AP5 and CNQX application in the locus coeruleus while in hippocampal CA3 neurons and the medial habenula the currents are smaller still: even recruiting more fibres, the largest P2X currents in CA3 neurons averaged just 37pA. The total excitatory P2X inputs to these cells is therefore small compared to the excitatory input provided by glutamate fibres: this suggests that the physiological role of purinergic inputs is probably something other than driving the cells to firing threshold. The P2X currents in CA3 neurons had relatively slow rise times and long latencies to onset which is consistent with the receptors being located towards the periphery of the synapse, possibly to detect high synaptic activity. This hypothesis is consistent with the electron microscopic observations at the Schaffer collateral-CA1 synapse and the parallel fibre-Purkinje cell synapse which show that P2X receptors are located towards the periphery of the synapses (Rubio & Soto, 2001); whether this is true for CA3 neurons has not been investigated. It is also consistent with the release of ATP from Schaffer collateral fibres by high frequency stimulation (Wieraszko *et al.*, 1989) but is at odds with Pankratov's finding (Pankratov *et al*, 1998) that the P2X currents at Schaffer collateral synapses were only evoked at low stimulating frequencies (0.05 Hz).

Other clues to the role of fast purinergic transmission come from the spinal cord where ATP has been shown to be a co-transmitter with GABA (Jo & Schlichter, 1999). Co-transmission by a fast excitatory and a fast inhibitory transmitter might seem counterintuitive but Jo and Schlichter point out that, if an extracellular agent could selectively modulate the release of one transmitter, such a synapse might have the unusual property of switching between being inhibitory and excitatory. Another interesting potential property of this synpase is that it could produce a postsynaptic Ca^{++} signal through the P2X receptors whilst GABA simultaneously clamps the voltage close to the Cl^- reversal potential. Whether switching of the

synapse or voltage-clamped Ca^{++} signals occur has not yet been tested experimentally. If ATP co-transmission proves to be the rule throughout the CNS, the role of ATP might be understood in its interaction with its co-transmitters.

1.8 ATP and Glial-Neuronal / Neuronal-Glial signalling

The presence of P2 receptors on glia and the release of ATP by these cells has already been discussed. In recent years there has been an increasing interest in the role of glial cells in the CNS. One area which has received some attention is that of intercellular signalling both from neurons to glia and from glia to neurons.

It has been known for a few years that glutamate released from astrocytes can elevate Ca^{++} in nearby neurons (Parpura *et al.*, 1994). In the retina, a spreading glial Ca^{++} wave can modulate the firing of neurons as it passes by (Newman & Zahs, 1998). Recently Haydon and colleagues have shown that astrocytes can modulate glutamate release through receptors located on, or close to, the presynaptic terminals (Araque *et al.*, 1998a;Araque *et al.*, 1998b); in both of these studies the signal from astrocytes to neurons was carried by glutamate acting at metabotropic and NMDA receptors. Currently there are no reports that ATP signals between astrocytes and neurons, but it is certainly a candidate molecule for such a role. As already discussed, ATP is released by astrocytes, it is implicated in the propagation of Ca^{++} waves, and there are presynaptic P2 receptors on neuronal terminals ideally situated to detect any astrocytic ATP release at the synapse.

ATP is also a candidate molecule for signalling from neurons to glia. Application of ATP can trigger glutamate release from astrocytes as well as initiate Ca^{++} waves. It is therefore entirely possible that neuronally released ATP can function similarly.

1.9 Extracellular ATP

The presence of cell surface P2 receptors throughout the CNS strongly implies that, under certain physiological or pathophysiological conditions, they are exposed to extracellular ATP or another nucleotide agonist. There are little data on actual extracellular ATP concentrations but there are data on extracellular adenosine: estimates from several studies put the background concentration between 25 and 250nM (Dunwiddie & Diao, 1994;Ballarin *et al.*, 1991). As ATP is rapidly metabolized to adenosine (see below) this puts an upper limit on extracellular ATP of approximately 250nM, about 4 times lower than the EC_{50} of the most sensitive adenine nucleotide sensitive P2 receptors. It follows that these receptors are unlikely to be tonically activated by background extracellular ATP.

1.9.1 Neuronal ATP release

Stimulation-evoked ATP release was first reported over 40 years ago (Holton, 1959). In several CNS nuclei, including the medial habenula (Sperlágh *et al.*, 1998b) and the hypothalamus (Sperlágh *et al.*, 1998a), field stimulation released ATP in a Ca^{++}-dependent manner; this was largely TTX-dependent indicating that ATP was released from neurons. Similarly, in the hippocampus, stimulation of the Schaffer collaterals released ATP by a Ca^{++}-dependent machanism (Wieraszko *et al.*, 1989); the authors suggest that ATP is co-released with glutamate, although they did not show that release is TTX-sensitive. (It should also be noted that in similar experiments Hamann and Attwell (Hamann & Attwell, 1996) detected ATP release in the habenula and hippocampus which was largely TTX-insensitive and which they suggest was due to cell electroporation. The reasons for the differences between the groups are not clear.) Experiments using synaptosomes prepared from several different brain regions have also demonstrated a Ca^{++}-dependent release of ATP (White, 1978;Potter & White, 1980;Sawynok *et al.*, 1993). In some of these studies there is evidence that ATP is co-released with a second transmitter: with noradrenaline in the hypothalamus (Sperlágh *et al.*, 1998a) and with acetylcholine in the cortex (Richardson & Brown, 1986).

1.9.2 Pathophysiology and ATP release

ATP is a ubiquitous intracellular molecule with a cytoplasmic concentration of some 3-5mM (Traut, 1994;Moyer & Henderson, 1985); it is therefore not surprising that ATP is released from cells during certain pathological conditions. Hypoxia-induced ATP release has been demonstrated from several different cell types including erythrocytes (Bergfeld & Forrester, 1992) and cardiac myocytes (Forrester & Williams, 1977) consistent with a proposed role of ATP in the control of vascular tone. More recently, increased release of ATP has been shown from hippocampal slices rendered hypoxic for a 5 minute period (Juranyi *et al.*, 1999).

Shear stress can release ATP from epithelial and endothelial cells. In blood vessels this is a physiological mechanisms involved in controlling vascular tone. However, it can also be released from epithelial cells of tubes (such as the ureter and gut) and sacs (such as the urinary and gall bladder) when they are distended (Burnstock, 1999b). Burnstock has proposed that this ATP acts on $P2X_{2/3}$ receptors of subepithelial sensory nerves to initiate nociceptive signals. As the medial habenula sits on the border of the third ventricle, separated from the cerebrospinal fluid by a thin layer of epithelial cells, these observations in the periphery raise the intriguing possibility that a similar epithelial-neuronal signal may occur in this nucleus. The epithelial cells might, for example, release ATP as a result of increased intraventricular pressure.

Finally, because the intracellular ATP concentration is high, any damage to the cell membrane will inevitably result in ATP release into the extracellular space and the activation of local P2 receptors.

1.10 Extracellular UTP

Althougth there are uridine nucleotide-selective P2Y receptors, there is relatively little data on the physiological release of UTP. This is probably because a sensitive and selective assay for UTP has only recently been developed (Lazarowski *et al.*, 1997). Using this assay, Harden and

colleagues first detected the release of UTP from cultured glial cells, in response to mechanical stimulation produced by changing the medium bathing the cells (Lazarowski *et al.*, 1997). In a follow up study they detected UTP release from different cells including several cultured epithelial cell types, platelets and neutrophils (Lazarowski & Harden, 1999); no release was detected from primary astrocytes or neurons. The stimulus was again mechanical (medium change); with the exception of platelets they did not try to induce release with any other form of stimulus. (Platelets also happen to be the only cell type in which UTP has been detected in a storage organelle (Goetz *et al.*, 1971) and UTP was released after challenge with thrombin (Lazarowski & Harden, 1999). Only one study has assayed for neuronal UTP release following electrical stimulation and detected none (Saiag *et al.*, 1998). There are insufficient data, therefore, to conclude whether there are neurons which release UTP in a Ca^{++} dependent manner appropriate to neurotransmission.

1.11 Ectonucleotidases

A criterion for establishing a compound as a neurotransmitter is that mechanisms exist for terminating the signal. Extracellular ATP is metabolised to adenosine with some or all of the following intermediates being formed along the way.

$$ATP \leftrightarrow ADP \leftrightarrow AMP \leftrightarrow Adenosine$$

The enzymes responsible for extracellular ATP degradation are broadly referred to as ectonucleotidases. Initially their existence was inferred from applying nucleotides to a tissue and detecting the metabolites although some of the enzymes have now been cloned. The best studied enzyme is the 5'nucleotidase which catalyses this last step in the above chain, forming adenosine from AMP. There are thought to be two intracellular versions of this enzyme as well as a membrane anchored ecto-5'nucleotidase and a soluble form derived from the latter (Zimmermann, 1992). The purified enzyme has a broad substrate specificity, hydrolysing

AMP, CMP, UMP, IMP and GMP with a Km in the low μM range for all of these. This seems to be a common feature of ectonucleotidases: a comparison of the cloned rat ecto-ATPase and ecto-Apyrase (Heine *et al.*, 1999) showed that, while both enzymes have a slight preference for adenine nucleotides, they are capable of hydrolysing guanine-, cytosine-, inosine-, and uridine nucleotides almost as effectively.

Ectonucleotidase activity has been detected in a wide range of tissues including both neurons and glia (Zimmermann, 1996). Different tissues probably possess different complements of these enzymes and consequently they may convert ATP to adenosine by different pathways. Kinetic analysis of ATP breakdown reveals that the accumulation of ADP, AMP and adenosine does not necessarily occur linearly over time. For example, at the cholinergic synapses in the striatum at least three ectonucleotidases are present: an ecto-ATPase, an ecto-ADPase and an ecto-5'-nucleotidase with Vmax ratios of 30:14:1 (James & Richardson, 1993). Both ATP and ADP inhibit the ecto-5'-nucleotidase and ATP also inhibits the ectoADPase. Consequently ATP is rapidly broken down to ADP which accumulates until the ADPase is relieved of the inhibition exerted by ATP. ADP is then metabolized to AMP but the conversion of AMP to adenosine is delayed by the inhibition of the ecto-5'-nucleotidase by ADP. Due to the nature of feedforward inhibition, the delay in forming adenosine from ATP is proportional to the initial ATP concentration; therefore, depending on the firing frequency and the consequent amount of ATP released, adenosine accumulation is more or less rapid. As adenosine inhibits acetylcholine release, the mechanism may work to disinhibit release at high frequencies. At some synapses both ATP and adenosine act presynaptically, with either the same or the opposing effects. The kinetics of conversion of ATP to adenosine introduces another level of complexity and subtlety to the presynaptic control of transmitter release.

Extracellular ATP is potentially cytotoxic; there are numerous reports that ATP kills cells (Steinberg & Di Virgilio, 1991), in some cases by acting at

the large pore-forming $P2X_7$ receptor but also simply because P2X receptors are Ca^{++} permeable. Therefore, in addition to terminating the ATP signal at purinergic synapses, ectonucleotidases probably have a more general role in protecting cells against the potentially toxic effects; this is supported by the finding that ecto-apyrase, ecto-ATPase and ecto-5'nucleotidase were upregulated in some areas of the hippocampus following an ischeamic shock (Braun *et al.*, 1998), when there was shown to be a concomitant increase in extracellular ATP. This would increase the rate of ATP breakdown as well as the accumulation of the neuroprotective agent adenosine.

1.12 Long-term Potentiation

Data is presented in this thesis showing a long-lasting potentiation of glutamate transmission which is induced by a novel protocol. Two different and well characterized forms of long-term potentiation (LTP) are briefly introduced below and are further discussed later in the thesis with particular reference to the results which are presented.

LTP is the most extensively studied form of synaptic plasticity in the mammalian CNS. Its importance lies partly in the hypothesis that it underlies certain forms of learning memory (Bliss & Collingridge, 1993). In the CA1 region of the hippocampus, LTP is dependent on the activation of NMDA receptors (Coan *et al.*, 1987;Collingridge *et al.*, 1983). The Mg^{++} block of this receptor is relieved by depolarization (Ascher & Nowak, 1988) and is responsible for two of the characteristic properties of LTP at this synapse: cooperativity and associativity (Bliss & Collingridge, 1993). This associativity provides a cellular mechanism for Hebb's correlate of classical conditioning. NMDA receptor-dependent LTP is widespread within the CNS. However, the medial habenula is unusual in that no NMDA receptor component to synaptic responses was detected and no NMDA receptor response was seen when glutamate was applied to the soma (Robertson *et al.*, 1999). If these synapses express a form of LTP, it is likely to be NMDA receptor-independent.

The mossy fibre synapses on to CA3 neurons express just such a form of NMDA receptor-independent LTP (Ascher & Nowak, 1988;Zalutsky & Nicoll, 1990). Once induced, mossy fibre LTP is believed to be expressed presynaptically, due to an increase in glutamate release (Weisskopf & Nicoll, 1995). There is evidence from several studies that a presynaptic elevation of cAMP is involved in the induction process (Weisskopf *et al.*, 1994;Hestrin *et al.*, 1990); however, other aspects are controversial, particularly whether a postsynaptic Ca^{++} rise is necessary. The issue is still disputed (Mellor & Nicoll, 2001) even though a recent study has suggested a possible resolution to some of the conflicting studies (Yeckel *et al.*, 1999): long-lasting high frequency stimulation (L-HFS) activates postsynaptic metabotropic glutamate receptors with the release of Ca^{++} from postsynaptic stores and subsequent LTP induction; in contrast, a brief tetanus (B-HFS) requires that the postsynaptic cell is coincidentally depolarized so that there is a Ca^{++} influx through VDCCs. These two induction protocols are therefore non-Hebbian and Hebbian respectively. Under normal physiological conditions granule cells are though not to fire prolonged high frequency bursts necessary to induce non-Hebbian LTP (Henze *et al.*, 2000). This form of LTP may therefore be associated with pathological firing patterns of granule cells such as occurs during epileptic activity.

1.13 LTP and ATP

There is some evidence that ATP plays a role in LTP. Several studies have previously demonstrated that LTP can be induced in hippocampal CA1 neurons by the pairing of low frequency stimulation with the application of ATP (Wieraszko & Seyfried, 1989;Wieraszko & Ehrlich, 1994;Fujii *et al.*, 1999). In none of the studies was the effect blocked by P2 receptor antagonists (although only a limited number were tested). The slowly hydrolysable analogue of ATP, ATPγS, also induced LTP but with a slower timecourse to ATP (Wieraszko & Seyfried, 1989) suggesting that ATP hydrolysis is required to produce LTP. In support of this hypothesis, Chen et al (Chen *et al.*, 1996) demonstrated the presence of an ecto-protein

kinase on the surface of cultured hippocampal pyramidal neurons which phosphorylated surface proteins. This phosphorylation was blocked by an antibody directed against the catalytic domain of the ecto-protein kinase. Finally, they showed that the antibody did not affect the induction of LTP but did prevent its maintenance, whilst an antibody directed against the non-catalytic domain of the ecto-protein kinase did not affect any aspect of LTP. This work was in the mouse. Fujii et al (Fujii *et al.*, 1999), working in the guinea pig, induced LTP by pairing low frequency stimulation with ATP application; in their experiments, an ecto-protein kinase inhibitor blocked the expression of LTP, possibly indicating a species difference between mice and guinea pigs . It has previously been demonstrated that ATP is released from the Schaffer Collaterals by high frequency stimulation (Wieraszko *et al.*, 1989) which could be the source of ATP for the physiological induction of LTP.

1.14 The Medial Habenula

The motive behind the experiments described in this thesis was to investigate some roles of ATP in fast synaptic transmission in the CNS. The nucleus chosen for these experiments was the medial habenula, a CNS nucleus which has received relatively little attention from neuroscientists. In this lab, initial interest in the medial habenula stemmed from the discovery of fast purinergic currents (Edwards *et al.*, 1992); further experiments investigated the properties of these currents as well as those of the glutamatergic inputs (Gibb & Edwards, 1996;Robertson & Edwards, 1998;Robertson *et al.*, 1999). The work presented in this thesis builds upon and extends previous work from the laboratory: hence the choice of this nucleus.

One advantage of working in the medial habenula is that there is obviously much to discover. However, there are a number of disadvantages. The description of the internal anatomy of the medial habenula and the afferent and efferent connections which it makes is incomplete, and its physiological role is not well understood. This makes it harder to match electrophysiological findings to the anatomy or to understand the results

presented in this thesis in the context of medial habenula physiology. The following paragraphs introduce the current knowledge of this nucleus.

1.14.1 Neuronal Cells of the Medial Habenula

Paxinos and Watsons' Atlas of the Rat Brain recognises just one cell type in the medial habenula at the light microscope level with Nissl staining; Tokunaga and Otani (Tokunaga & Otani, 1978) also reported that this nucleus comprises a single type of neuron. A recent study (Andres *et al.*, 1999) at the light and electron microscope level describes five subnuclei of the medial habenula based on criteria including cellular and synaptic morphology. Four of the subnuclei comprise small, densely packed neurons with only small size differences and the authors admit that the delineation of the subnuclei is not always clear. For the electrophysiologist looking down an infrared microscope the medial habenula would appear as a largely homogeneous neuronal population.

Different techniques, however, reveal subsets of neurons in this nucleus. Neurochemistry reveals two distinct populations of medial habenula neurons: the dorsal neurons stain for Substance P (Kawaja *et al.*, 1990;Ronnekleiv & Kelly, 1984) whilst the ventral neurons stain for choline acetyl-transferase (ChAT) (Kawaja *et al.*, 1990;Sperlagh *et al.*, 1998) or acetylcholinesterase (AChE) (Villani *et al.*, 1983) and are presumed to be cholinergic. These cell types also appear to be distinguishable under the electron microscope where the cholinergic neurons have a much better developed rough endoplasmic reticulum (Villani *et al.*, 1983). Both neuronal types project from the medial habenula via the fasciculus retroflexus to the interpeduncular nucleus; studies were unable to detect axon collaterals from either of these projections making connections onto medial habenula neurons (Sperlagh *et al.*, 1998;Kawaja *et al.*, 1988). Furthermore, no interneurons have been detected. This suggests that the medial habenula may separate afferent inputs into two parallel paths to the interpeduncular nucleus, one cholinergic and one using Substance P, with little or no interaction between the two.

1.14.2 Ultrastructure of Synaptic Terminals

Tokunaga and Otani describe four distinct types of terminals at the electron microscope level (Tokunaga & Otani, 1978). Type I terminals have small clear vesicles and are probably excitatory; type II have pleomorphic vesicles and may be inhibitory; type III have larger, dense-core vesicles which are mixed with clusters of small, clear vesicles; and type IV have dark-core granules similar to neurosecretory nerve terminals. Synapses are mainly on dendrites with few on somata or axons. The type III endings deserve additional comment because they resemble the endings thought to belong to purinergic nerves which are seen in peripheral organs (Burnstock, 1975): if they do represent ATP releasing neurons, the fact that terminals have a heterogeneous population of vesicles suggests that ATP may be a co-transmitter in the medial habenula; the small clear vesicles suggest that its co-transmitter might be glutamate (but see below).

Andres et al (Kawaja *et al.*, 1990) also describe terminals which seem to correspond to type I and type II endings. In this study the type I endings are restricted mainly to the dorsal medial habenula and the type II endings are found in the ventral region. The type I endings probably arise from the posterior septal afferents (Kawaja *et al.*, 1990).

Both of these studies describe synapses which are unusually tightly ensheathed by glial cells which wrap themselves many times around the pre- and postsynaptic elements.

1.14.3 Afferent and Efferent Connections

When studying the neuronal connections of a nucleus, there must be a certain caution when interpreting both positive and negative results. For example, lesioning nucleus A and finding degenerating terminals in nucleus B is not necessarily conclusive proof of a connection between the two: the explanation for the effect could lie with fibres passing through, but not originating from, nucleus A. On the other hand, the failure to show a connection between two nuclei using anterograde or retrograde tracers

might be due to a failure of uptake at the somata or terminals. The evidence for connections is therefore more convincing when two or more different techniques (lesioning, tract tracing, electrophysiology etc) point the same way.

1.14.4 Triangular and Septofimbrial Afferents

The major afferent projections to the medial habenula come from two nuclei of the posterior (postcommissural) septum: the ipsilateral triangular septal (TS) and septofimbrial (SFi) nuclei (Staines *et al.*, 1988;Kawaja *et al.*, 1990;Sperlágh *et al.*, 1998b). Nearly every cell in these two nuclei projects to the medial habenula; the SFi nucleus projects to the rostral medial habenula while the TS nucleus projects to the caudal end (Herkenham & Nauta, 1977). Fibres from both nuclei terminate throughout the dorsal-ventral extent of the medial habenula (Staines *et al.*, 1988) and, therefore, presumably innervate both the Substance P and cholinergic neurons. The terminals of these neurons synapse primarily on spines and also on dendrites with no synapses detected on the soma (Kawaja *et al.*, 1990). Axo-axonic contacts have also been described in electron microscope studies (Kawaja *et al.*, 1990); these are of interest as they represent one potential mechanism for a purinergic modulation of glutamatergic transmission as discussed above.

What is the transmitter between these septal nuclei and the medial habenula? The evidence points to more than one, either because there are subsets of neurons or because they make use of co-transmitters.

While glutamate is the major excitatory transmitter in the medial habenula, the origin of the glutamatergic fibres has been surprisingly neglected: perhaps there has been a tacit assumption that the most likely source of the glutamate input is the main afferent projection i.e. the TS and SFi nuclei. The evidence either way appears to be limited to a single study: Sperlagh et al (Sperlágh *et al.*, 1998b) found a set of neurons in the TS nucleus which project to the medial habenula and stain for calretinin which is

believed to be a marker for glutamatergic neurons. Some of these afferents may, therefore, be glutamatergic.

Evidence for a purinergic projection from the septal nuclei is stronger. Staines et al (Staines *et al.*, 1988) found subsets of septal neurons by staining for adenosine deaminase (ADA), which might be a marker for purinergic neurons. In the rostral SFi nucleus virtually all the neurons to the medial habenula were ADA positive whilst in the caudal region and in the TS nucleus approximately half of the projecting neurons stained. The ADA positive neurons project via the stria medullaris to the medial habenula where their terminals are restricted to the dorsal part of the nucleus, the region of the Substance P neurons. Furthermore, lesion of the TS nucleus reduced by 81% the amount of ATP released in the medial habenula by field stimulation (Sperlágh *et al.*, 1998b); there was no detectable transneuronal damage in the medial habenula and given the apparent absence of interneurons and axon collaterals in this nucleus, the authors concluded that the TS nucleus is the most likely source of the purinergic afferents. Whilst there is electron microscopic evidence for ATP- and glutamate-containing vesicles in the same terminals in the medial habenula (see above), it has been shown that ATP and glutamate do not act postsynaptically as co-transmitters (Robertson & Edwards, 1998). The possibility that ATP is co-released with glutamate, and only acts presynaptically, is discussed in relation to glutamate plasticity in chapter 7.

1.14.5 The Nucleus of the Diagonal Band: source of GABA afferents?

The nucleus of the diagonal band is also part of the posterior septum which projects to the medial habenula and there is evidence that this is the source of the GABA afferents: kainic acid lesions of the diagonal band nucleus reduce glutamic acid decarboxylase levels in the medial habenula by 40% (Contestabile & Fonnum, 1983). It is not known whether there is an additional GABA projection to the medial habenula.

1.14.6 Other Afferent Projections to the Medial Habenula

Other afferents which are reported to project to the medial habenula include a noradrenergic projection from the superior cervical ganglion (Chafetz & Gage, 1983), a dopaminergic projection from the ventral tegmentum (Phillipson & Pycock, 1982), a serotonergic projection from the median raphé (Björklund *et al.*, 1972) and a projection from the pineal gland (Patrickson & Smith, 1987).

Despite the fact that the medial habenula is one of the strongest staining nuclei in the CNS for nicotinic receptors (Goldman *et al.*, 1986;Clarke *et al.*, 1985;Rainbow *et al.*, 1984), there is no conclusive evidence for a cholinergic projection to this nucleus.

1.14.7 Physiology of the Medial Habenula

The medial habenula has been reported to play a role in the control of various physiological functions ranging from sexual behaviour through avoidance learning to fluid intake (Sutherland, 1982). However, the results of different studies often appear contradictory, possibly because of differences in the experimental protocols, and there is currently no clear understanding of medial habenula physiology. Several lines of evidence point to an involvement in the sleep / wake cycle: the interpeduncular nucleus sends large projections to the median and dorsal raphe (Shibata & Suzuki, 1984) and the dorsal and dorsolateral tegmentum (Groenewegen & Van Dijk, 1984), areas implicated in the control of the sleep / wake cycle (Quattrochi *et al.*, 1989); metabolic activity in the interpeduncular nucleus does not drop during sleep or anaesthesia (Herkenham, 1981); and lesions of the fasciculus retroflexus disrupted the number and duration of sleep episodes as well as some aspects of REM sleep (Haun *et al.*, 1992). Normal sleep patterns were restored by transplants of fetal habenula neurons (Haun *et al.*, 1992)which reinnervated the interpeduncular nucleus largely in a topographically appropriate manner (Eckenrode *et al.*, 1992).

This possible physiological role of the medial habenula is discussed in the final chapter when considering the facilitation of glutamate transmission presented in Chapter 4.

Chapter 2

Methods

2.1 Rats

Experiments were performed on Sprague-Dawley rats (21 days old, unless otherwise stated). Both males and females were used.

2.2 Preparation of medial habenula slices

Rats were decapitated and after hemi-section along the midline and removal of the cerebellum each hemisphere was rapidly transferred to ice cold Krebs solution (see below for composition) and allowed to cool for between 2 and 10 minutes. The front third of each hemisphere was removed and the lateral portion trimmed allowing them to be glued to the tissue block of a Camden Vibra-slice (Loughborough, U.K.) with the mid-line surface facing upwards. The slicing chamber containing the tissue block had previously been partially filled with Krebs solution (approximately 1cm deep) and stored overnight in a freezer so that the base was covered with a frozen layer. Ice-cold Krebs was added to the chamber to completely cover the tissue.

The medial habenula lies on the midline bordering the 3rd ventricle and is delineated by the stria medullaris above and the fasciculus retroflexus below, making it easily identifiable (fig 2.1, A). The medial surface of the habenula is covered with a thin layer of epithelial cells which was removed by cutting the thinnest possible slice from the surface. Thereafter, 200μm thick parasagittal slices were cut using either stainless steel or carbonsteel blades (Campden Instruments Ltd) and the medial habenula dissected out using fine hypodermic needles (Becton Dickinson). Two slices of the medial habenula were routinely taken although in a few experiments only the most medial slice was used to ensure that slices contained exclusively medial habenula neurons; it is stated in the results where only the medial

slice has been used. A Pasteur pipette (cut off to have a tip diameter of approximately 2mm and then fire polished) was used to transfer slices to an incubation chamber filled with Krebs and bubbled with 95% O_2 / 5% CO_2. This was stood in a tissue bath (Grant Instruments Ltd): slices were incubated at 35°C for 1 hour and thereafter at room temperature (20-22°C). All recordings were made at room temperature.

2.3 Solutions and Drugs

2.3.1 Extracellular solution

The composition of the standard extracellular (Krebs) solution was (in mM): NaCl 125, $NaHCO_3$ 26, Glucose 25, KCl 2.4, NaH_2PO_4 1.1, $MgCl_2$ 1, $CaCl_2$ 2; pH 7.4 when bubbled with 95% O_2 / 5% CO_2. This solution was made up on the evening before the experiment and stored overnight in the refrigerator at ~ 4°C.

2.3.2 Intracellular solution

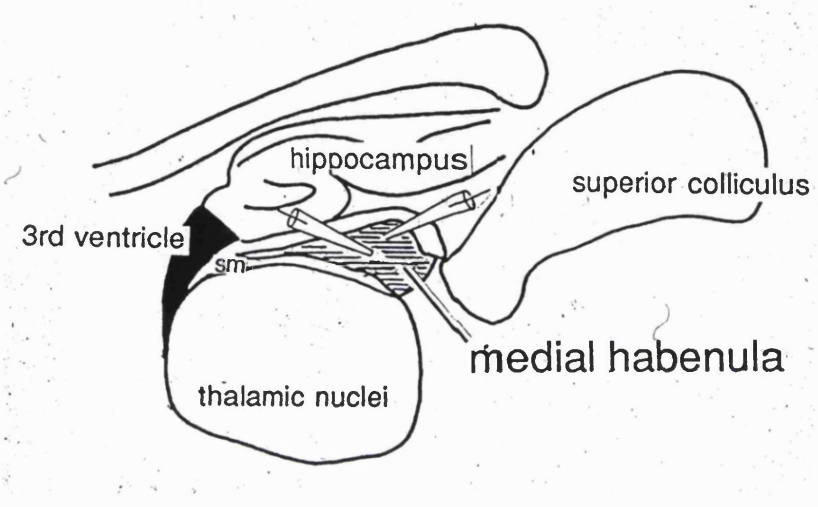
A CsCl based intracellular solution was used; the composition was (in mM): CsCl 140, Hepes 5, EGTA 10, $CaCl_2$ 2, Mg_2ATP 2, pH 7.4 with CsOH. **The junction potential between this intracellular solution and the Krebs soution was measured as +2.5mV and calculated as +3.7mV: this was not corrected for. Note that the intracellular solution contained no GTP and therefore does not support the functioning of postsynaptic G-protein coupled receptors. Intracellular solution was stored as 1ml frozen aliquots.**

2.3.3 Drugs

All the drugs used were water-soluble. Most of them were stored in frozen aliquots at 1000x times the concentrations commonly used in experiments; exceptions were UTP, UDP and bicuculline: to avoid possible decomposition, these were stored as solids and made up as solutions on the day of the experiment. Prior to experimenting, appropriate amounts of each drug were added to the Krebs solution to make the desired

Fig 2.1 Medial Habenula

A. Schematic diagram of the medial habenula as seen in the sagittal plane following transection of the cerebral hemispheres along the midline. The medial habenula is clearly delineated by the white matter tracts of the stria medullaris (sm) and the fasciculus retroflexus (not shown), making it relatively simple to dissect out. Figure reproduced from Edwards *et al.*, 1992.


hippocampus
superior colliculus
3rd ventricle
sm
medial habenula
thalamic nuclei

concentrations. Drugs were bath applied using the perfusion system described below.

Drugs were obtained from the following sources: TTX, bicuculline, NBQX, NF279 and RB2 from Tocris; UTP, UDP, 7-chlorokynurenate and α,β-methyleneATP from Sigma; PPADS from RBI.

2.4 Perfusion of slices

Single slices were transfered to a perspex recording chamber (volume approximately 1ml). Slices were held in position by the nylon threads of a U-shaped grid placed on top of them. The grid was made from 0.04″ diameter platinum wire (World Precision Instruments) bent into a U-shape and flattened in a vice; nylon threads from stockings were stretched across the grid and glued to it to make between 5 and 10 parallel fibres.

The recording chamber was perfused continuously at a rate of approximately 1ml/min with Krebs bubbled with 95% O_2 / 5% CO_2. Perfusion was by a gravity system using 50ml reservoirs mounted above the recording chamber on the side of the Faraday cage; the outflow from each reservoir could be turned on and off allowing for switching between them when applying drugs. Solution was removed from the chamber by a suction mechanism: a "sucker" was made from a borosilicate glass microhaematocrit tube (Modulohm A/S, Denmark) heated in a bunsen flame, bent into an L-shape and drawn out at the tip. This was attached by plastic tubing to a vacuum pump (Medcalf Brothers Ltd) which sucked solution out of the chamber and collected it in a buckner flask. A second Buchner flask, containing anyhdrous copper sulphate crystals, was connected in series with the collecting flask to prevent solution being sucked into the pump.

In some experiments a recirculating system was used whereby the solution was pumped out of the chamber and back in to the perfusing reservoir using a cartridge pump (Masterflex).

2.5 Visualizing cells

The recording chamber was mounted on the stage of an upright Olympus microscope (Olympus) with Nomarski optics; this was connected to an IR-CCD camera (Hitachi Denshi, Japan) allowing for viewing on a black and white monitor (Hitachi). Cells were viewed under either a 40x or 60x water immersion lens (Olympus, Japan; fig 2.2, A).

2.6 Patch-clamp recordings

Recordings were made variously from one of two rigs in the lab. These were set up to be as similar to each other as practically possible but there were inevitably differences in the make or model of some pieces of apparatus.

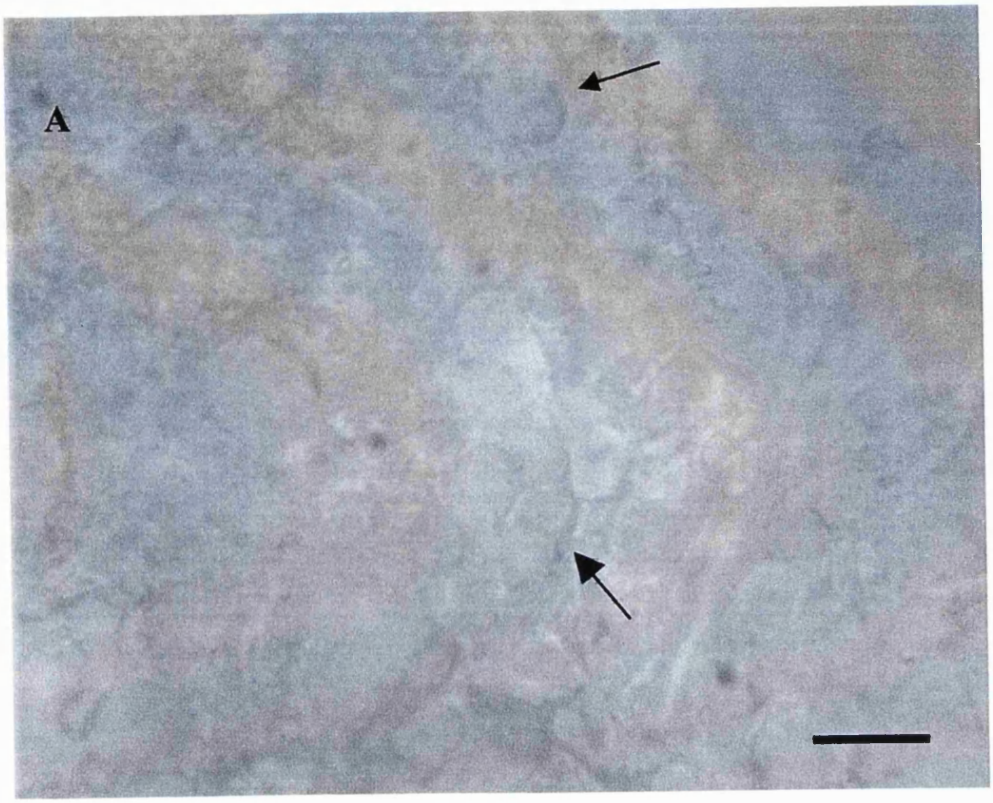
An upright Olympus microscope sat in the centre of an air table (Newport). The headstage for the patch-clamp amplifier (Axopatch 1D , Axon Instruments, USA) was mounted on a micromanipulator (Narishige) positioned beside the microscope. A second manipulator with both coarse and fine control (Sutter Instruments) was positioned on the other side of the microscope for stimulating electrodes or fast drug applicators. The airtable and apparatus on it was enclosed within a Faraday cage.

Thick-walled borosilicate glass electrodes (1B150F-3, World Precision Instruments) were pulled using a Narishige electrode puller and filled with intracellular solution (see above for composition) using microfillers (World Precision Instruments) to give a resistance of 4-5 MΩ. Whole-cell voltage clamp recordings were made with an Axopatch 1D patch clamp amplifier (Axon Instruments CA. USA). An electrode was inserted into the electrode holder with a silver chloride-coated silver wire forming the electrical contact between the solution and the headstage. The wire was rechlorided when necessary by immersing in bleach. A plastic tube connected to the side of the electrode holder allowed positive or negative pressure to be applied by sucking or blowing gently. The electrode was

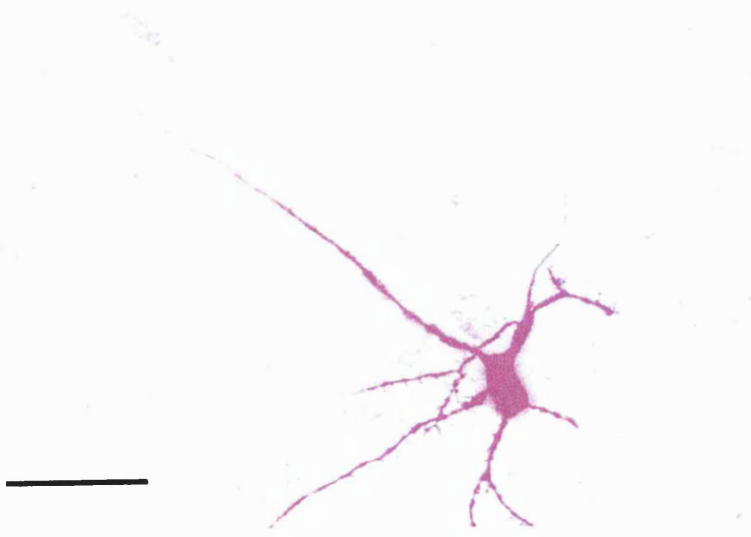
Fig 2.2 Medial Habenula Neurons

A. 200μ slice of the medial habenula viewed through a 60x lens, exactly as viewed when patching cells. The large-headed arrow points to a group of viable cells; the small-headed arrow points to another viable cell, probably the cell of choice in this image. Scale bar is 20μ.

B. Projection image of a medial habenula neuron filled with Lucifer Yellow (0.5mg/ml). The image was taken on an Olympus confocal microscope. Scale bar is 50μm.



B



visually guided towards a cell whilst maintaining positive pressure to clear away any surface material. Once the electrode was touching the cell the positive pressure was relieved and negative pressure applied to form a seal. The seal resistance was determined from the size of the ionic current flowing in response to a 5 or 10mV voltage step (the transient capacitative current were compensated as far as possible). The electrode was hyperpolarized to –70mV and, when the seal resistance was greater than 1GΩ, further suction was applied to break through into the whole cell configuration.

The membrane potential was clamped at -70mV and the series resistance was monitored regularly throughout the recording by injecting a small voltage pulse (5 or 10mV): recordings were rejected if the size of the current response altered by more than 15% and could not be recovered.

2.7 Recording and Evoking Currents

2.7.1 Glutamate receptor-dependent currents (Epscs)

When recording glutamate currents, the extracellular solution contained the $GABA_A$ antagonist bicuculline (10μM in initial experiments, 20μM in later ones) and the NMDA antagonist 7-chlorokynurenate (10μM); in addition, for the recording of miniature glutamate currents (minis), the solution contained TTX (1μM).

Currents were evoked using a monopolar glass stimulating electrode, pulled in the same manner as the recording electrode and filled with either standard extracellular solution or 1M NaCl (as indicated). The electrode holder was attached to a second micromanipulator (Sutter) positioned beside the microscope on the air table.
The electrode was visually positioned in the slice and voltage pulses applied using an isolated pulse stimulator (Grass SDG or AM Systems). Typically a pulse duration of 140μs was used and the voltage increased

until a current was evoked; if this failed to evoke a current, the electrode was moved to a new position and the procedure repeated.
The protocol used in a number of experiments was the so-called minimal stimulation technique. This is an attempt to record currents from as few presynaptic fibres as possible and, ideally, from a single one. To achieve this, currents were recorded using the lowest voltage at which a response could be measured. Once such a response was found, the stimulating voltage was increased by a few volts to ensure that this did not alter the response by recruiting additional fibres, before returning the voltage to a level between this and the original value. (Note that, if increasing the voltage only very slightly recruited additional fibres, a different input was searched for). In the following sections this is referred to as minimal stimulation and the currents thus evoked as minimally-evoked currents.
In those experiments where minimal stimulation was not necessary, the stimulus strength was increased beyond the minimal voltage required to evoke a current, in order to recruit more fibres and increase the signal-to-noise ratio. In the following sections this is referred to as supraminimal stimulation.

2.7.2 P2X receptor-dependent currents

When recording P2X currents, the extracellular solution contained bicuculline (10μM), 7-chlorokynurenate (10μM) and the AMPA receptor antagonist, NBQX (10μM).

The protocol for evoking P2X currents was the same as that used for glutamate currents. Minimal stimulation was not a requirement but, in practice, once a current was found increasing the voltage further usually failed to recruit more fibres.

2.8 Data acquisition and storage

Currents were digitised at 44kHz and recorded at a bandwidth of 10kHz (4 pole Bessel filter) on a modified video recorder (Vetter, U.S.A.) for long-

term storage on video tape (Fuji). They were further filtered at 2kHz (Frequency Devices, 8 pole Bessel filter) and sampled at 10kHz with either a CED1401 or digidata 1200 interface (Axon Instruments) using either the winWCP program for capturing evoked currents or the winWCD program for capturing minis (J Dempster, University of Strathclyde Electrophysiology Software): WCD captures a continuous record of the membrane current; WCP captures a chosen number of records of a specified length, either continuously or when triggered to do so by a specific event (usually the stimulus artefact). Data were routinely sampled online and backed up to CD-ROM for long-term storage.

2.8.1 Spontaneous TTX-insensitive Synaptic Currents

To capture spontaneous, TTX-insensitive glutamate currents (minis) a continuous record of the membrane current over a period of 10 minutes was recorded with WCD. A detection algorithm implemented in WCD was run on this record, searching for possible minis; the algorithm was relatively crude and searched for events which were larger than twice the standard deviation of the noise and remained above this level for at least 2ms. Each time such an event was detected, a record of it was made automatically and written to a WCP file for further analysis.
All the events detected by this algorithm were visually examined to exclude noise or other artefacts. Records were examined blind i.e. the examiner was not aware as to whether the records were recorded in control or drug solutions. To be included as a mini, an event had to have a fast rise time (in the order of 1-2ms) and an approximately exponential decay. Mini parameters (rise times, amplitudes and decay time constants) were measured in WCP and the data exported to the statistical package Prism for further analysis (statistics, graph plotting).

2.8.2 Evoked Currents

Evoked currents were captured using WCP; this was set to detect the stimulus artefact and capture a 200ms record beginning 10ms before the artefact.

Current parameters were measured in WCP and exported to Prism for further analysis.
When minimal stimulation was used, failures were detected by eye as they were clearly distinguishable from events.

2.9 Data Analysis and Statistics

2.9.1 Control and Test Data Sets

A control data set was collected before the application of a drug. The drug was then bath applied and the test data set collected after the drug effect had reached a maximum and stabilized – this usually occurred within approximately 5 mins or less. When recording spontaneous currents or minimally-evoked currents it was not always possible to determine drug effects on line, so a drug was applied for at least 10 minutes before the test data set was collected to try and ensure that the drug reached equilibrium within the slice.

2.9.2 Measuring current parameters

Current parameters were measured automatically by routines written into WCP; two cursors were positioned around the current defining the section of the record to be analysed and, in the case of evoked currents, a third cursor on the stimulus artefact defined time t = 0. The baseline (current = 0 amps) was determined for each record by averaging a number of data points on an inactive section (ie where no currents occurred).
Rise times were measured from 10%-90% of the peak amplitude; latencies to onset were measured from the t = 0 cursor to the 10% rise point; amplitudes were measured from the baseline to the peak.
Decay time constants were measured for both individual currents and for averaged signals by fitting the decaying phase of the signal with either a single or double exponential curve with the formulae:

$f(x) = Ae^{-x/\tau}$ single exponential

$f(x) = Ae^{-x/\tau 1} + Be^{-x/\tau 2}$ double exponential

For the single exponential, A is a constant = f(x) when x = 0 (i.e. the maximum point on the curve) and the time constant, τ, is the value of x when f(x) = 0.36*A (i.e. the curve has decreased to ~ 1/3 of its maximum). The double exponential is the sum of two such single exponentials where B and $\tau 2$ have the same meanings as A and τ respectively.
The cursors defining the region to fit the curve to were positioned so as to give the best fit as judged by eye.

2.9.3 Determining Quantal Amplitude

The quantal amplitude, q, of glutamate currents was determined by making the simplifying assumption that the underlying distribution of current amplitudes is described by a Poisson distribution. The first term of the Poisson distribution (e^{-m}, where m is the mean quantal content) gives the probability of zero releases (P_0); this can be independently determined from the experimental data by counting the number of failures:

$$P_0 = e^{-m} = \text{number of failures / number of events.}$$

Taking logs and rearranging: m = log (number of events / number of failures). The quantal amplitude is then calculated by dividing the mean current amplitude (measured in the experiment) by the mean quantal content (q = mean current amplitude / m).

2.9.4 Discriminating between a real and an apparent presynaptic effect

In the following chapters two experimental approaches have been used to try to distinguish between pre and postsynaptic actions of drugs: analysis of mini frequency and analysis of current failures. It is possible to misinterpret a postsynaptic effect as a presynaptic effect if the postsynaptic effect reduces small amplitude currents to below the detection threshold or enhances even smaller currents to above the detection threshold. To try to eliminate such errors, the following analysis was applied (fig 2.3). For evoked currents, it was hypothesized that the change in failures was entirely due to a postsynaptic effect. If the mean amplitude of currents in the drug was, for example, 1.5 x control mean amplitude, the entire control

amplitude distribution was multiplied by 1.5 to give a hypothetical amplitude distribution. The number of currents in a particular bin was then compared between the theoretical and the actual curves.

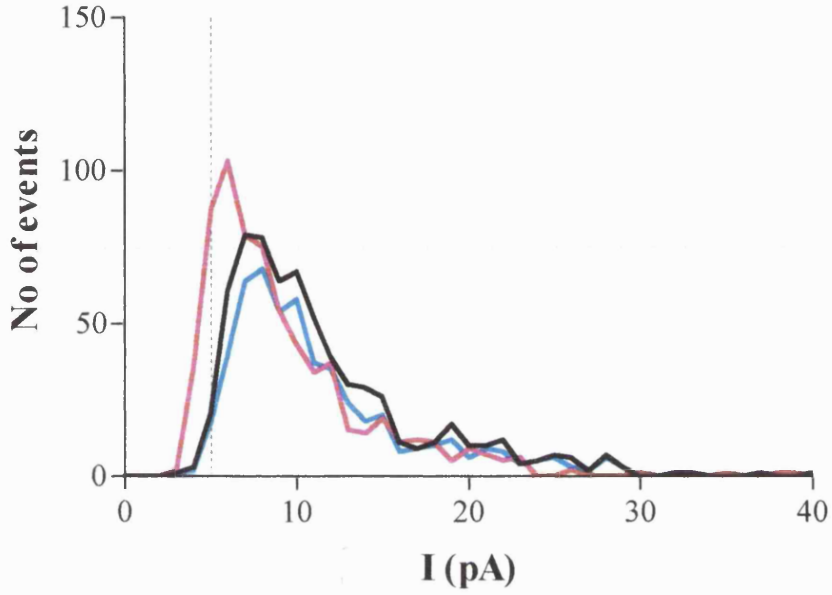
Similar analysis was applied to minis except the method of calculating the theoretical distribution was as follows. The smallest amplitude current in control was measured and this value taken as the detection threshold (YpA). The difference in the number of minis in control and in the drug was counted (X) and the amplitude of the Xth largest control mini was measured. The control amplitude distribution was multiplied by Y/X to give the theoretical distribution. The actual distribution in the drug and the theoretical distribution were compared using the Kolmogorov-Smirnov test.

2.9.5 Statistical Analysis

Various statistical tests were used as appropriate. The majority of the statistical analysis was carried out using GraphPad Prism; the Kolmogorov-Smirnov tests were carried out using an add on package for Excel. Values quoted in the text are the mean ± standard error of mean. For individual cells, mean current amplitudes were measured in one of two ways: for purinergic currents, which had low signal to noise ratios, an average of 100 events was made in WCP and the peak amplitude of this averaged trace was measured. For other currents, the amplitude of 100 individual events was measured and the mean of these values calculated. While the first method generally gave a slightly lower value due to some variability in the latencies, the choice of measurement did not affect the *comparison* of mean current amplitudes in different recording conditions. To assess the effect of a drug, the mean current amplitudes were compared in the different recording conditions. Raw data values were analysed using the Wilcoxon signed rank test. Normalized data were analysed with a one-sample t-test or a repeated measures ANOVA, as appropriate. The statistical test used is stated with the results.

Fig 2.3 Distinguishing between a pre and post synaptic effect of a drug on current amplitudes

In this example the black line is the actual amplitude distribution of the control currents and the blue line is the actual amplitude distribution of the currents in the drug. The dotted black line is the noise; in reality currents below this amplitude go undetected although for this explanation they are shown as if they could be detected. The red line is the *theoretical* distribution of the current amplitudes in the drug assuming the hypothesis that the drug acts postsynaptically to reduce current amplitudes by, in this example, 20%. Note that such an effect would cause many more currents to go undetected. The red and the blue curves can now be compared statistically (Kolmogorov-Smirnov test) and the hypothesis accepted or rejected accordingly.


150
100
50
0
No of events
0
10
20
30
40
I (pA)

Chapter 3

Properties of Medial Habenula Neurons and Glutamatergic Currents

In total, evoked glutamate currents were recorded in over 130 cells, using either minimal or non-minimal stimulation. The stimulating electrode was moved around the slice and the stimulating voltage increased until a current was evoked. Although it was not studied systematically, slices appeared to fall into one of two groups with respect to the ease with which currents were evoked: in one group currents could be evoked from almost any electrode placement within the field of view with a stimulating voltage often less than 10V; in the other group, the stimulating electrode typically had to be moved to several different positions within the slice before a current was evoked. Currents were evoked at 1Hz, although in a few experiments additional frequencies were used to study frequency-dependent effects. The properties of glutamate currents described in this chapter were measured in cells where minimal stimulation was used.

3.1 Cell Properties

Cells had a capacitance of 16.9 ± 2 pF and an input resistance of 1.3 ± 0.16 GΩ (n=12).

3.2 Properties of Evoked Glutamate Currents

Examples of evoked glutamate currents are shown in fig 3.1.

3.2.1 Current Failures

The failure rate was 47 ± 5% (n=18).

3.2.2 Latency to Onset

The mean latency to onset of the currents was 2.6 ± 0.3ms (n=7). 4/7 cells showed a range of latencies similar to the red columns in fig 3.2,A, with an interquartile range of less than 0.5ms; in the other 3 cells the range was considerably broader (eg fig 3.2, B, black columns) with an interquartile

Fig 3.1 Glutamate currents in a medial habenula neuron

From top left, horizontally, nine consecutive events recorded in a medial habenula neuron. The cell was clamped at –70mV and currents were evoked at 1Hz in the presence of 7-chlorokynurenate (10μM) and bicuculline (20μM). Minimal stimulation was used and failures (marked with an asterisk) are clearly distinguishable from events.

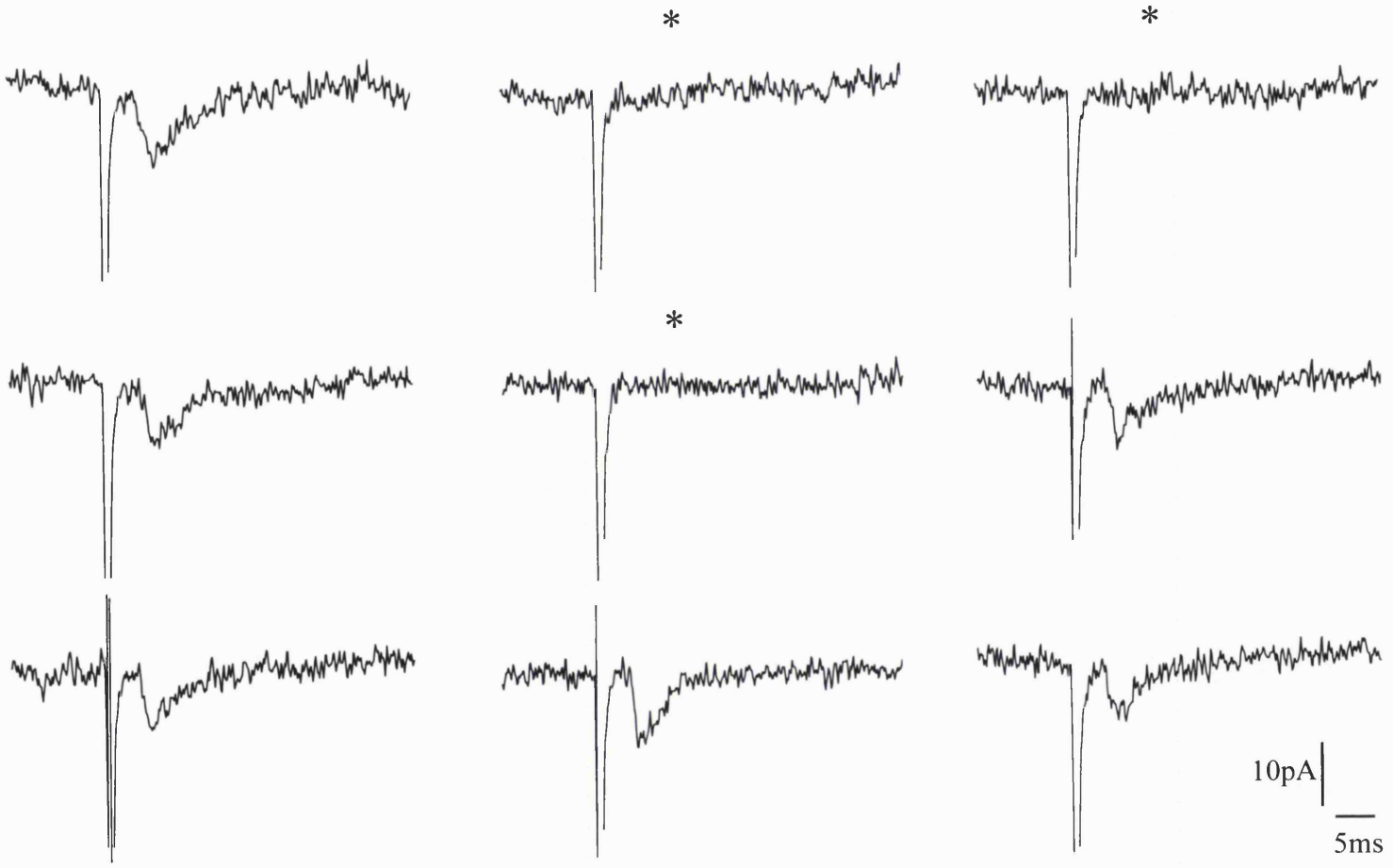
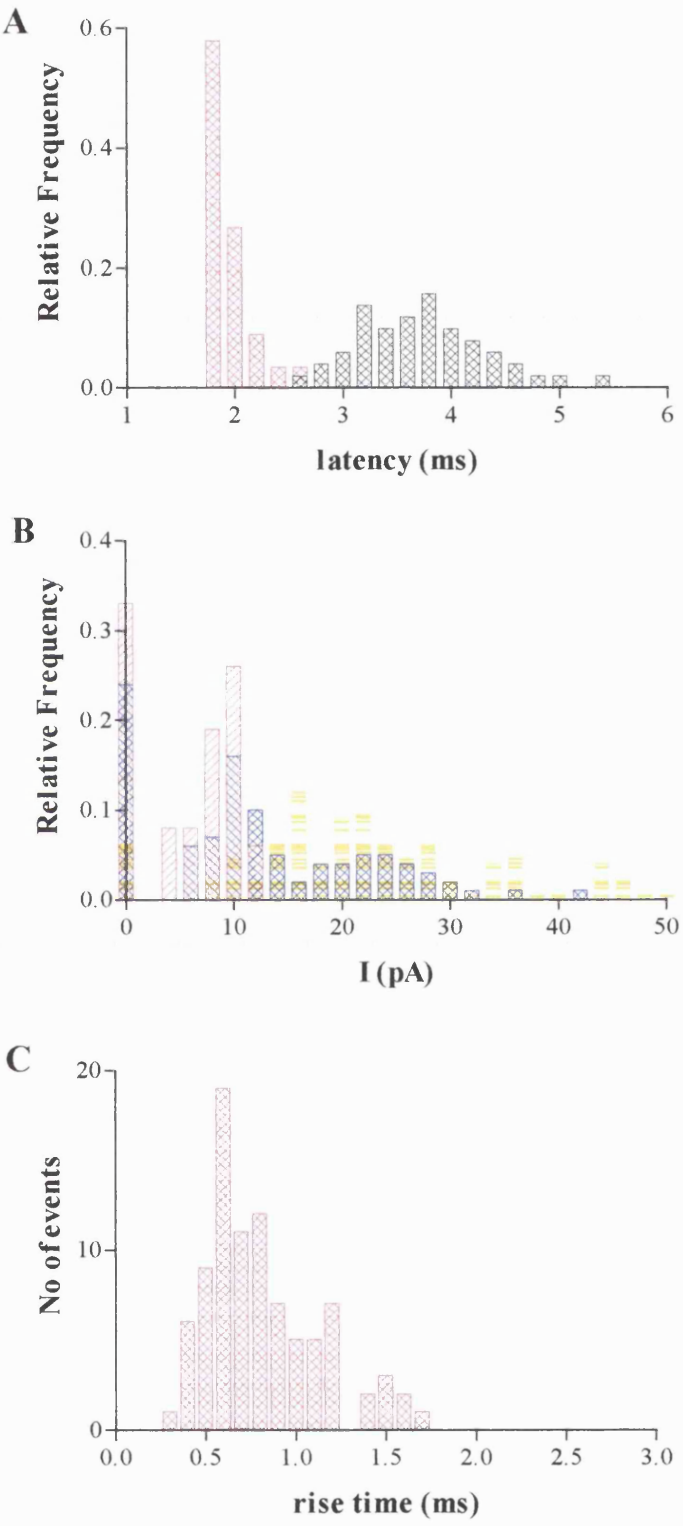

*
*
*
10pA
5ms

Fig 3.2 Kinetic properties of glutamate currents

A. Latency to onset of glutamate currents in two different cells. 4/7 cells had a relatively narrow spread of latencies (eg red columns), probably indicative of monosynaptic connections. The other three cells had a broader spread of latencies (eg black columns) with larger mean value, possibly indicative of polysynaptic connections.

B. Amplitude histograms from three different cells. 6/7 cells had amplitude distributions with a skew towards lower amplitudes and a modal peak current amplitude of 10-12pA; in contrast, the cell with the red columns had a relatively narrow spread of peak current amplitudes. The blue columns are from a cell with an apparently bimodal distribution.

C. Current rise times from one typical cell: the modal rise time was 0.6ms and the majority of the currents (75%) had rise times of less than 1ms.


A
Relative Frequency
0.6
0.4
0.2
0.0
1
2
3
4
5
6
latency (ms)
B
Relative Frequency
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
I (pA)
C
No of events
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
rise time (ms)

range of approximately 1ms. Despite the spread of latencies seen in all cells, only one cell showed the appearance of two distinct peaks in the latency histogram.

The longer latency currents may be polysynaptic. Consistent with this is the fact that, between cells, there is a significant correlation between latency to onset and the number of failures ($r=0.82$, $p=0.03$, Spearman correlation coefficient).

3.2.3 Current Amplitudes

The mean current amplitude (including failures) was 14.4 ± 2.5pA ($n=7$). In 6/7 of cells the amplitude histograms were skewed with a modal peak amplitude between 8 and 16pA (eg fig 3.2, B, yellow columns); one cell had a narrower, more symmetrical amplitude histogram (eg fig 3.2, B, red columns); one cell had an amplitude histogram which appeared to be bimodal (fig 3.2, B, blue columns).

3.2.4 Rise times

Currents had fast rise times (mean = 0.9 ± 0.1ms, $n=7$, fig 3.2, C).

3.2.5 Decay time constant

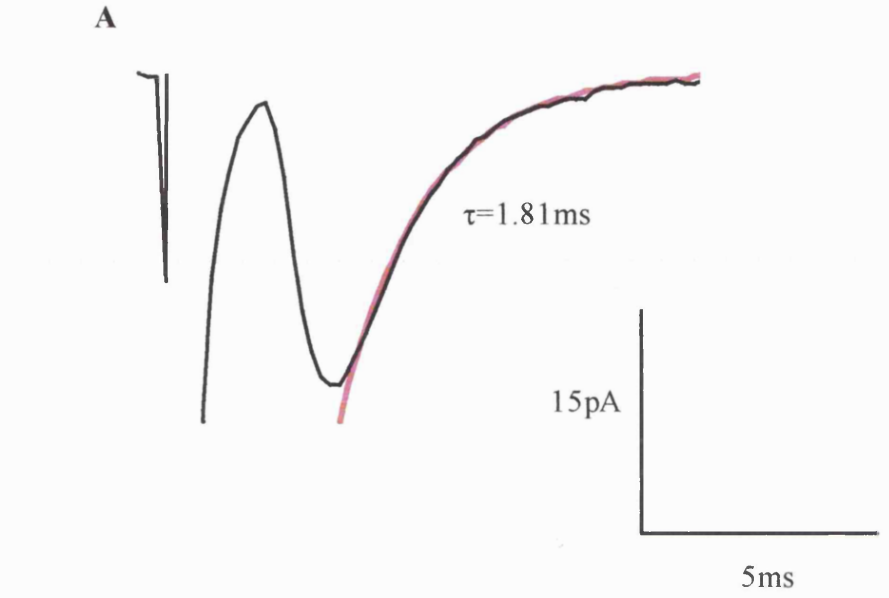
The decay phase of the currents was well fitted with a single exponential decay function (fig 3.3). The time constant of decay was 2.2 ± 0.3ms ($n=7$).

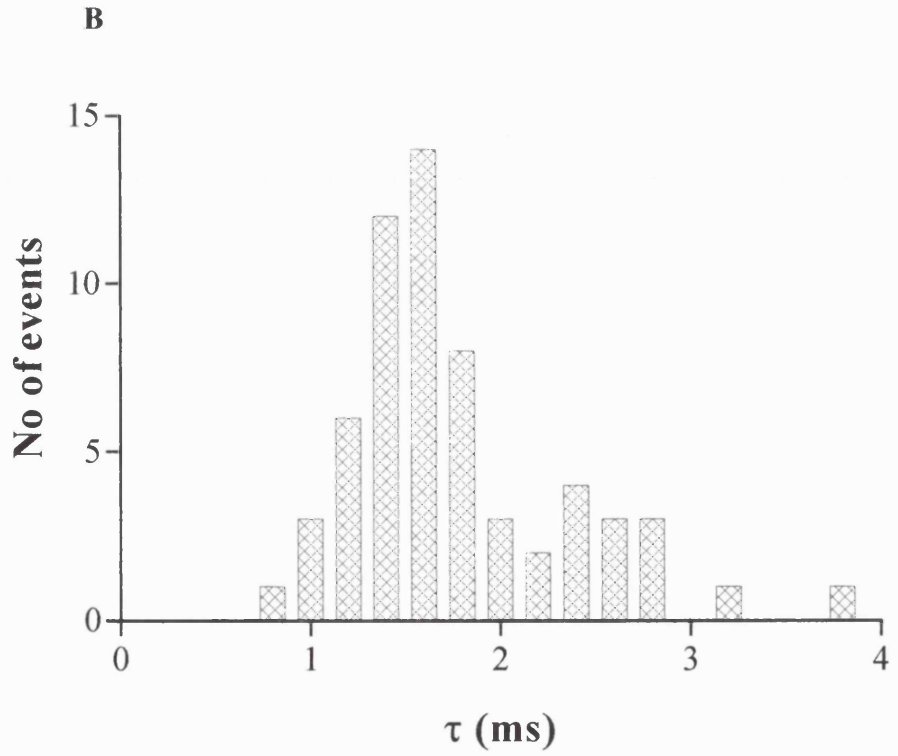
The rise times and decay time constants reported above are in agreement with the values previously obtained using a fast applicator to mimick synaptic currents on these neurons (Robertson *et al.*, 1999). There were no differences in the kinetic properties of currents from cells of the medial or the lateral of the two slices.

Fig 3.3 Decay phase of glutamate currents

A. Averaged current (average of 100 events) fitted with a single exponential curve (red line) with a τ of 1.8ms

B. Decay time constants for the individual currents averaged in A.


A
τ=1.81ms
15pA
5ms


B
No of events
15
10
5
0
0
1
2
3
4
τ (ms)

3.3 Properties of miniature glutamatergic currents

3.3.1 Mini amplitudes

Figure 3.4 is an example of glutamate minis recorded from a medial habenula neuron. The mean amplitude of glutamate minis was 14.6 ± 0.9pA (n=7 cells). Amplitude distributions were typically skewed towards lower amplitude events with a modal amplitude of 6-12pA (fig 3.5, A).

3.3.2 Rise times

Minis had fast rise times (mean = 1.2 ± 0.1ms, n=7).

3.3.3 Decay time constant

In each cell approximately 600 minis were averaged and the decay phase of the resulting averaged trace was well fitted with a single exponential curve with a decay time constant, τ = 3.7 ± 0.4ms (n=5, fig 3.5, B).

3.3.4 Frequency of glutamate minis

There was a considerable variation in the frequency of minis between cells (mean = 3.1 ± 1Hz, n=13).

3.3.5 Block by CNQX

CNQX (10μM) reduced the frequency of minis by 98.7 ± 0.8% (n=3), presumably through a postsynaptic block of AMPA receptors. This confirms that nearly all minis were glutamatergic.

3.4 Discussion

The cell capacitance and input resistance are relatively low and high respectively indicating that these are small neurons. This is consistent with their appearance under the microscope: the soma are approximately 10μm in diameter and, from the occasional cell which has been filled with fluorescent dye, the dendritic tree appears not to be extensive. For comparison, the input resistance of CA1 pyramidal was 200-900MΩ (Hestrin *et al.*, 1990).

Fig 3.4 Glutamate minis recorded in a medial habenula neuron

One continuous second of recording from a medial habenula neuron, showing spontaneous miniature glutamatergic currents. The cell was clamped at –70mV and the currents recorded in 7-chlorokynurenate (10μM), bicuculline (20μM) and TTX (1μM).

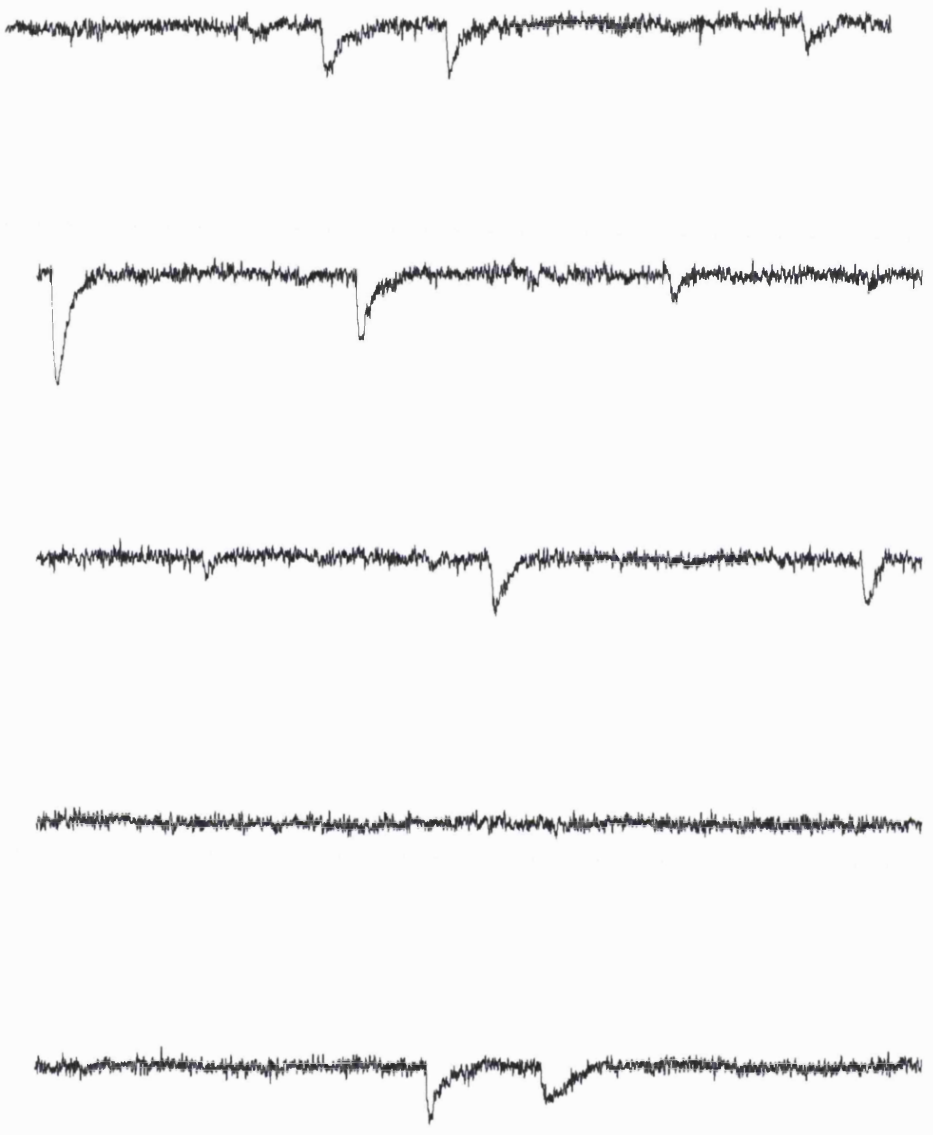
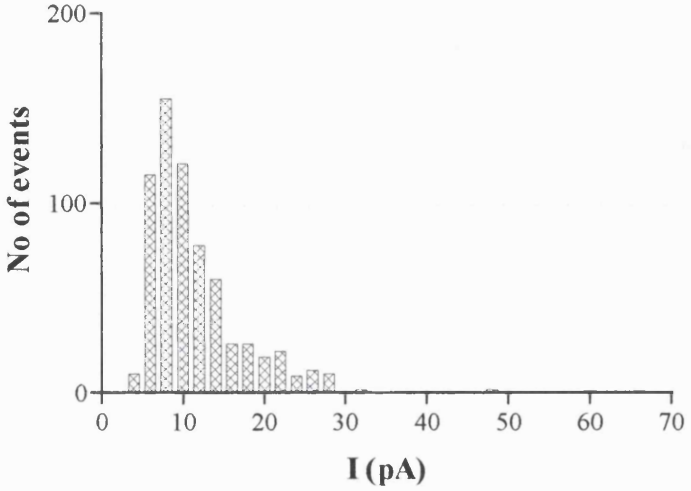

10pA
50ms

Fig 3.5 Amplitude and decay time constants of glutamate minis

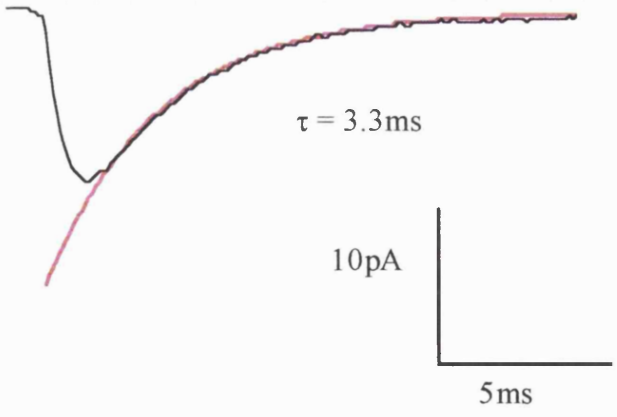
A. Amplitude distribution of glutamate minis from a typical cell (n=676 events).

B. Averaged trace of the glutamate minis from the same cell. The decay phase was fitted with a single exponential function with a time constant, $\tau = 3.3$ms.

A



B



The kinetic properties of medial habenula glutamate currents have been described before (Robertson & Edwards, 1998) and are in agreement with the results presented here. Note that, in our laboratory, these currents have previously been confirmed as glutamatergic as they were blocked by the AMPA receptor antagonist CNQX. P2X antagonists could not be included in the recording solution, but glutamate epscs can be clearly distinguished from purinergic apsc due to their different decay kinetics.

The rise times of these currents are much faster than those measured in CA1 pyramidal cells (Hestrin *et al.*, 1990) although they do not quite approach those measured in cortical stellate cells, where the synapses are on or close to the soma (mean 20-80% rise time = 0.4ms, (Stern *et al.*, 1992)); nonetheless, their speed indicates that they are relatively close to the soma and are therefore likely to be well voltage-clamped. In most cells the latency to current onset was indicative of monosynaptic connections. This is consistent with the anatomical data which suggests that the glutamate input is from the posterior septal nuclei (Sperlágh *et al.*, 1998b). In a few cells the latency was indicative of polysynaptic connections although the delay could be accounted for by slower action potential conductance in some afferents. However, the correlation between latency and failure rate is consistent with a polysynaptic connection in some cells. If this is true, there must be intrinsic excitatory neurons within the medial habenula; these could be either glutamatergic or purinergic.

In most cells the amplitude histograms of the evoked currents were skewed with a modal amplitude between 8 and 16pA, similar to the quantal amplitude recorded in cortical stellate cells (Stern *et al.*, 1992) and CA3 pyramidal cells (Jonas *et al.*, 1993). This compares with the modal amplitude for minis of 6-12pA. Assuming that the mini amplitude represents the release of one quantum, this suggests that the modal quantal content of the evoked currents is either one or two. In most cells the largest unitary currents were some 40-50pA, suggesting that the number of release sites is probably less than 10; in a few cells the narrow spread around the modal amplitude suggests that it can be as few as one or two.

Chapter 4

The Effect of Uridine Nucleotides on Glutamatergic Transmission

This section contains the results of the series of experiments investigating the effects of the uridine nucleotides UTP and UDP on glutamatergic transmission. These are agonists at a subset of P2Y receptors and are inactive at P2X receptors (see Introduction and table 1); they are therefore useful pharmacological tools for distinguishing between the P2 receptor subtypes. It should be noted, however, that the $P2Y_2$ and $P2Y_4$ receptors are equisensitive to UTP and ATP, and the latter may be the physiological ligand.

4.1 Effects of UTP on glutamate transmission

The initial hypothesis was that UTP inhibits glutamate transmission by a presynaptic mechanism involving P2Y receptors. Currents were evoked using minimal stimulation so that changes in failures could be measured to test for a possible presynaptic effect. In addition, the coefficient of variation and the quantal amplitude of the currents were determined as these parameters are also indicative of the locus of action of an effect.

Having determined the effects on failures, some subsequent experiments used non-minimal stimulation as this gives a higher signal-to-noise ratio and also makes it easier to monitor drug effects during the course of an experiment.

As an additional test for a receptor-dependent effect, the non-selective P2 receptor antagonist RB2 was used to try and bock the effects of UTP.

4.1.1 High concentrations of UTP inhibit glutamate transmission presynaptically

UTP (200μM) was tested on minimally-evoked glutamate currents; currents were evoked at different frequencies (0.5, 1 and 2Hz) to test for frequency-dependent effects; 100 events were recorded at each frequency.

UTP significantly increased the percentage of failures (absolute increases in failures: 0.5Hz, 17 ± 7; 1Hz, 19 ± 8; 2Hz, 14 ± 2; n=5 cells, p=0.005, 2-way ANOVA, fig 4.1, A). In 2/3 cells held long enough the effect partially or fully reversed. The analysis revealed no interaction between the drug and the stimulation frequency i.e. UTP was equally effective at all three frequencies: in subsequent experiments, therefore, currents were evoked only at 1Hz.

To confirm that UTP was inhibiting glutamate transmission, the change in the average current amplitudes (including failures) was measured in the same cells as the failures were counted (fig 4.2). In all 5 cells UTP significantly reduced the average current amplitude (p=0.04, 2-way ANOVA); UTP was equally effective at all 3 frequencies tested.

In these experiments, both the medial and lateral slices of the medial habenula were used. All of the following experiments with UTP used the medial slice only.

4.1.2 Low concentrations of UTP potentiate glutamate transmission presynaptically

In contrast to the inhibition caused by 200μM UTP, lower concentrations (10 and 30μM) potentiated glutamate currents in a dose-dependent manner (fig 4.3,B; 10 μM: mean increase = 42 ± 11 %, n=10, p<0.001; 30 μM: mean increase = 51 ± 11 %, n=5, p<0.001, repeated measures ANOVA). Increasing the UTP concentration to 200μM reduced the currents towards, but not below, the control values (n=2, fig 4.3, A); current amplitudes increased again on switching back to 10μM UTP (n=2).

In six of these cells minimal stimulation was used and failures were counted. The effect of UTP mirrored its effect on averaged current amplitudes (fig 4.4): 10μM significantly reduced the number of failures (n=6, p=0.03, one-tailed t-test), 30μM caused a further reduction (n=3) and 200μM increased failures back towards the control value.

Fig 4.1 UTP inhibits glutamate currents presynaptically

UTP (200μM) increased the percentage of currents which failed at 3 different frequencies (n=5/5; p=0.005, 2-way ANOVA; large error bars reflect the spread of % failures between different cells). This effect was reversible.

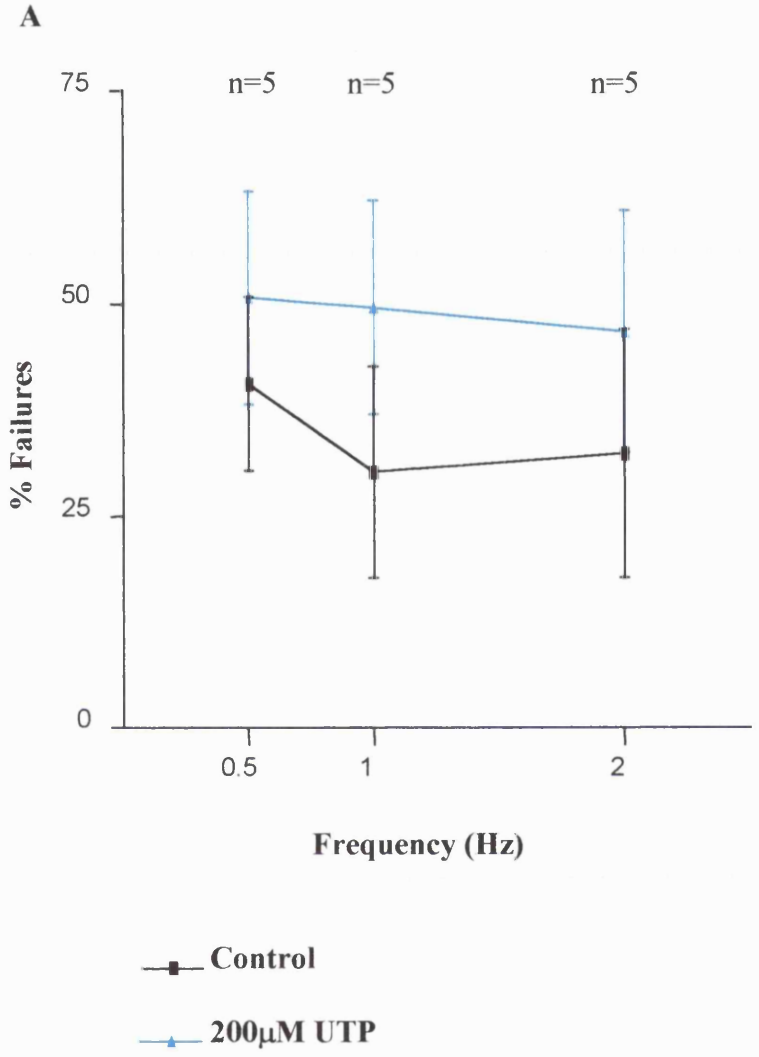
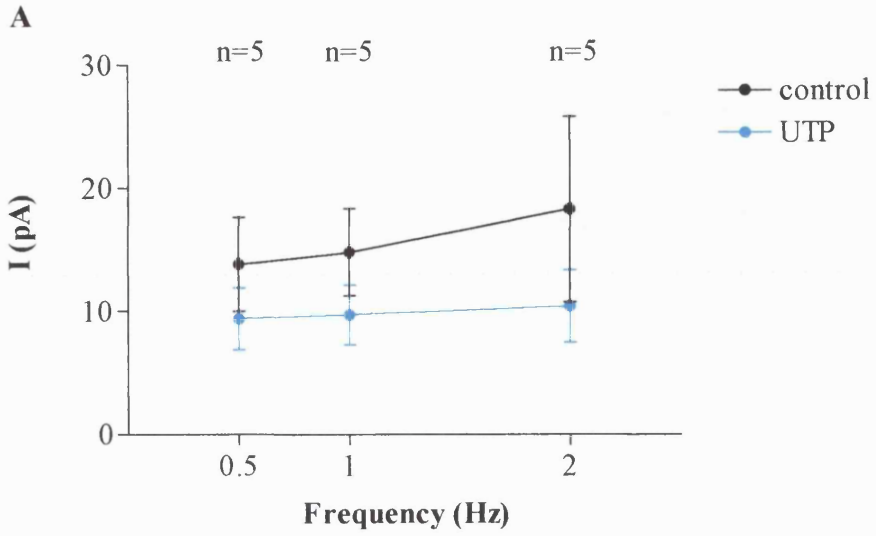

A
75
50
25
0
n=5
n=5
n=5
% Failures
0.5
1
2
Frequency (Hz)
Control
200μM UTP

Fig 4.2 The effect of 200μM UTP on glutamate current amplitudes

A. Data from the same cells as in fig 4.1. The effect of UTP on failures was mirrored by its effect on mean current amplitudes which were significantly reduced at all three frequencies ($p=0.04$, 2-way ANOVA).

B. Averaged current traces from one of the cells in A (black is control, blue is UTP). In this cell the mean current amplitude was reduced by 18%. Each trace is the average of 100 events.


A
n=5
n=5
n=5
30
20
10
0
I (pA)
0.5
1
2
Frequency (Hz)
control
UTP

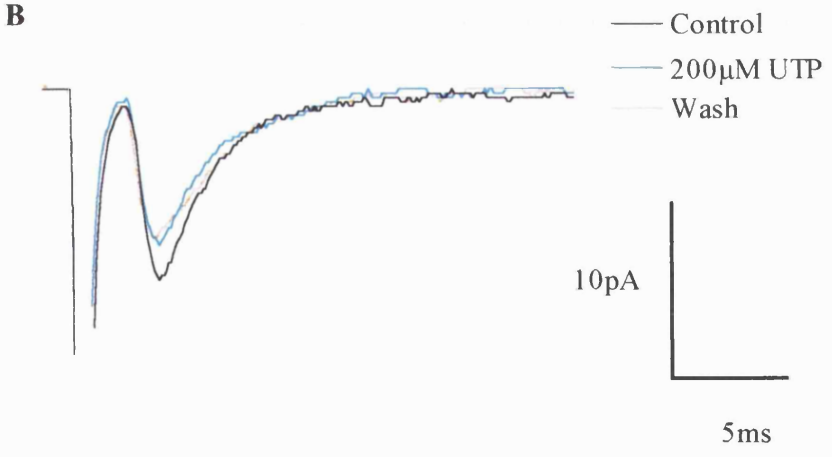
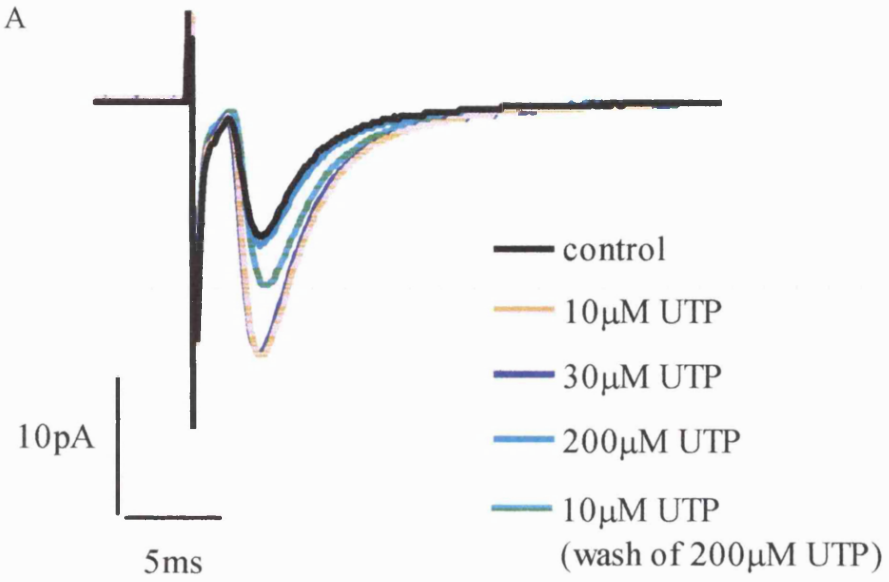

B
Control
200μM UTP
Wash
10pA
5ms

Fig 4.3 Dose-dependent effects of UTP on glutamate currents

A. Dose-dependent effects of UTP on glutamate currents from a single medial habenula neuron. Traces are averages of 100 currents, evoked at 1Hz. 10μM UTP (red) facilitated the current amplitude by 187 %, 30μM (dark blue) caused no further facilitation, 200μM (light blue) reduced the current amplitude back to the control value and this inhibition reversed on returning to 10μM UTP (green).

B. Data from all cells. 10 μM and 30 μM UTP facilitated the glutamate currents (10 and 30μM: $p<0.001$, repeated measures ANOVA). Increasing the concentration to 200μM reduced the current amplitudes towards control values, an effect which reversed on returning to 10μM UTP.


A
control
10μM UTP
30μM UTP
200μM UTP
10μM UTP
(wash of 200μM UTP)
10pA
5ms

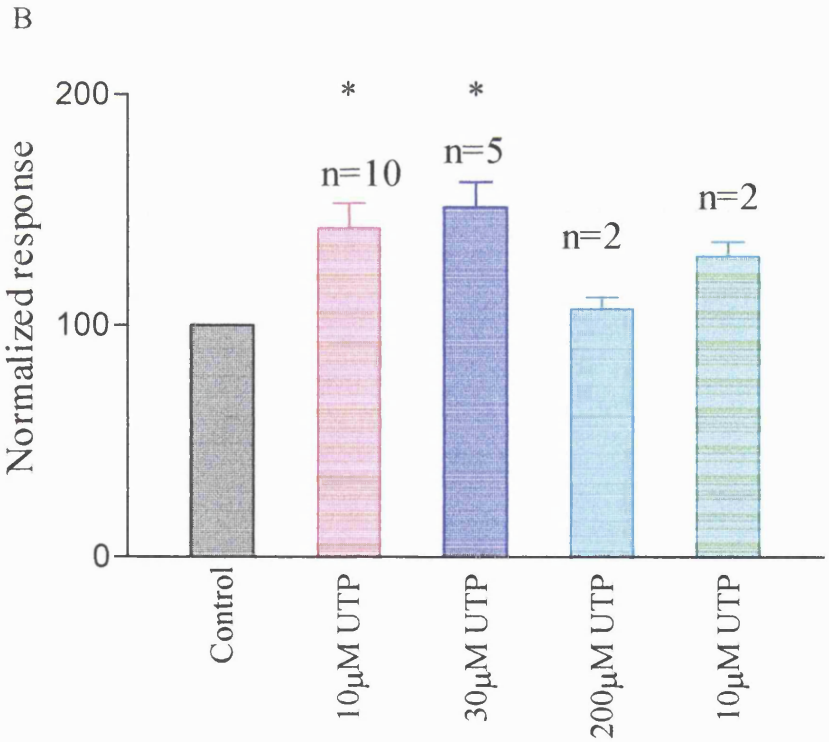
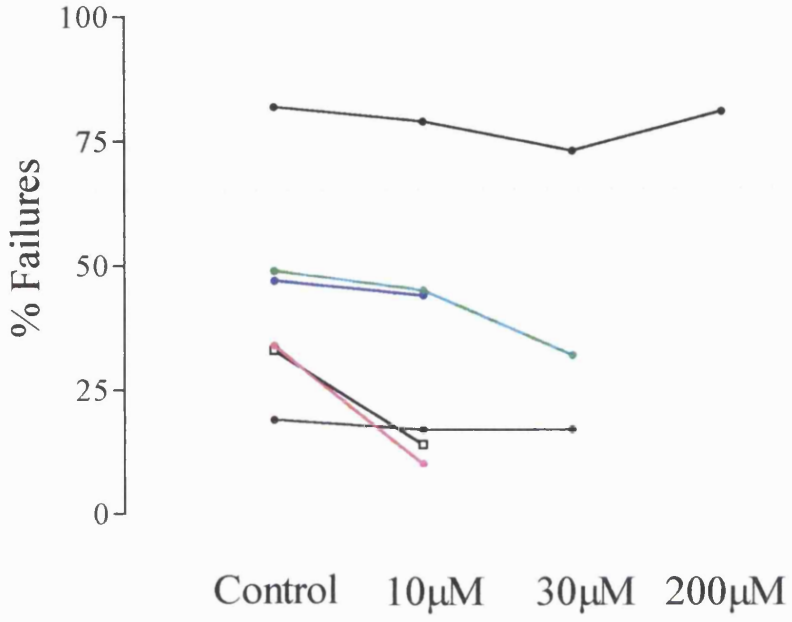

B
Normalized response
200
100
0
*
*
n=10
n=5
n=2
n=2
Control
10μM UTP
30μM UTP
200μM UTP
10μM UTP

Fig 4.4 Effect of UTP on current failures

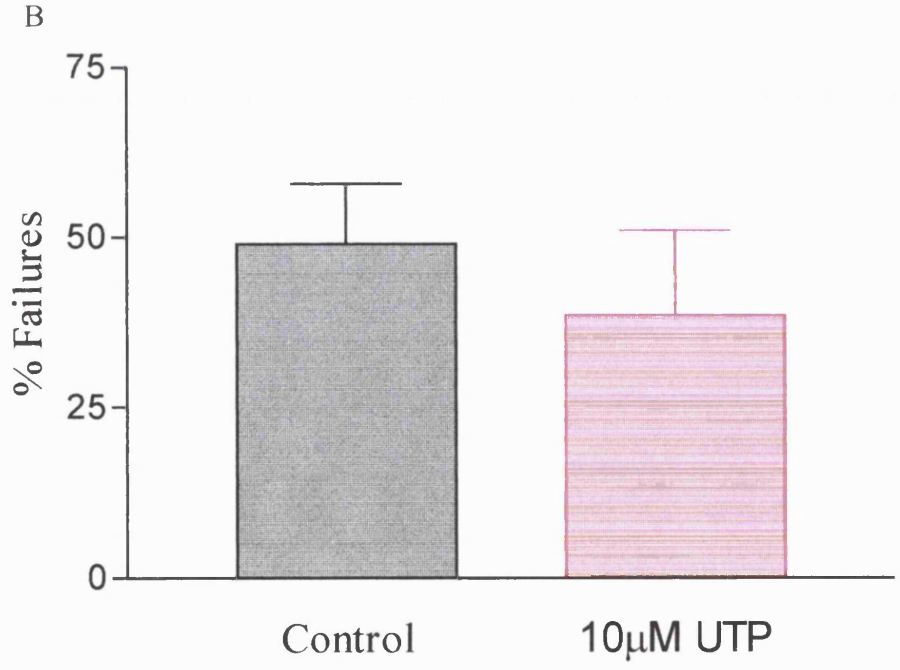
A. The effect of UTP on failures in individual cell. Data points from the same cell are joined with a line. Compared to control, the number of failures was increased by 10μM UTP and further increased by 30μM UTP. The effect of UTP on failures mirrored its effect on current amplitudes.

B. Summary of the data from A for 10μM UTP. Compared to control there was a significant decrease in the number of failures (n=6, p=0.03, one tailed t-test).

A


100
75
50
25
0
% Failures
Control
10μM
30μM
200μM

B


75
50
25
0
% Failures
Control
10μM UTP

To exclude the possibility of a postsynaptic effect being misinterpreted as a presynaptic effect, the actual amplitude distribution in UTP was compared with the predicted distribution assuming a purely post synaptic effect. In 5/6 cells there were fewer currents in a given bin in the actual distribution compared to the predicted number from the theoretical distribution.

Additional analysis was carried out to pinpoint the locus of action of UTP.

First, the coefficient of variation (CV = standard deviation of current amplitudes / mean current amplitudes) of the currents was measured in control and test conditions. The increase in the mean current amplitude correlated significantly with a decrease in the CV (Spearman r = -0.80, p=0.0069, Fig 4.5, A). When the ratio of $1/CV^2$ was plotted against the ratio of the mean current amplitudes for control and test currents, the point fell in the quadrant predicted for a presynaptic change.

Secondly the quantal amplitude was calculated in control and test conditions based on the assumption that the underlying amplitude distribution of the currents could be described by a Poisson distribution. Application of 10μM UTP caused no significant decrease in the quantal amplitude (n=6, p=0.06, Wilcoxon matched pairs test, fig 4.5, B). This is inconsistent with a postsynaptic potentiation.

4.1.3 Facilitation by UTP is long lasting

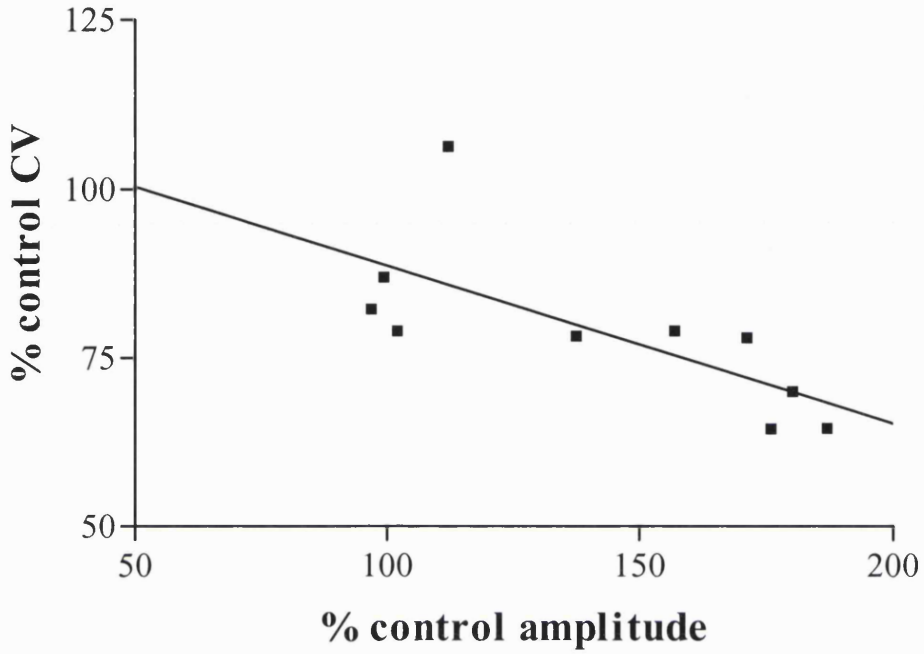
In cells in which 10μM UTP was applied and then washed out, the currents remained facilitated 50 minutes after switching back to the control solution (n=4, fig 4.6).

Fig 4.5 UTP reduces the coefficient of variation of glutamate currents

A. There was a significant correlation between the increase in current amplitude and decrease in CV caused by 10μM UTP (Spearman $r = -0.80$, $p=0.0069$). This is consistent with a presynaptic effect of UTP.

B. 10μM UTP caused no change in the quantal amplitude of glutamate currents ($n=6$, $p=0.06$, Wilcoxon matched pairs test. This is inconsistent with UTP causing a postsynaptic potentiation.

A


125
100
75
50
% control CV
50
100
150
200
% control amplitude

B

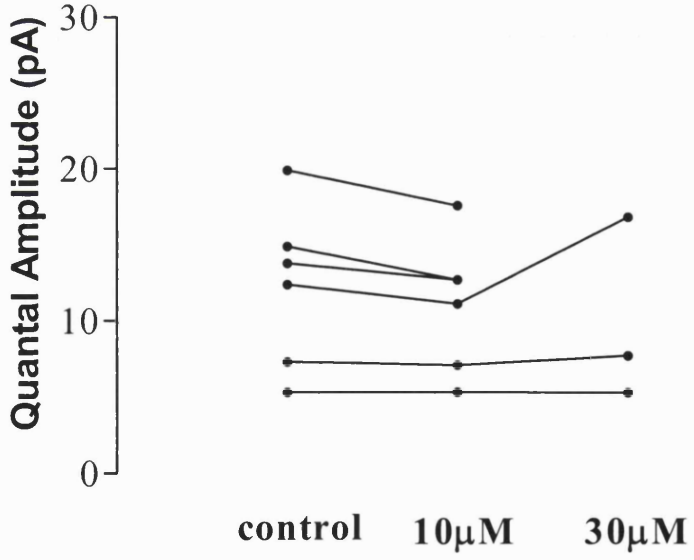
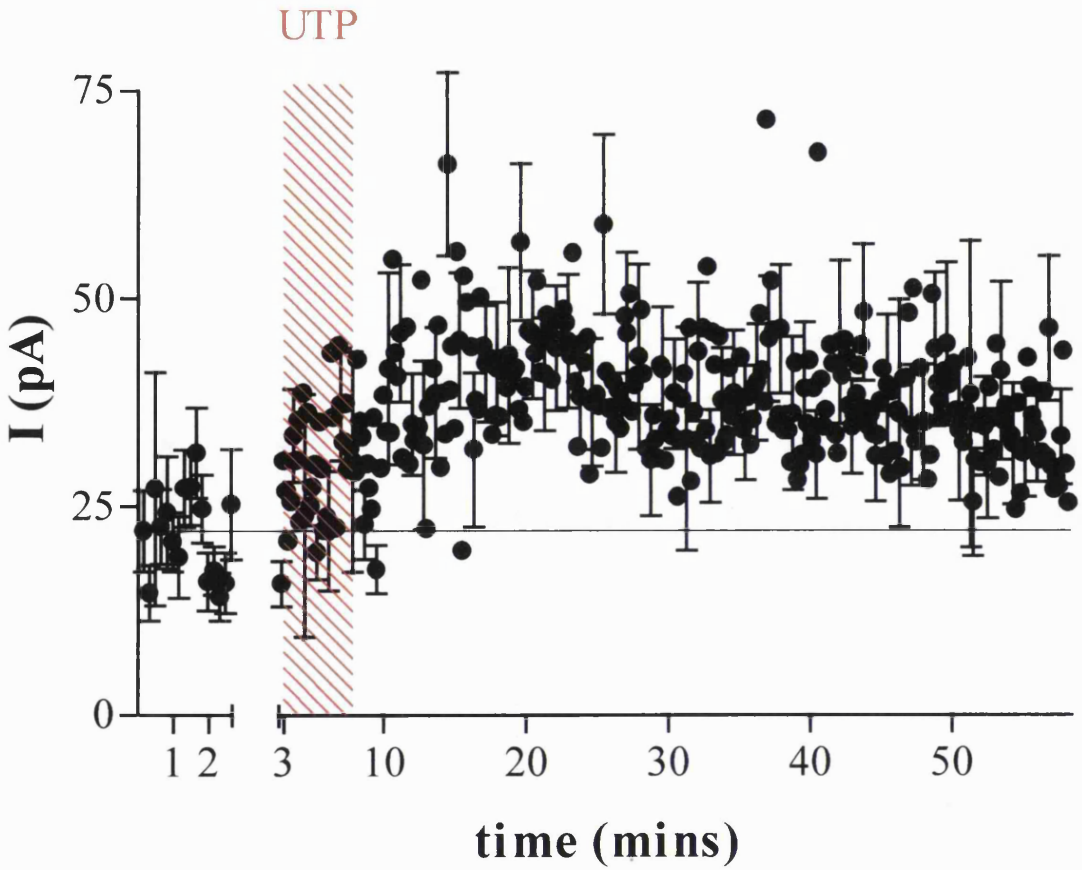

30
20
10
0
Quantal Amplitude (pA)
control
10μM
30μM

Fig 4.6 Long-lasting facilitation of glutamate currents by UTP

Each data point is the mean amplitude of 10 consecutive currents. The horizontal dashed line is the mean of the control current amplitudes. 10μM UTP was applied during the red striped section and then washed out for 50 minutes at which time currents were still potentiated compared to control currents. For clarity, the x-axis has been expanded over the control section and standard error bars are included on every 5^{th} point.


UTP
75
50
25
0
I (pA)
1 2
3
10
20
30
40
50
time (mins)

4.2 UTP and Reactive Blue 2

The non-selective P2 antagonist Reactive Blue 2 (RB2, 100μM) was used to try and block both the inhibitory and facilitatory effects of UTP. The effect of RB2 alone on glutamate currents varied between individual cells but across all cells there was no significant effect.

4.2.1 The inhibitory effects of UTP are potentiated by Reactive Blue 2

The data above showed that 200μM UTP inhibited glutamate currents. When the same concentration of UTP was applied in the presence of RB2 (100μM), rather than the inhibition being blocked, it was enhanced (fig 4.7, A). Currents were significantly smaller in UTP + RB2 compared to those in RB2 alone (control = 100%, RB2 = 106 ± 14%, RB2 + UTP= 38 ± 10%, $p<0.01$, repeated measures ANOVA, n=6); this is likely to be an underestimate of the inhibition as in four cells the currents were still decreasing in amplitude when washout of UTP began. In comparison, 200μM UTP alone (see above) inhibited glutamate currents by only 27 ± 11% (n=5); the inhibition by UTP was significantly greater with RB2 present ($p=0.047$, unpaired t-test).

This effect of UTP in the presence of RB2 was not reversible.

4.2.2 The facilitatory effects of UTP are blocked by Reactive Blue 2

RB2 was used to try and block the facilitation caused by 30μM UTP (fig 4.7, B).

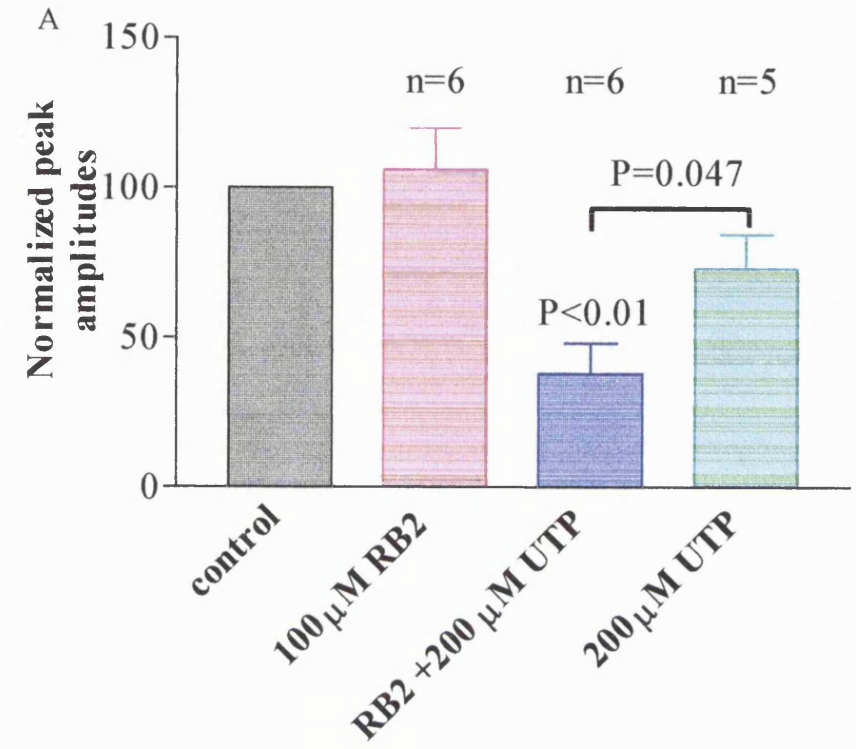
When UTP was applied in the presence of RB2, glutamate currents in UTP + RB2 were significantly smaller compared to those measured in RB2 alone: control = 100%, RB2 = 118 ± 23%, UTP + RB2 = 46 ± 19% (n=4, $p<0.05$, repeated measures ANOVA). Thus, not only did RB2 block the facilitation previously seen with 30μM UTP, but it appeared to unmask an inhibitory effect.

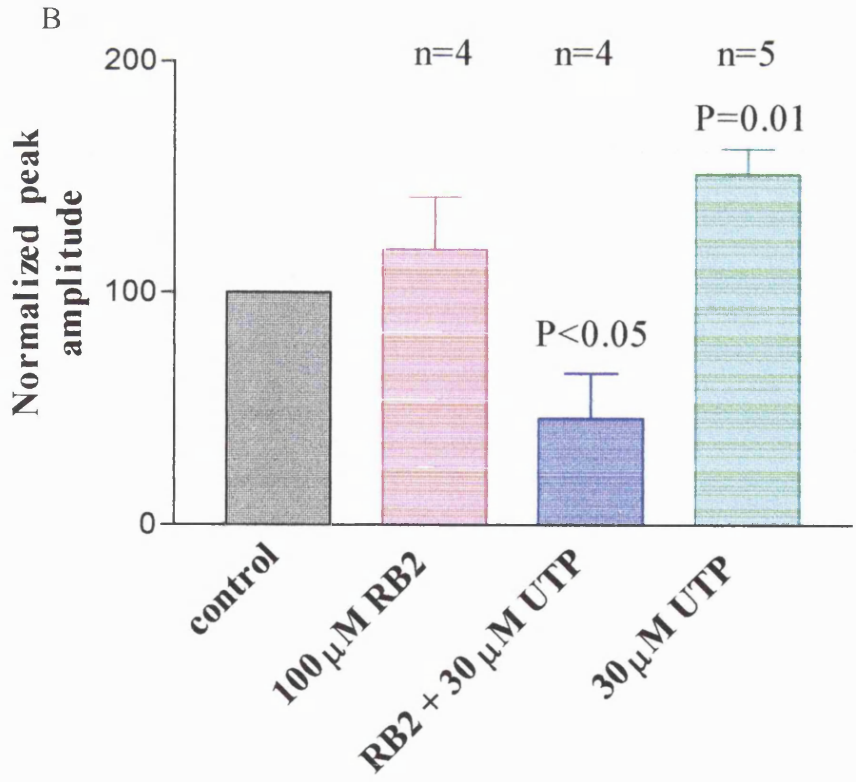
Fig 4.7 RB2 blocks facilitatory but not inhibitory effects of UTP

100μM RB2 alone had no significant effect on glutamate currents (red columns).

A. In the presence of RB2, 200μM UTP (blue column) inhibited glutamate currents ($p<0.01$, repeated measures ANOVA). For comparison, the green column shows the inhibition in the absence of RB2. The inhibition is slightly but significantly larger in the presence of RB2 ($p<0.05$, unpaired t-test)

B. In the presence of RB2, 30μM UTP (blue column) significantly inhibited glutamate currents ($n=4$, $p<0.05$, repeated measures ANOVA). For comparison, the green column is the data from fig 6.3, showing the facilitation of glutamate currents by 30μM UTP in the absence of RB2.


A
150
100
50
0
Normalized peak amplitudes
n=6
n=6
n=5
P=0.047
P<0.01
control
100 μM RB2
RB2 +200 μM UTP
200 μM UTP


B
200
100
0
Normalized peak amplitude
n=4
n=4
n=5
P=0.01
P<0.05
control
100 μM RB2
RB2 + 30 μM UTP
30 μM UTP

4.3 Effects of UDP on glutamate transmission

As outlined in the introduction, UTP is rapidly metabolized by a chain of enzymes and at least one of these metabolites, UDP, is an extracellular signalling molecule: it is necessary, therefore, to exclude the possibility that any effect attributed to UTP is not actually caused by a metabolite. To test this, the experiments performed with UTP were repeated using UDP: if an apparent UTP effect is really caused by a metabolite then the prediction is that UDP would cause a qualitatively similar effect.

4.3.1 200μM UDP facilitates minimally-evoked glutamate currents by a presynaptic mechanism

When tested under identical conditions as UTP (minimal stimulation, 0.5, 1 and 2Hz) UDP caused a decrease in the percentage of failures (fig 4.9); this was the opposite effect to 200μM UTP. The facilitation was significant (n=5, p=0.01, 2-way ANOVA) but there was no interaction i.e. the effect was not different across the three frequencies.

The effect of UDP was tested in a further 9 cells in which currents were evoked at just one frequency (0.5Hz: n=1 and 1Hz: n=8) using minimal simulation. The effect was the same, with UDP decreasing the number of failures in 8/9 cell (p=0.04, Wilcoxon signed rank test).

The change in the average current amplitude (including failures) was measured in the same cells in which failures were counted; the one cell in which failures increased was excluded from the analysis. UDP increased the average current amplitude by 41.6 ± 16.7 % (n=13, p=0.028, one sample t-test).

4.3.2 Dose-dependent effects of UDP

Having determined the effect of 200μM UDP under the same conditions as 200μM UTP had previously been investigated, the effect of lower doses (10 and 30μM) of UDP were tested. To determine whether the effect of UDP was altered by recruiting more fibres, each dose was tested on both

minimally- and supraminimally-evoked currents. These experiments were done on the medial of the two slices only and the effect of 200μM UDP was also tested.

200μM UDP potentiated glutamate currents evoked with supraminimal stimulation in all 9 cells tested (fig 4.8, A): the mean increase was 47.4 ± 6.9 % (p=0.0001, one sample t-test).

At minimal stimulation 200μM UDP also significantly potentiated glutamate transmission (fig 4.8, A): the mean increase was 37 ± 15.8 % (n=9, p=0.048, one sample t-test). To further confirm that the effect of UDP was independent of the stimulus protocol, in 5 cells common to both data sets minimally- and supraminimally-evoked currents were interleaved in the same recording: UDP had the same effect on each set of currents.

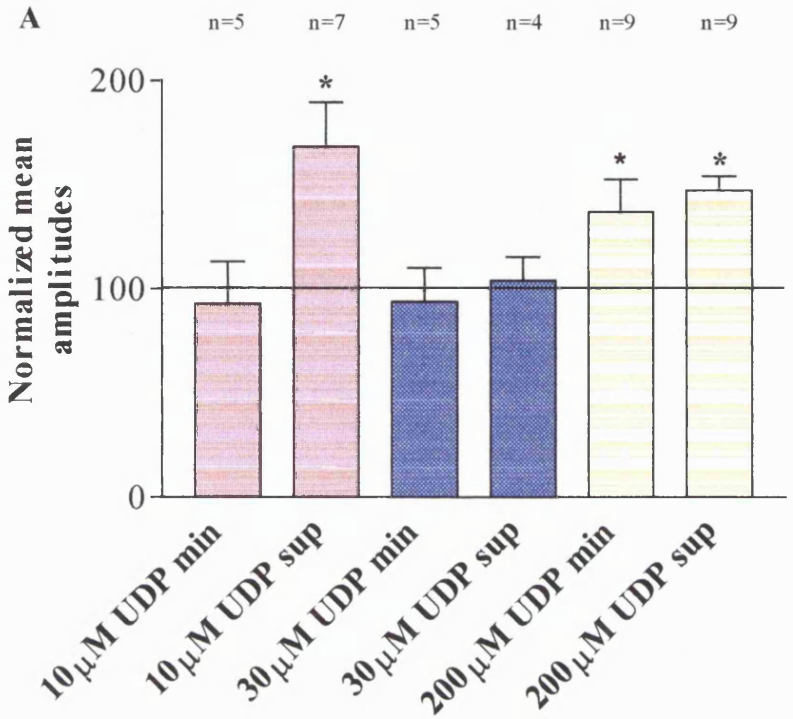
When currents were evoked using minimal stimulation, the facilitation of the current amplitudes was accompanied by a significant decrease in the number of failures (p=0.04, paired t-test, fig 4.9, A): this is indicative of a presynaptic effect. As additional tests of the locus of action of UDP, the coefficient of variation and the quantal amplitude of the current amplitudes were determined (fig 4.9, B and C). The facilitation of the currents was accompanied by a decrease in their coefficient of variation and there was a significant inverse correlation between these two parameters (Spearman r = -0.79, p=0.03). Furthermore, UDP did not alter the quantal amplitude (p=0.9, paired t-test). This analysis is consistent with UDP acting presynaptically.

Lower doses of UDP (10 and 30μM) were also tested (fig 4.8). When using minimal stimulation, the effect of both 10 and 30μM UDP varied between cells but overall was not significantly different to control. However, when supraminimal stimulation was used, the dose response curve was U-shaped, with 10μM UDP potentiating the currents even more than 200μM (68.6 ± 21.2 %, n=7, one sample t-test, fig 4.8,A).

Fig 4.8 Potentiation of glutamate currents by UDP and block by RB2

A. Dose-dependent effect of UDP on glutamate currents. UDP (10, 30 and 200μM) was tested on glutamate currents evoked with either minimal or supraminimal stimuli. 200μM UDP significantly enhanced minimally- and supraminimally-evoked currents (yellow columns, p= 0.048 and P=0.0001 respectively, one-sample t-tests). 10μM UDP also enhanced supraminimally evoked currents (red column, right, p=0.018, one-sample t-test) but not minimally-evoked currents (red column, left). Currents evoked in the presence of 30μM UDP (blue columns) were not significantly different from control.

B. RB2 blocks the UDP-induced facilitation of glutamate currents. Glutamate currents recorded in the presence of RB2 (100μM) or RB2 + UDP (200μM) were not significantly different from control values (n=5, p=0.7, one-way ANOVA).


A
n=5
n=7
n=5
n=4
n=9
n=9
200
100
0
Normalizæd mean amplitudes
*
*
*
10 μM UDP min
10 μM UDP sup
30 μM UDP min
30 μM UDP sup
200 μM UDP min
200 μM UDP sup

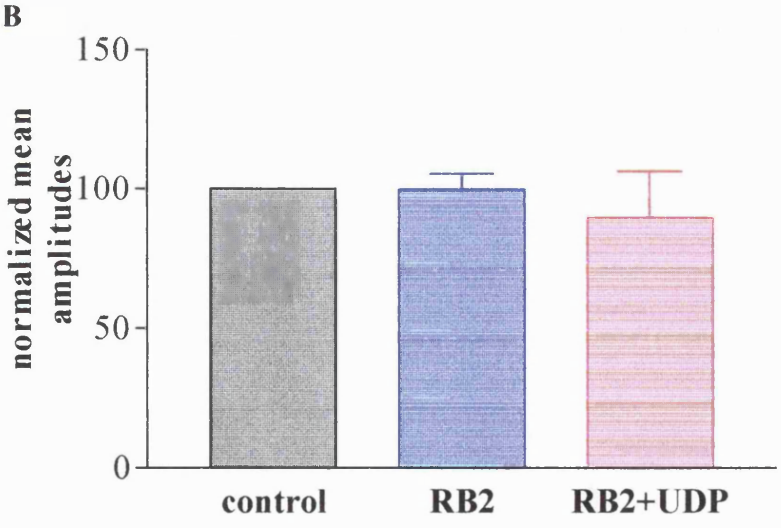
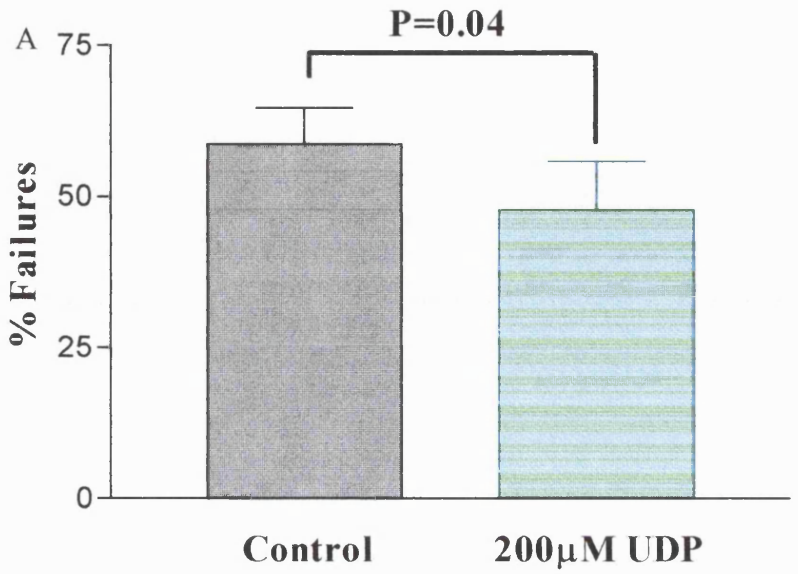

B
150
100
50
0
normalizæd mean amplitudes
control
RB2
RB2+UDP

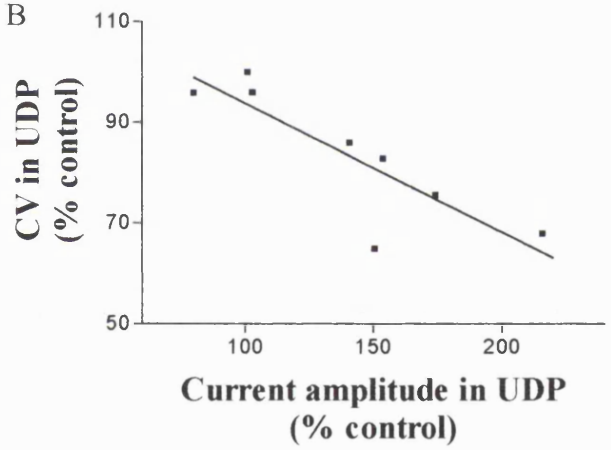
Fig 4.9 Presynaptic facilitation of glutamate currents by UDP

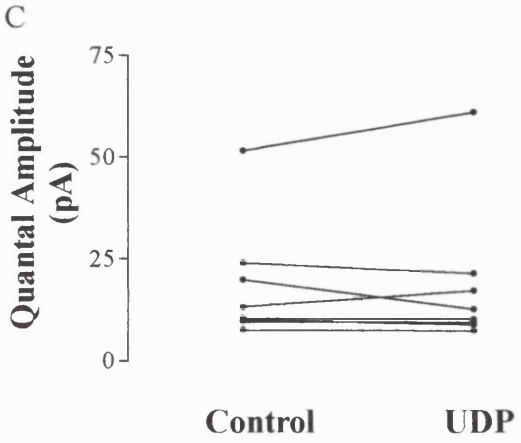
A. The facilitation of glutamate currents by 200μM UDP was accompanied by a significant decrease in the number of failures (n=8, p=0.04, paired t-test).

B. In the same cells as UDP decreased the number of failures, it also decreased the coefficient of variation of variation (CV) of the current amplitudes: there was a significant inverse correlation between the effect of UDP on current amplitudes and its effect on their CV (Spearman r = -0.79, P=0.03).

C. 200μM UDP had no significant effect on the quantal amplitude of glutamate currents.


A
P=0.04
75
50
25
0
% Failures
Control
200μM UDP

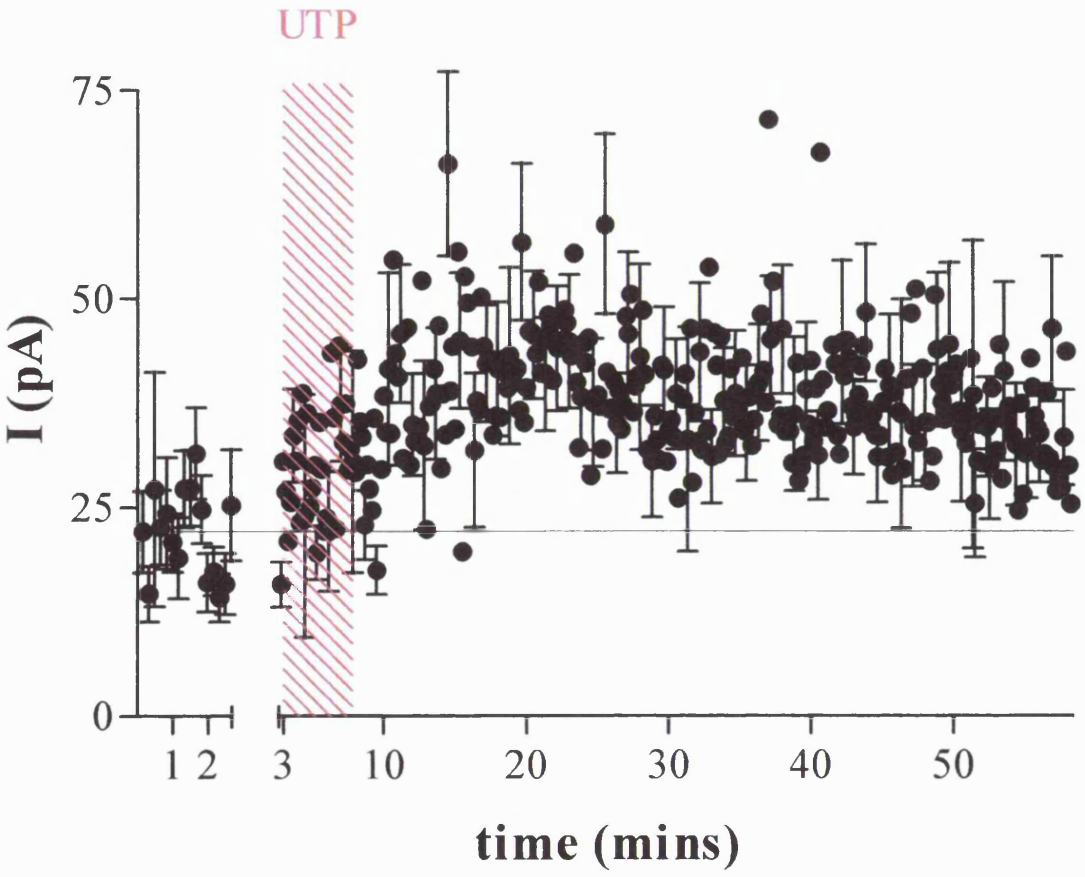

B
110
90
70
50
CV in UDP
(% control)
100
150
200
Current amplitude in UDP
(% control)


C
75
50
25
0
Quantal Amplitude
(pA)
Control
UDP

96

Fig 4.10 Long-lasting facilitation of glutamate currents by UDP

Each data point is the mean amplitude of 10 consecutive currents. The horizontal dashed line is the mean of the control current amplitudes. 200μM UDP was applied during the red striped section and then washed out for nearly 50 minutes at which time currents were still potentiated compared to control currents. For clarity, the x-axis has been expanded over the control section and standard error bars are included on every 5^{th} point.


UTP
75
50
25
0
I (pA)
1 2
3
10
20
30
40
50
time (mins)

4.3.3 The facilitation of glutamate currents by UDP is long-lasting

Six cells in which 200μM UDP potentiated glutamate currents were held for long washouts of the drug (>20 minutes). In two cells the effect reversed within 20 minutes or less; however, in four cells the effect did not reverse: this includes two cells in which the current amplitudes were still potentiated over 45 minutes after washing out the UDP (fig 4.10).

4.4 RB2 blocks the facilitation of glutamate currents by 200μM UDP

RB2 (100μM) was tested to see if it blocks the facilitation of glutamate currents by 200μM UDP (fig 4.8B): glutamate currents measured in the presence of RB2 or in the presence of RB2 + UDP were not significantly different from control values (n=5, p=0.7, repeated measures ANOVA).

4.5 Discussion

From the results presented in this section the following conclusions can be drawn.

1. Low concentrations of UTP (10μM and 30μM) potentiate glutamate release by a presynaptic mechanism.
2. A higher concentration of UTP (200μM) inhibits glutamate release by a presynaptic mechanism.
3. A high concentration of UDP potentiates glutamate release by a presynaptic mechanism.
4. The potentiation by both UTP and UDP is inhibited by the P2 antagonist RB2.
5. The inhibition by UTP is not blocked by RB2.
6. The potentiation by both UTP and UDP is long-lasting.

In the following paragraphs the validity of these conclusions is considered along with a model which is consistent with the results.

4.5.1 Potentiation of glutamate transmission by UTP

4.5.1.1 Presynaptic Effect

10μM and 30μM UTP potentiate glutamate transmission. The concomitant change in failures indicates that UTP acts presynaptically to produce this effect. Calculations predicting the apparent effects of a postsynaptic change on the apparent number of current failures showed that such an effect is unable to fully account for the changes seen in these experiments. To further confirm the presynaptic locus of the effect, the coefficient of variation of the current amplitudes in control and in UTP were compared: the change in coefficient of variation decreased in UTP as is predicted for a presynaptic effect. However, this form of analysis must be treated with caution as it is based on a binomial model of release probability, with the simplifying assumption that release probability (p) and quantal amplitude (q) are uniform across all release sites. It is, therefore, only possible to say that the change in the coefficient of variation is consistent with the conclusion that the potentiation is presynaptic.

An additional confirmation of a presynaptic locus could be provided by testing the effect of UTP on the paired-pulse ratio of glutamate currents. A presynaptic potentiation predicts that the ratio would decrease, whilst no change in the ratio would be compatible with a postsynaptic effect

Whilst a presynaptic increase in release probability is the simplest interpretation of the results, it cannot be completely discounted that other mechanisms underlie the potentiation. Either the addition of postsynaptic receptors to silent synapses (an entirely postsynaptic effect) or the formation of completely new synapses (a pre and post synaptic effect) could result in a decrease in failures.

4.5.1.2 Long-lasting Potentiation

The potentiation by 10μM UTP lasted for over 50 minutes. It is unlikely that the UTP had not washed out of the slice as it was possible to reverse the inhibitory effect of 200μM UTP. This is, therefore, a genuinely long-lasting potentiation.

As discussed in the Introduction, several studies have previously demonstrated that LTP can be induced in hippocampal CA1 neurons by the application of ATP (Wieraszko & Seyfried, 1989;Wieraszko & Ehrlich, 1994;Fujii *et al.*, 1999). However, the evidence suggests that ATP does not act at a P2 receptor but rather acts as a phosphate donor for an ecto-protein kinase. The UTP-induced potentiation described in this section therefore differs from this previously described ATP-induced LTP in that it is blocked by a P2 antagonist, RB2; this argues in favour of a receptor-dependent effect. However, as it is not yet known whether UTP acts as a phosphate donor for ecto-protein kinases and as there is some evidence that RB2 might inhibit such an enzyme in the vas deferens (Bultmann et al., 2002), it can not be totally excluded that UTP is acting in such a manner. Nontheless, this phenomenon immediately raises a number of questions and suggests further avenues of research; these are discussed in the final chapter.

4.5.1.3 Interactions between inhibition and potentiation

When 200μM UTP was applied to naïve slices, currents were inhibited by 27% relative to the control amplitudes; however, when 200μM UTP was applied to slices previously exposed to 10μM and 30μM UTP, currents were similarly inhibited by 30% but relative to their amplitudes in 30μM UTP. In both protocols the slice was exposed to 10μM and 30μM UTP prior to 200μM; why therefore was inhibition not the same, relative to control amplitudes, in each protocol? A possible explanation could lie with the difference in time course of the drug applications. One could propose a time window during which the facilitatory mechanism can be reversed but at the end of which a molecular switch is thrown making the process

irreversible (at least by higher UTP concentrations). Under this hypothesis, the inhibitory pathway would actually reverse or inhibit the facilitatory pathway rather than simply mask it. A full understanding of these effects may emerge with experiments to elucidate the intraterminal pathways involved. Some candidate pathways are discussed in chapter 7.

4.5.1.4 P2X or P2Y receptor

The potentiation was blocked by the P2 antagonist, RB2, demonstrating that UTP was acting through a P2 receptor. RB2 does not distinguish between P2X and P2Y receptors (table 1); both uridine nucleotides are generally considered to be selective for P2Y receptors although there are reports that UTP can activate $P2X_1$ and $P2X_3$ receptors at concentrations of 10μM and above (McLaren *et al.*, 1998;Robertson *et al.*, 1996). What is the probability that UTP is acting at a P2X rather than a P2Y receptor in the experiments reported here? The studies in which UTP showed weak agonist activity at $P2X_1$ and $P2X_3$ receptors were carried out on acutely dissociated cells (smooth muscle cells and DRG neurons) where there is less ectonucleotidase activity than in a slice: it is reasonable to assume that a considerably higher concentration would be required to produce the same effect in a slice. In addition, both the $P2X_1$ and $P2X_3$ receptors desensitize rapidly and recover from desensitization only very slowly: following bath application of UTP, all the receptors in the slice would rapidly desensitize and remain so. It is unlikely, therefore, that UTP acted through either of these P2X receptors; however, the possibility that the application of UTP leads to a very brief activation of P2X receptors which is responsible for the potentiation cannot, at present, be entirely eliminated.

Of the uridine nucleotide-sensitive P2Y receptors, $P2Y_4$ and $P2Y_6$ are blocked by RB2 whilst $P2Y_2$ is not. The $P2Y_6$ receptor is unlikely to underlie the potentiation by either UTP or UDP because its sensitivity to these nucleotides is in the nM range (Nicholas *et al.*, 1997;Filippov *et al.*, 1999); it is therefore unlikely to require as much as 200μM UDP to

produce a consistent facilitation (see below). The most probably candidate receptor is therefore $P2Y_4$.

RB2 was selected as an antagonist because, from the literature, it seemed the one most likely to block effects of UDP. As discussed in chapter 5, it is possible that RB2 has some antagonistic activity at AMPA receptors. However, because current amplitudes in the presence of the nucleotides + RB2 have been compared to current amplitudes in RB2 alone, the conclusions drawn in this section about RB2's effects against UTP and UDP remain valid.

4.5.2 Inhibition of glutamate transmission by UTP

200μM UTP inhibits glutamate transmission: the accompanying increase in failures indicates that this is also a presynaptic effect. However, this inhibition was not blocked by RB2 and until a block of this effect by one of the other P2 antagonists is demonstrated, it can not be concluded for certain that it is receptor-dependent. However, the simplest interpretation is that this inhibition occurs via a P2Y receptor, but a different one to that responsible for the potentiation seen at lower concentrations:
the best candidate is the RB2 insensitive $P2Y_2$ receptor.

4.5.3 Potentiation of glutamate release by UDP

200μM UDP potentiates glutamate transmission by a presynaptic mechanism. The evidence that this is presynaptic is also based primarily on the decrease in failures and supported by the coefficient of variation analysis. In respect of these analyses, the arguments and caveats which have already been addressed for the UTP experiments have the same relevance to these experiments.

Similar to the potentiation seen with 10μM UTP, this UDP-induced potentiation is long-lasting: in 4 cells there was no reversal of the effect in over 50 minutes of wash. However, in two cells the effect did reverse within approximately 10 minute of washing, arguing that this

concentration of UDP can be washed out of the slice. (The effects of 200μM UTP also reversed confirming that it is possible to wash high concentrations out of the slice).

At lower concentrations (10μM and 30μM) the effect of UDP varied from potentiation, through no effect, to inhibition. Unexpectedly, however, when currents were evoked with a supraminimal stimulus the effect of 10μM UDP was consistently to potentiate transmission. The nature of this potentiation has not yet been further investigated: at present the locus of the effect (pre- or postsynaptic) remains to be determined, as does its sensitivity to RB2 and it's reversibility.

As stated above, the UDP-induced potentiation is unlikely to be mediated by the $P2Y_6$ receptor. The simplest explanation is that UDP acts at the same receptor as UTP but with lower potency, as suggested in the model below.

4.5.4 An All-embracing Model ?

Is it possible to construct a model incorporating UDP, UTP and P2Y receptors to account for the effects which have been described? The answer is yes and there are many possibilities because of the number of variable parameters: for example, there are three uridine sensitive P2Y receptors; UTP and UDP are interconvertable; RB2 antagonises $P2Y_4$ and $P2Y_6$ receptors as well as inhibiting ectonucleotidases. However, the most parsimonious model (illustrated in fig 4.11) is one in which there is one presynaptic facilitatory receptor, the RB2-sensitive $P2Y_4$ receptor and one presynaptic inhibitory receptor, $P2Y_2$. At low UTP concentrations facilitation predominates whilst inhibition predominates at higher concentrations. This is reminiscent of some forms of LTP and LTD where the magnitude of the Ca^{++} influx determines whether transmission is depressed or potentiated (Teyler *et al.*, 1994), except that LTP is favoured by high Ca^{++} concentrations and vice versa.

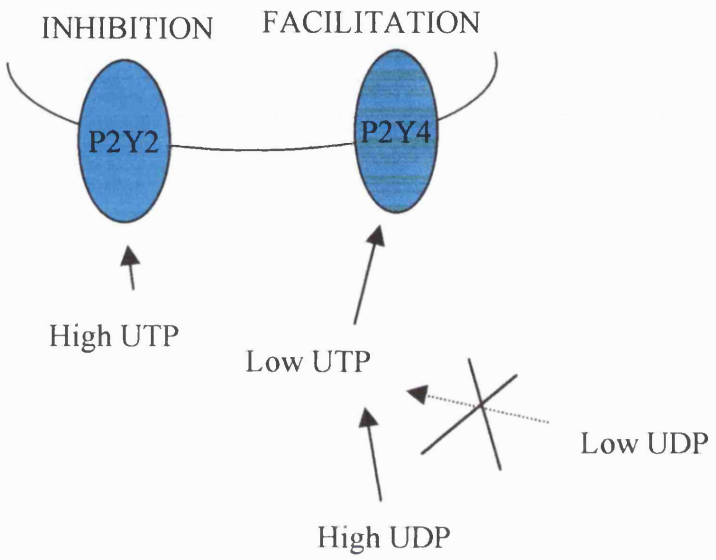
Whilst the $P2Y_4$ receptor is more sensitivity to UTP than UDP, a high UDP concentration also activates it, either directly (fig 4.11, B) or through conversion to UTP (fig 4.11, A); this accounts for the potentiation by 200μM UDP; in contrast, lower concentrations of UDP are insufficient to activate the receptor directly and also produce insufficient UTP to activate it. In fact, if the effect of UDP is primarily, or exclusively, via conversion to UTP and a subsequent action at the $P2Y_4$ receptor, this might partially explain the variable effect of UDP (ranging from facilitation through no effect to inhibition) seen with lower concentrations: 200μM might be sufficient UDP to produce UTP at all synapses; lower concentrations of UDP might produce varying amounts of UTP, perhaps depending on the set of ectonucleotidases present at a particular synapse. An alternative explanation, also depending on the ectonucleotidases, is that UDP acts directly at the $P2Y_4$ receptor but that synapses vary in the activity of the ecto-ADPase which metabolises dinucleotides: consequently synapses would be exposed to varying amounts of UDP.

An unexpected result is the different effects of 10μM UDP depending on the stimulating protocol (a variable effect with minimal stimulation but a consistent facilitation with supraminimal stimulation). A possible explanation, which requires no additional receptors, is that UDP increases the threshold for stimulating fibres (fig 4.12); this hypothesis would also explain why inhibition is sometimes seen. A minimal stimulating protocol is necessarily close to threshold for stimulating a single fibre, such that increasing this threshold may prevent the fibre from firing in response to every stimulus; this is effectively a presynaptic inhibition. Consequently there will be a balance between the facilitation and this inhibition and a varied effect depending on which is the larger. In contrast, fibres stimulated with a supraminimal stimulus may be less susceptible to a change in the stimulating threshold because the stimulus is not by necessity close to threshold and, therefore, facilitation will predominate.

Fig 4.11 Candidate model for the effects of uridine nucleotides on glutamate transmission

High concentrations of UTP inhibit glutamate transmission through a presynaptic $P2Y_2$ receptor while low concentrations facilitate transmission through a presynaptic $P2Y_4$ receptor. UDP acts in one of two ways: by conversion to UTP which subsequently acts at the $P2Y_4$ receptor (A) or by acting directly on the $P2Y_4$ receptor (B). Low concentrations of UDP are unable to activate the $P2Y_4$ receptor and also produce insufficient UTP to do so.

A


INHIBITION
FACILITATION
P2Y2
P2Y4
High UTP
Low UTP
Low UDP
High UDP

B

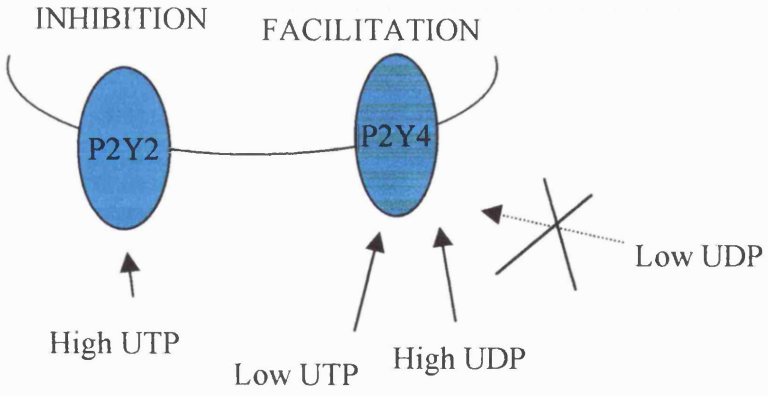

INHIBITION
FACILITATION
P2Y2
P2Y4
Low UDP
High UTP
Low UTP
High UDP

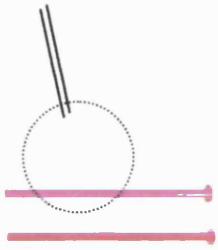
Fig 4.12 Possible effect of UDP on fibre threshold

A schematic representation of one possible model to explain the differing effects of UDP depending on the stimulus protocol. The stimulating electrode (parallel lines) stimulates any fibres lying within the dotted black circle. With minimal stimulation the stimulated fibre is close to threshold (A); applying UDP increases the threshold (fibre now lies on the edge of the circle) such that the some stimuli fail to induce an action potential. With supraminimal stimulation (B), the stimulating voltage is sufficiently above threshold that the fibres are still reliably stimulated even when this threshold is increased by UDP.

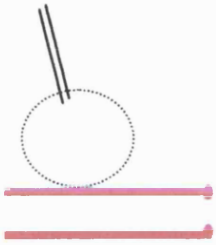
A

Minimal Stimulation

Control: every stimulus induces an AP



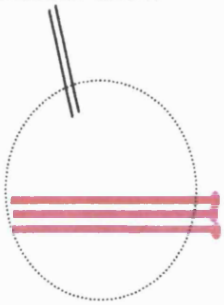
UDP: some stimuli may fail to induce an AP



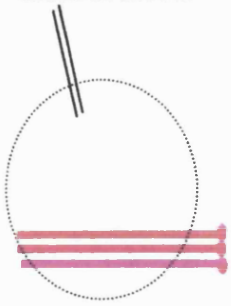
B

Supraminimal Stimulation

Control: every stimulus induces an AP



UDP: every stimulus induces an AP



In all of these models UTP and / or UDP act on P2Y receptors located on the presynaptic terminals; the arguments in favour of a presynaptic mechanism have already been discussed. However, the data do not exclude the possibility that the P2Y receptors are located somewhere other than on the presynaptic terminal (e.g. the postsynaptic cell or glial cells) and their activation leads to the release of an additional messenger which acts presynaptically. Nonetheless, the most likely model remains one in which activation of a presynaptic $P2Y_4$ receptor induces long-lasting potentiation of glutamate transmission. If a presynaptic $P2Y_2$ receptor is sufficiently activated in parallel, the potentiation is prevented and reversible inhibition predominates.

Chapter 5

THE EFFECT OF P2 RECEPTOR ANTAGONISTS ON GLUTAMATE TRANSMISSION

In Chapter 4 data were presented showing that exogenously applied uridine nucleotides exert a presynaptic control over glutamate release. In this section the question of P2 receptor-mediated control of glutamate transmission is further examined by studying the effect of two compounds acting at P2 receptors: PPADS, and α,β-methyleneATP. PPADS is an antagonist acting at some, but not all, P2X and P2Y receptors (see Introduction, table 1). α,β-methyleneATP is an agonist at the $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors; $P2X_1$ and $P2X_3$ rapidly desensitize so that α,β-methyleneATP effectively behaves like an antagonist, whilst it behaves as an agonist at the non-desensitizing $P2X_{2/3}$ receptor. It also acts as an antagonist at the non-desensitizing $P2X_4$ receptor. Importantly, it does not act at P2Y receptors so it can be used to distinguish between the two receptor families. Otherwise the pharmacological tools available are limited: there is no compound - either agonist or antagonist – which is active at all subunits of one type and inactive at all the other type of subunits.

The hypothesis being tested is that there are presynaptic P2 receptors modulating glutamate release. The use of antagonists tests the hypothesis that these receptors are tonically or intermittently activated by an endogenous ligand, presumably either ATP or another nucleotide. Two different experimental protocols were used, both of which can distinguish between pre- and postsynaptic effects: the first was to evoke glutamate currents using minimal stimulation so that changes in failures could be measured; the second was to record spontaneous miniature glutamate currents (minis) so that changes in their frequencies could be measured.

5.1 The effect of PPADS on evoked glutamate currents

PPADS (30μM) was tested on minimally-evoked glutamate currents (n=5 cells, fig 5.1, A); 100 events were counted in each condition. There was no significant effect of PPADS on current failures (p=0.9, Wilcoxon matched pairs test) although there was a significant increase in failures in one of the five cells.

5.2 The effect of PPADS on glutamate minis

PPADS (30μM) was tested on glutamate minis (n=8, fig 5.2, A). In 7/8 cells PPADS reversibly decreased the mini frequency by up to 50%. In one cell there was a large increase in frequency which increased further when PPADS was washed out. Excluding this cell from the analysis, PPADS significantly reduced the mini frequency ($p<0.01$, repeated measures ANOVA).

To determine the effect of PPADS on individual cells, the interevent intervals were compared in control and test conditions (fig 5.2, B). In 5 cells there was a significant shift towards longer interevent intervals in PPADS (Kolmogorov-Smirnov test), indicating that it was indeed reducing the mini frequency in these cells. In some cells, this was accompanied by a small but significant shift towards smaller amplitude minis. In individual cells, the cumulative distribution curves of amplitudes in control and in PPADS overlapped at low amplitudes and deviated at larger amplitudes (fig 5.2, C).

To confirm that a postsynaptic change in amplitude was not causing an apparent change in frequency the hypothetical amplitude distribution resulting from a purely postsynaptic effect was compared to the actual amplitude distribution in PPADS (see Methods). The two curves were always found to be significantly different (Kolomogorov-Smirnov test), showing that a purely postsynaptic effect of PPADS is insufficient to account for the observed changes in frequency.

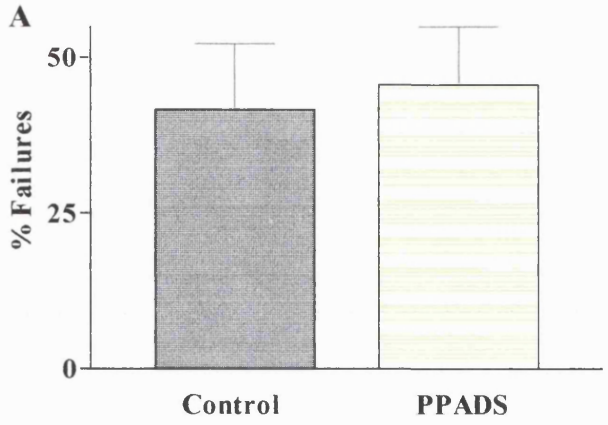
Fig 5.1 The effect of PPADS and αβmethyleneATP on evoked glutamate currents

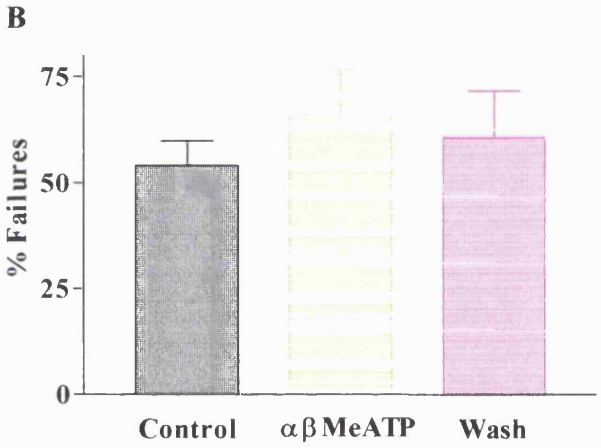
Currents were evoked with minimal stimulation and 100 events were analysed in control and test conditions.

A. Across all cells PPADS caused no significant change in failures (n=5, p=0.9, Wilcoxon matched pairs test). Analysis of individual cells showed that PPADS significantly increased failures in one cell.

B. αβmethyleneATP also caused no significant change in failures (n=6, p=0.3, Wilcoxon matched pairs test).

C. Data from B, but showing the change in failures in each cell. Using a Chi-squared test, analysis of individual cells showed that αβmethyleneATP significantly increased failures in three of the six cells (red lines) but had no effect in the other three (black lines).


A
50
25
0
% Failures
Control
PPADS


B
75
50
25
0
% Failures
Control
αβMeATP
Wash

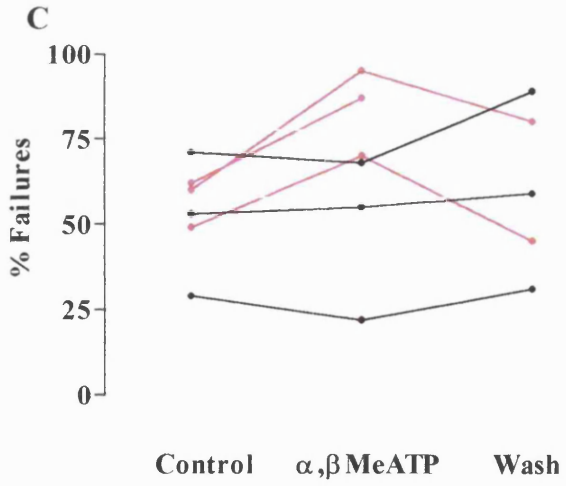
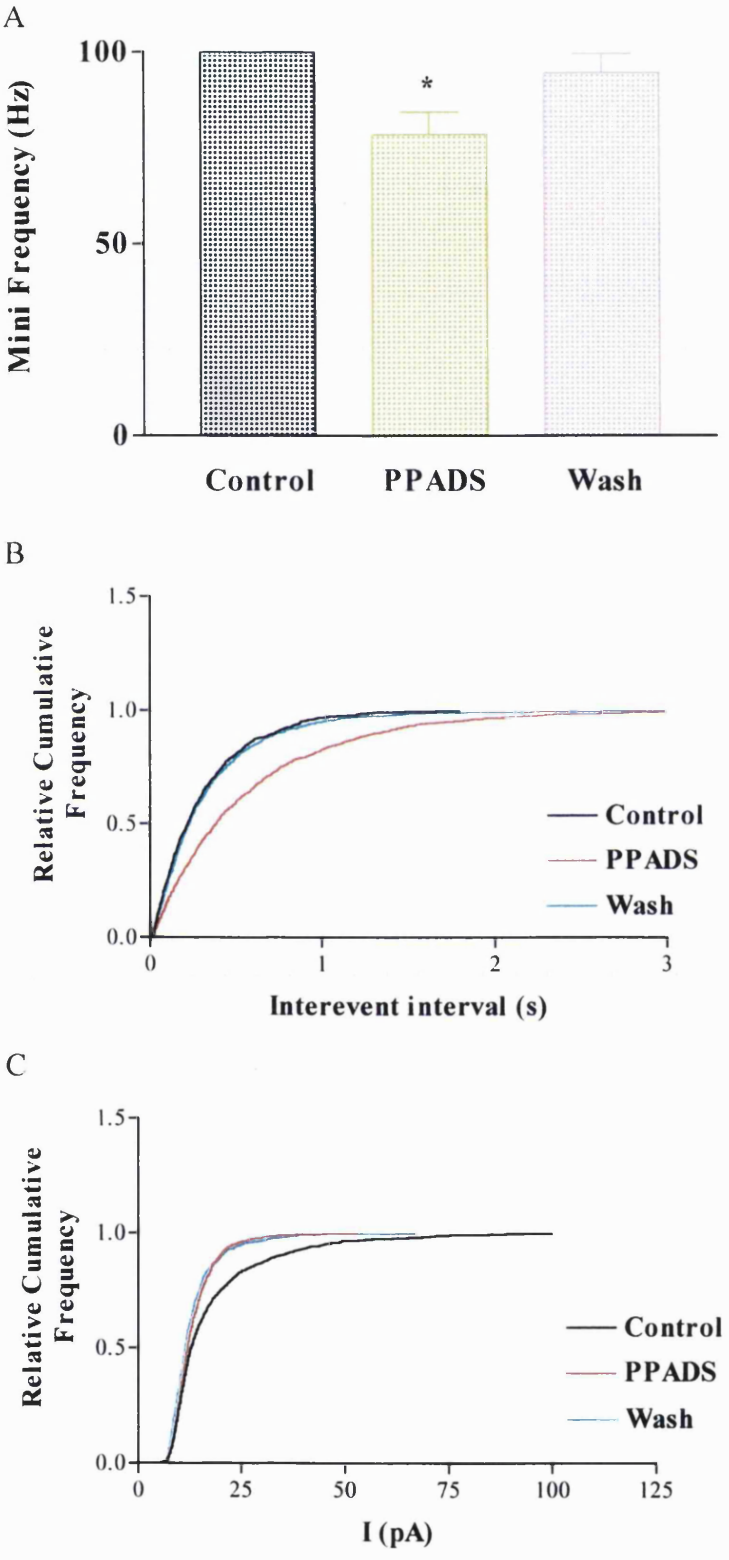

C
100
75
50
25
0
% Failures
Control
α,βMeATP
Wash

Fig 5.2 The effect of PPADS on glutamate minis

A. PPADS reversibly decreased mini frequency in 7/8 cells (middle column). Excluding the one cell in which the frequency increased (and didn't reverse) this effect was significant ($p<0.05$, repeated measures ANOVA).

B. In this cell PPADS reduced the occurrence of minis, as is evident from the shift towards longer interevent intervals (control is black, PPADS is red, wash is blue). This effect reversed.

C. In the same cell PPADS induced a shift towards smaller amplitude minis (control is black, PPADS is red, wash is blue); however, the cumulative distribution curves overlap at low amplitudes and deviate at larger amplitudes, indicating a possible selective reduction in larger amplitude events. Note that whilst the effect on frequency reversed, the effect of PPADS on amplitudes did not, which argues against the second effect causing the first.


A
100
50
0
Mini Frequency (Hz)
*
Control
PPADS
Wash
B
1.5
1.0
0.5
0.0
Relative Cumulative Frequency
0
1
2
3
Interevent interval (s)
Control
PPADS
Wash
C
1.5
1.0
0.5
0.0
Relative Cumulative Frequency
0
25
50
75
100
125
I (pA)
Control
PPADS
Wash

5.3 The effect of α,β-methyleneATP on evoked glutamate currents

α,β-methyleneATP (100μM) was tested on minimally-evoked glutamate currents (100 events in each condition, n=6 cells, fig 5.1, B). Across all cells it had no significant effect on current failures (p=0.3, Wilcoxon matched pairs test); however, the six cells comprised three in which there was no change in failures and three in which α,β-methyleneATP markedly increased failures in a reversible manner (fig 5.1, C) ; the effect of α,β-methyleneATP did not depend on the number of failures in control conditions. To confirm an effect of α,β-methyleneATP in these cells, all the cells were analysed individually using a Chi-squared test: in three of the six cells there was a significant increase in failures in α,β-methyleneATP compared to control ($p<0.01$ in all three of these cells).

5.4 The effect of RB2 on evoked glutamate currents

RB2 is not the antagonist of choice for investigating the effects of P2 receptors on glutamate transmission, as it is reported to block glutamate receptors at concentrations of 60μM and above (Nakazawa *et al.*, 1995). However, in the course of experiments investigating the effects of UTP and UDP (chapter 4), data were collected on the effects of RB2 per se on glutamate transmission. The effects were variable and difficult to interpret, possibly reflecting a combination of a direct effect on glutamate receptors and an effect at a P2 receptor. For this reason, these data are not explicitly reported in this section. It should be noted, however, that none of the data involving RB2 contradicts any of the findings in this section. The rationale behind the use of RB2 to investigate the effects of uridine nucleotides and the interpretation of the results is discussed in chapter 4.

5.5 Discussion

From the results presented in this section it can be concluded that presynaptic P2 receptors are involved in controlling glutamate release at some synapses within the medial habenula; furthermore, as α,β-methyleneATP is inactive at P2Y receptors, there may be a presynaptic P2X receptor on some glutamate terminals, as well as the P2Y receptor

postulated in chapter 4. As PPADS is an antagonist, a corollary of this conclusion is that there is sufficient extracellular ATP at some synapses to activate P2 receptors. The variability between cells suggests either that not all glutamate synapses are subject to this control or that P2 receptors influence glutamate release in a complex manner involving more than one mechanism.

In the following subsections, the results for PPADS and α,β-methyleneATP are discussed separately before some models are proposed which are consistent with the data.

5.5.1 Presynaptic Effect

The conclusion that the receptors are presynaptic is based on changes in failures (evoked currents) and changes in frequency (minis). The pitfalls in interpreting failures have been discussed in chapter 4; they are not repeated here but they are equally applicable to these data. A purely postsynaptic effect was excluded by analysis showing that the mini amplitude distribution predicted by a postsynaptic effect was significantly different from the actual amplitude distribution in PPADS. It should be noted that when the amplitude distribution of minis is skewed, it is possible to have a change in this distribution due to a change in release probability; for example, this might occur if large minis resulted from multivesicular release.

5.5.2 Glutamate Release and α,β-methyleneATP

As α,β-methyleneATP is inactive at P2Y receptors, the rational behind its use was to try and determine whether P2X receptors modulate glutamate release. In 50% of the cells, α,β-methyleneATP inhibited glutamate release; the question is whether it blocked a facilitatory effect of ATP or acted as an agonist to produce an inhibition. α,β-methyleneATP has recently been reported to antagonize the $P2X_4$ receptor (Jones *et al.*, 2000). As this receptor does not desensitize, it could mediate a tonic facilitation of glutamate transmission in the presence of extracellular ATP; blocking

the receptor would cause an inhibition. The $P2X_1$ and $P2X_3$ receptors rapidly desensitize (and remain so for 10s of seconds); they are unlikely, therefore, to be involved in a tonic facilitation of glutamate release by background ATP. These could, however, be involved in facilitating glutamate release throughout the control period if they were regularly activated by a brief elevation in extracellular ATP too short to desensitize them. Presumably the resulting Ca^{++} transient in the terminal would have to coincide with the Ca^{++} influx through VDCCs in order that they add to give a larger increase than is achieved by action potential-dependent influx alone. In the event that a purinergic afferent made an axo-axonic contact with a glutamate terminal, such a mechanism could conceivably occur, especially if the purinergic axon were stimulated along with the glutamate axon.

The $P2X_{2/3}$ heteromer produces a sustained current in the prolonged presence of an agonist. It is possible that α,β-methyleneATP acts through this receptor to produce inhibition although it would be unusual for a Ca^{++}-permeable receptor to inhibit transmitter release and at other synapses presynaptic P2X receptors facilitate release. It is possible, though, that α,β-methyleneATP, acting on the $P2X_{2/3}$ receptor, alters the amplitude or time course of the action potential, either by depolarizing the terminals to a subthreshold level or by activating a Ca^{++}-dependent K^+ current.

α,β-methyleneATP blocks ectonucleotidases (Chen & Lin, 1997). It could be acting likewise here to increase the background ATP concentation; in this case, there would be a presynaptic P2 receptor inhibiting glutamate release. For the reasons already discussed, this is more likely to be a P2Y receptor than a P2X receptor.

The fact that α,β-methyleneATP only inhibited glutamate release in half of the cells tested suggests one of two explanations: either glutamate afferents are heterogeneous with approximately half of them possessing the P2 receptor; or synapses vary as to whether or not they are exposed to ATP under the experimental conditions.

5.5.3 Glutamate Release and PPADS

The effect of PPADS on minis was to decrease their frequency; this indicates that there is likely to be a PPADS-sensitive presynaptic P2 receptor facilitating glutamate release. The mean inhibition by PPADS was 21%, the same as the percentage of cells in which PPADS inhibited the evoked currents. This is consistent with approximately 20% of the glutamate afferents to these cells possessing the presynaptic P2 receptor and spontaneous release from these afferents being predominantly P2 receptor-dependent. Could this be the same receptor as mediates the facilitation by UTP and UDP? Given that the uridine nucleotides produced long-lasting facilitation, it is unlikely that PPADS could reverse such an effect over the time course of a few minutes; the receptor must therefore be different. Alternatively, could PPADS and α,β-methyleneATP both block the same $P2X_1$, $P2X_3$, or $P2X_4$ receptor? As the data currently stand, the answer is yes. There is no significant difference between PPADS inhibiting evoked currents in 1 in 5 cells and α,β-methyleneATP inhibiting them in 3 in 6 cells (P=0.3, Chi Squared test)

5.5.4 Extracellular ATP

If there are presynaptic P2 receptors facilitating glutamate release then it follows that there is extracellular ATP activating these receptors. However, it is not necessarily true that all glutamate synapses are exposed equally to ATP; the concentration at any particular synapse will depend on the source of released ATP and the activity of the ectonucleotidases. Consequently synapses will differ in their sensitivity to P2 antagonists. This offers an alternative explanation for the different effect of PPADS on evoked currents and minis: instead of proposing that 20% of the synapses possess the presynaptic P2 receptor it could be proposed that only 20% of the synapses are exposed to ATP.

It is not necessarily the case that extracellular ATP levels remain constant within a discrete extracellular compartment such as a synaptic cleft. Any one of neuronal, glial and epithelial cells could release ATP

spontaneously, rhythmically or in response to extracellular stimulation. In particular, when evoking glutamate currents it is not known which other neurons and glial cells (if any) are concomitantly stimulated. This introduces the possibility - perhaps even the likelihood - of differences in experimental conditions which are not controlled for and lead to different results.

Varying background concentrations of ATP may be partly responsible for the large differences in basal mini frequency between cells. It is possible that some cells have a higher basal mini frequency because more synapses are exposed to ATP. As ATP is released from damaged cells, the overall amount of extracellular ATP may well differ between slices, depending on their health at the time of recording. However, as there was no correlation between the magnitude of the PPADS effect and the basal mini frequency it is unlikely that ATP alone is responsible for the differences. It is likely that other factors relating to the health of the slice are involved, such as depolarization of terminals due to an accumulation of extracellular K^+. Such explanations assume that the basal frequency is determined by differences in experimental conditions which it is not possible to control. It is also possible that the recordings were made from different populations of cells within the medial habenula, with differences in their glutamate inputs. The frequency of minis depends on release probability and the number of release sites; cells with a larger glutamate input will, on average, have a higher mini frequency.

The probability that extracellular ATP levels are non-homogeneous within a slice and vary between slices highlights a difficulty in using antagonists alone to study the question of release-modulating P2 receptors. Their inhibitory effects on ectonucleotidases also add to the complexity of interpreting the results. The use of agonists (as in the previous section) circumvents some of these problems and may be considered a preferable experimental approach.

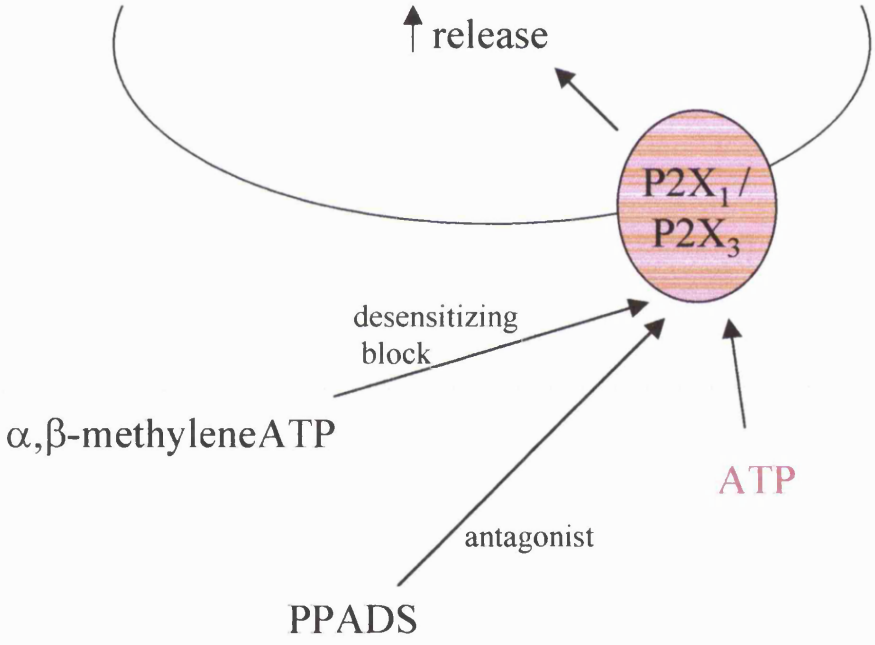
5.5.5 Model

A simple model, consistent with the data, is one in which some, or all, glutamate afferents possess a presynaptic $P2X_1$ or $P2X_3$ receptor. This is illustrated in fig 5.3. Transient ATP elevation briefly activates a $P2X_1$ or $P2X_3$ receptor to increase the release probability and, consequently, the mini frequency; this effect is blocked by PPADS. If, and only if, the Ca^{++} transient resulting from the ATP signal is coincident with the action potential-induced Ca^{++} transient, will it facilitate evoked glutamate release; α,β-methyleneATP (by desensitizing the receptor) and PPADS (by antagonism) block this effect. The failure of either compound to inhibit evoked release is due to either a lack of the presynaptic receptor on the afferent fibre stimulated, a lack of an ATP signal coincident with the presynaptic action potential or a chronic desensitization of the receptor by prolonged exposure to ATP.

It is also possible that α,β-methyleneATP antagonizes a presynaptic $P2X_4$ in which case it is not necessary to hypothesize that the receptor is activated by brief ATP transients. However, this receptor is not antagonized by PPADS (Buell *et al.*, 1996;Jones *et al.*, 2000), although it can not be discounted that PPADS hits a different receptor to α,β-methyleneATP, either P2X or P2Y. It should be possible to determine whether there is a $P2X_1$ or $P2X_3$ receptor present by using the selective antagonists such as NF279 or TNP-ATP. In terms of synaptic plasticity, it would be interesting to know for certain whether any of these glutamate terminals possess P2X receptors; some possible implications for plastic mechanisms are discussed further in the final discussion. The effect of α,β-methyleneATP argues for the involvement of a P2X receptor but it remains possible that it acted by inhibiting an ectonucleotidase. Nonetheless, the data presented in this section make it hard to escape the conclusion that there is a P2 receptor located on some glutamate afferents additional to the P2Y receptor (possibly $P2Y_4$) postulated in the previous section.

Fig 5.3 A model for the effects of PPADS and α,β-methyleneATP on glutamate transmission

Glutamate afferents possess either a $P2X_1$ or a $P2X_3$ receptor through which ATP facilitates glutamate release. This effect can be blocked by both PPADS, acting as an antagonist and α,β-methyleneATP, by desensitizing the receptors. For either compound to have an effect, the receptor must be present on the terminal and be exposed to brief elevations of extracellular ATP which are too short to desensitize the receptor.


release
P2X1 / P2X3
desensitizing block
α,β-methyleneATP
ATP
antagonist
PPADS

Chapter 6

Kinetic and Pharmacological Properties of Purinergic Currents

Purinergic epscs were recorded from 15 cells. Typically the stimulating electrode had to be moved to several different positions within the slice before the currents were found and the voltages required to evoke currents were high (between 30 and 100V) compared to those required to evoke glutamatergic epscs. Currents were evoked at 0.5Hz. No attempt was made specifically to stimulate single fibre inputs but, in spite of the high voltages frequently required, current failures were often seen, suggesting that single fibres or, at most, only a few fibres were stimulated. The currents were typically only a few pA in amplitude (see below) and consequently the signal-to-noise ratio was low. For this reason averages of 100 currents were made and the properties of these averaged currents are reported.

6.1.1 Current Failures

In most cells failures were apparent (fig 6.1); however, due to the small amplitude of the currents it wasn't always possible to count failures reliably. Among those cells where currents and failures could be unambiguously distinguished from one another, the failure rate was 23.3 ± 4.6% (n=7).

6.1.2 Decay time constant

The decay phase of the averaged current was well fitted in all cells with a single exponential function (fig 6.2, A) allowing the time constant to be computed: this was 15.3 ± 1.2ms (n=15). In 4 cells the decay phase appeared, judging by eye, to be better fit by a double exponential decay with a fast τ_1 (2.3 ± 0.8ms) and a slower τ_2 (19.2 ± 1.9ms) (fig 6.2, B); although two of these cells also had the fastest rise times it was not generally true that the faster rising currents were better fit with double exponential decay functions.

Fig 6.1 Purinergic Currents Recorded from a Medial Habenula Neuron.

From top left to bottom right, 9 consecutive events recorded in a medial habenula neuron. The cell was clamped at –70mV, currents evoked at 0.5Hz and recorded in NBQX (20μM), bicuculline (10μM) and 7-chlorokynurenate (10μM). Note that in two of the nine traces shown the currents fail (marked with *).

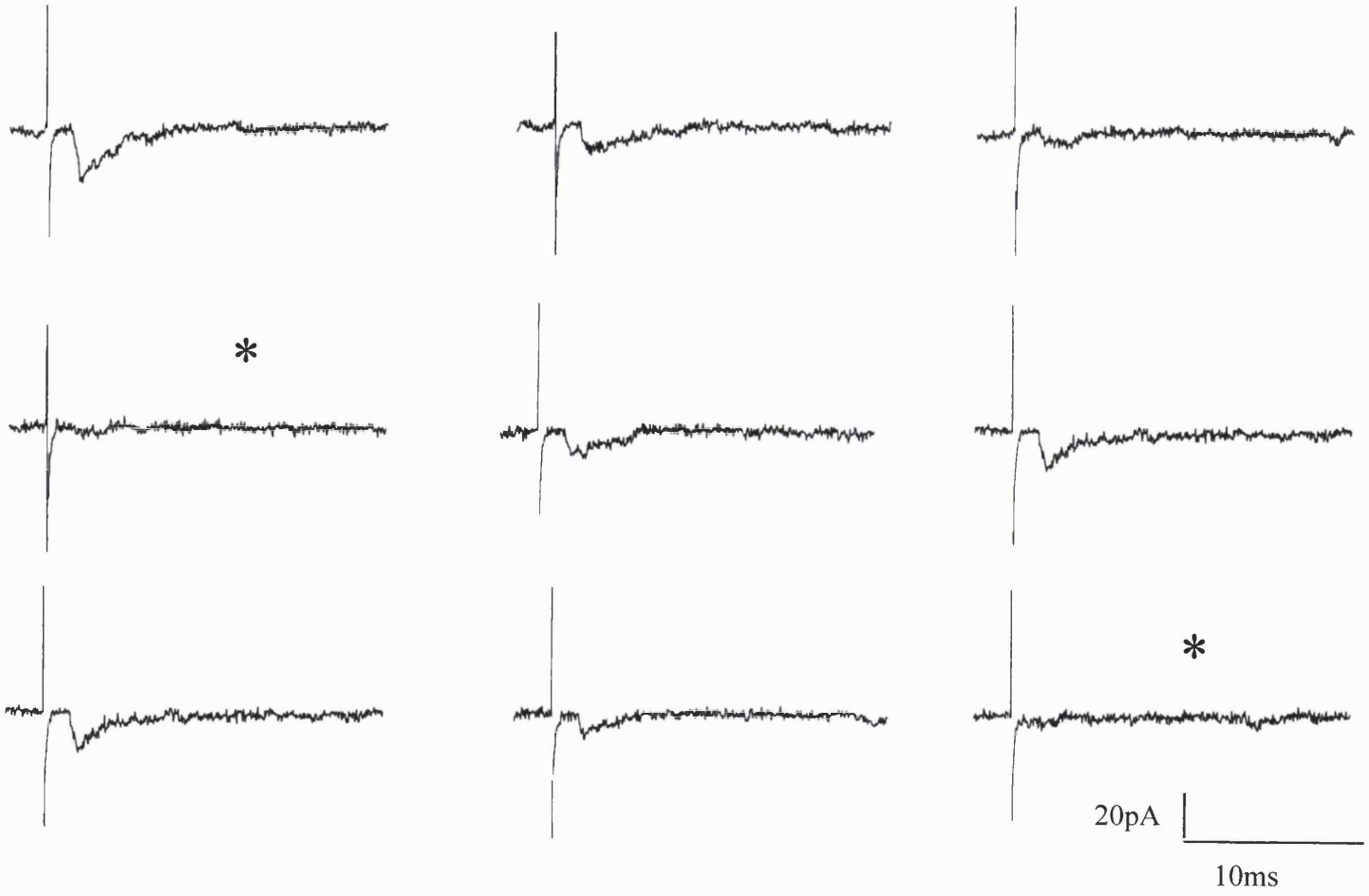
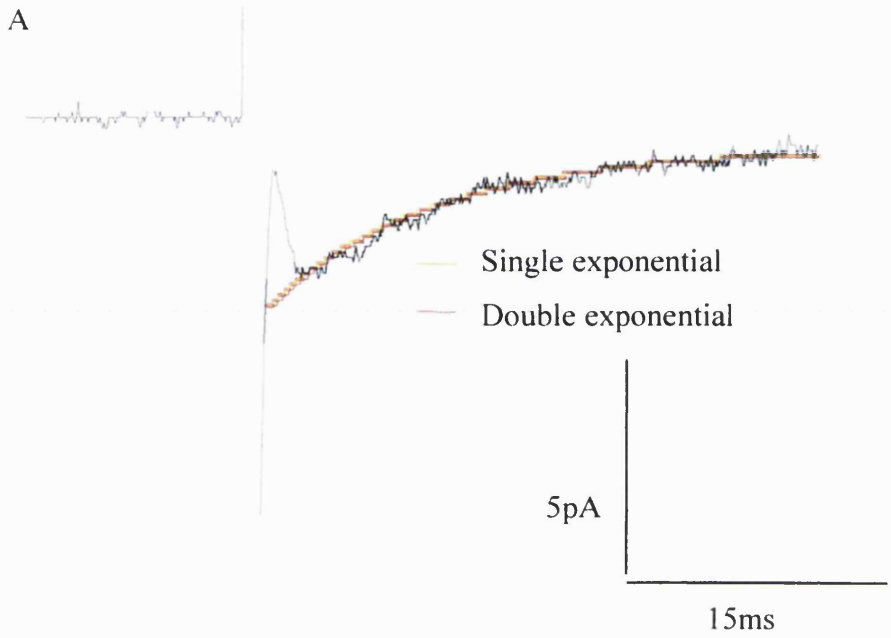
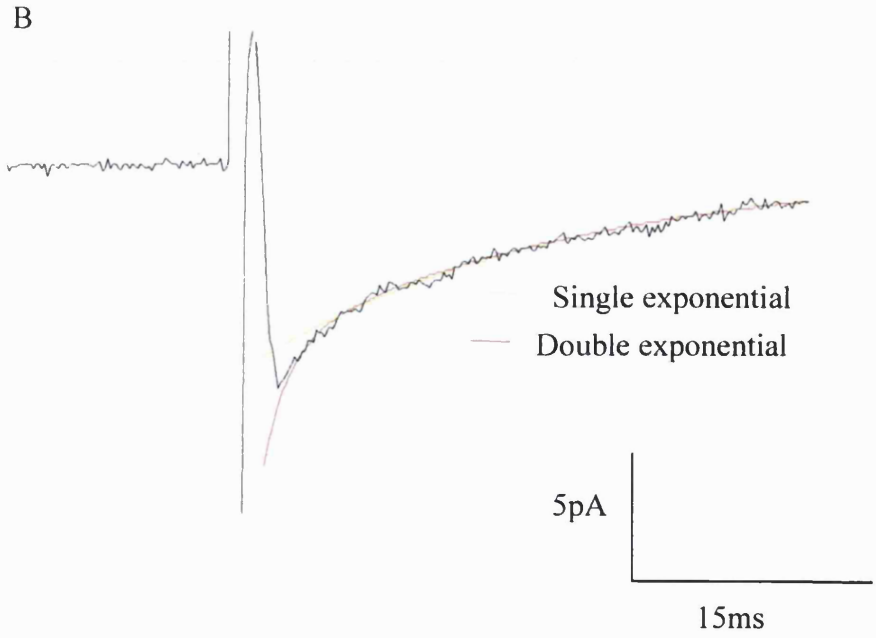

*
*
20pA
10ms

Fig 6.2 Decay phase of purinergic currents

A. The decay phase of this current was fitted almost equally well with a single exponential curve (red line, τ=14.8ms) as with a double exponential curve (yellow line, τ_1=13.4ms, τ_2=15ms). The majority of the currents fell into this category.

B. In this cell the decay phase of the current was better fit by a double exponential curve (red line) with τ_1=1.8ms and τ_2=22.8ms rather than a single exponential curve (yellow line) with τ=21.7ms.


A
Single exponential
Double exponential
5pA
15ms


B
Single exponential
Double exponential
5pA
15ms

6.1.3 Current amplitudes

The average current amplitudes, including failures, was 13 ± 2.6pA (n=13). Amplitude distributions in individual cells ranged from those with narrow distributions and a modal amplitude at or below 10pA (fig 6.3, A, red columns) to those with broader distributions and a less clearly defined modal amplitude (fig 6.3, A, blue columns).

6.1.4 Rise times

The rising phases of the averaged currents were fitted with a Gaussian curve with a mean time constant of 1.6 ± 0.2ms (n=15). Slight differences in the latency to onset of individual currents may result in an overestimate of the rise time measured from the averaged trace: when fitted to individual currents the mean time constant was faster (1.1 ± 0.1, n=3, fig 6.3, B).

6.1.5 Latency to onset

In 10/15 cells the rising phase of the current began before the stimulus artefact had returned completely to the base: it is therefore not possible to accurately measure the latency to onset of these currents but they are in the region of 1-2 ms, consistent with them being monosynaptic. In 3 cells the latency was between 3 and 7 ms, possibly indicating that these currents are polysynaptic.

6.2 Pharmacology of Purinergic epscs

In order to try to elucidate subunit composition of the P2X receptors underlying the purinergic currents, the recently developed P2X antagonist NF279 was tested; this compound is reportedly selective for $P2X_1$ (Kd=19nM) and $P2X_2$ (Kd=360nM) subunits (Rettinger *et al.*, 2000).

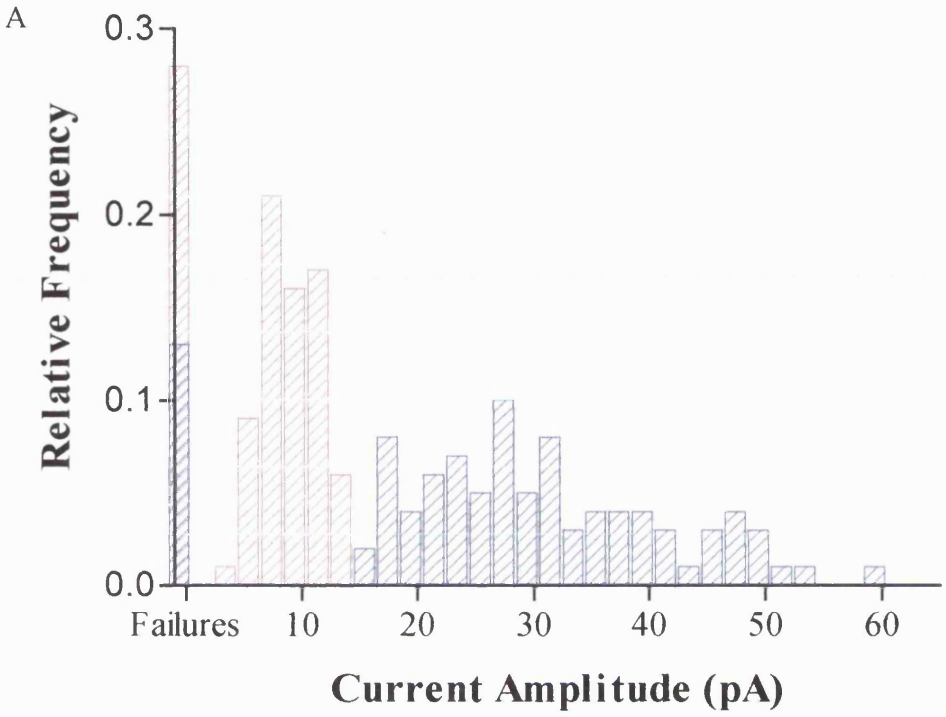
6.2.2 Inhibition by NF279

At concentrations greater than 100nM NF279 inhibited the currents in a dose-dependent manner (fig 6.4, A); this inhibition was reversible (fig 6.4, B) and the maximum inhibition of 47% was reached with 10μM NF279.

Fig 6.3 Apscs: Amplitudes and Rise Times

A. Amplitude distributions in individual cells ranged from those with narrow distributions and a modal amplitude at or below 10pA (red columns) to those with broader distributions and a less clearly defined modal amplitude (blue columns).

B. Distribution of rise times from a typical cell.


A
0.3
0.2
0.1
0.0
Relative Frequency
Failures
10
20
30
40
50
60
Current Amplitude (pA)

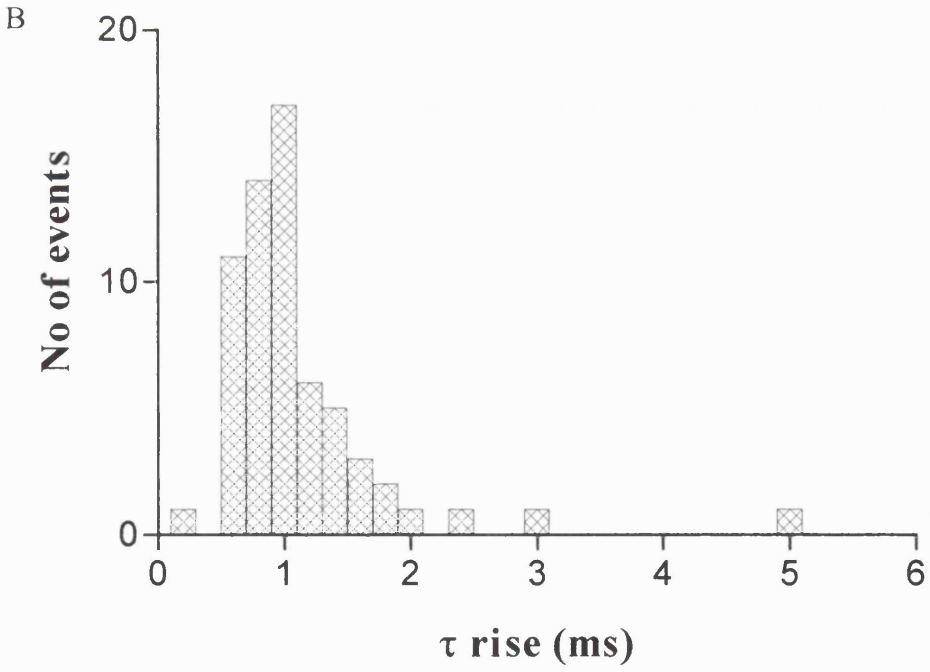
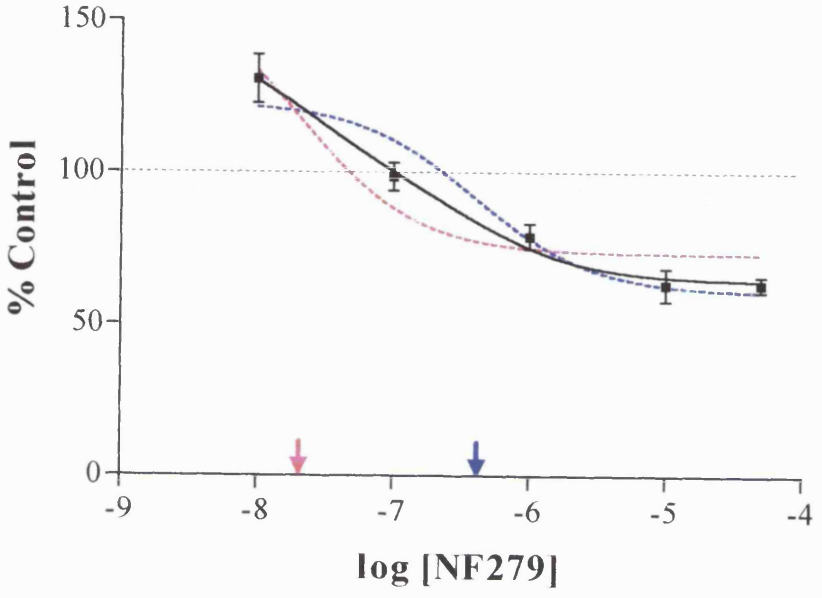

B
20
10
0
No of events
0
1
2
3
4
5
6
τ rise (ms)

Fig 6.4 Concentration dependent facilitation and inhibition of purinergic currents by NF279

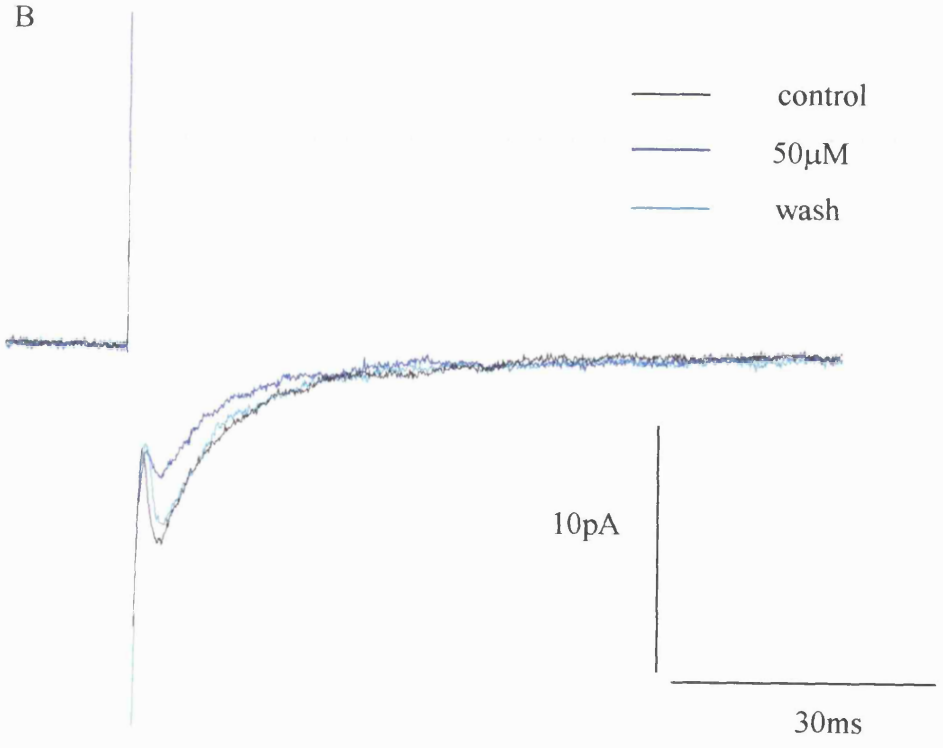
A. NF279 caused dose-dependent facilitation and inhibition of the purinergic currents. Data is shown with standard error bars. n is between 4 and 6 for each data point except 50μM NF279 where n=2. The horizontal line at 100% represents control responses. The red arrow is at –7.7, the log IC_{50} for inhibition of the $P2X_1$ receptor; the blue arrow is at –6.4, the log IC_{50} for inhibition of the $P2X_2$ receptor. The data points were best fit with the sum of three sigmoidal curves (black line) modelling a facilitatory effect and inhibition of postsynaptic $P2X_1$ and $P2X_2$ subunits. The data points were less well fit with a sum of two curves which modelled facilitation and inhibition of a postsynaptic $P2X_1$ receptor only (red curve) or a sum of two curves modelling facilitation and inhibition of a postsynaptic $P2X_2$ receptor only. See text for further details of the equations.

B. Averaged currents from a single cell in which 50μM NF279 reversibly inhibited apscs by 39% (Control is black, NF279, is dark blue, wash is light blue. Each trace is the average of 100 currents).

A


150
100
50
0
% Control
-9
-8
-7
-6
-5
-4
log [NF279]

B


control
50μM
wash
10pA
30ms

6.2.3 Facilitation by 10nM NF279

In contrast to the inhibitory effect of NF279, at the lower end of the dose-response curve 10nM NF279 facilitated currents in 5/5 cells (fig 6.4, A) (mean increase = 30.8 ± 8.0%, n=5).

To try to elucidate possible mechanisms underlying this facilitation, changes in failures were looked at in four cells where it was possible to count them clearly: in three cells the increase in the average current amplitude was accompanied by a decrease in failures.

The amplitudes of the evoked currents (excluding failures) were measured to determine whether there was a shift in the current amplitudes in 10nM NF279. Two cells showed a small, but significant shift towards larger amplitude currents (p=0.0098 and p=0.0097, Kolmogorov-Smirnoff test).

6.2.4 Fitting Dose-Response Curves to the Data: $P2X_1$, $P2X_2$ or both?

To try and determine which P2X subunits NF279 might be blocking, attempts were made to fit the data points with three different curves.

The first curve was the sum of a facilitatory curve and a single inhibitory curve with a log IC_{50} = -7.7 (log IC_{50} for the $P2X_1$ receptor). This is the red curve in figure 6.4,A and it has the equation

$$y = 100 + \frac{(TOP-100)}{1+10^{LogEC50 - x}} - \frac{(100-BOTTOM)}{1+10^{-7.7 - x}}$$

where TOP is the plateau of the facilitatory curve, $LogEC_{50}$ is the log of the EC_{50} for the facilitatory curve and BOTTOM is the plateau of the inhibitory curve. The final values assigned to these free parameters were: TOP = 174, $LogEC_{50}$ = -8.9 and BOTTOM = 0. Visual inspection of the curve reveals a poor fit (R^2=0.78) suggesting that a model with NF279 blocking only $P2X_1$ postsynaptically is not a good one to account for the observed dose-response curve.

The second curve was also the sum of a facilitatory curve and a single inhibitory curve being identical to the equation above except that –7.7 was replaced with –6.4, the log IC_{50} for the $P2X_2$ receptor. This is the blue curve in figure 6.4,A. The final values assigned to the free parameters were: TOP = 123, $LogEC_{50}$ = -16, and BOTTOM = 37. The fit was also poor (R^2=0.77), again suggesting that a model with NF279 blocking only a postsynaptic $P2X_2$ receptor is not a good one.

The third curve was the sum of a facilitatory curve and two inhibitory curves with log IC_{50}s of –7.7 and –6.4 (log IC_{50} of $P2X_1$ and log IC_{50} of $P2X_2$ respectively). This is the black curve in figure 6.4,A and has the equation

$$y = 100 + \frac{(TOP-100)}{1+10^{LogEC50 - x}} - \frac{(100-BOTTOMA)}{1+10^{-7.7 - x}} - \frac{(100-BOTTOMB)}{1+10^{-6.4 - x}}$$

The final values assigned to the free parameters were: TOP = 147, $LogEC_{50}$ = -16, BOTTOMA = 52 and BOTTOMB = 65. The curve is close to fitting all the data points (R^2=0.84), suggesting that a model with NF279 blocking postsynaptic $P2X_1$ and $P2X_2$ receptors is the most consistent with the data.

6.3 Discussion

The results from this section demonstrate that the fast purinergic epsc in the medial habenula is partially blocked by the $P2X_1$ and $P2X_2$ receptor antagonist NF279. Fitting curves to this data shows that both receptors are likely to underlie purinergic currents. As the block was incomplete, other P2X subunits are probably also involved. Unexpectedly the data revealed an additional facilitatory effect of NF279.

The kinetic properties of the purinergic epscs have been described before (Edwards *et al.*, 1992; Edwards *et al.*, 1997) the results presented here are consistent with these previous studies. The kinetic and pharmacological properties of the currents are discussed along with some possible

implications of this data for purinergic transmission in the medial habenula.

6.3.1 Properties of purinergic epscs

The fast rise time of the purinergic epsc is typical of an ionotropic receptor mediated current. While the decay phases of the majority of currents were well fitted with a single exponential function with a time constant of 15ms, in some cells the currents were better fitted with a double exponential function, suggesting that there might be a heterogeneous population of synapses in terms of the receptor subunit composition.

The purinergic input appears to be sparse as finding currents typically required several electrode placements and increasing the stimulating voltage rarely increased the current amplitude. Furthermore, the mean current amplitude is just 13pA: given an input resistance of 1.3GΩ, a synaptic current of 13pA would cause a depolarization of 16mV which would be attenuated at the soma; this may be insufficient to bring a cell to firing threshold although this has not been tested in current clamp. Although minimal stimulation of glutamate fibres elicits epscs of a similar amplitude to apscs (chapter 3) more glutamate fibres can be recruited by higher stimulating voltages resulting in currents of several hundred pAs. This suggests that the primary role of purinergic epscs is probably not to fire cells. As P2X receptors are the only Ca^{++}-permeable ligand-gated receptors present on medial habenula neurons (Robertson *et al.*, 1999), it is tempting to speculate that they underlie essential synaptic Ca^{++} signalling in these cells. Furthermore, as P2X receptors vary in their Ca^{++} permeabilities (Evans *et al.*, 1996), the nature of a synaptic Ca^{++} signal may depend on the subunit composition of synaptic P2X receptors.

There is a discrepancy between the apparently sparse purinergic input to habenula neurons as detected by the electrophysiology and the anatomical observation of a large putative purinergic input from the posterior septal nuclei (Staines *et al.*, 1988). This suggests that the majority of the

purinergic input underlies something other fast synaptic transmission with habenula neurons. In chapter 7 it is suggested that one other role might be related to the axo-axonic synapses in the habenula.

6.3.2 Where are the purinergic cell bodies?

In the majority of cells the latency to onset of the currents was consistent with a monosynaptic connection which could come from within or from outside the habenula; however, in three cells the latency was in excess of 3ms, suggesting a polysynaptic connection and thus implying that the somata of these purinergic afferents lie within the medial habenula; if this were the case, the transmitter at the first synapse could not be glutamate (the recordings were carried out in CNQX) and therefore might be ATP itself. It is also possible, however, that the long latencies were due to slowly conducting fibres rather than synaptic delay.

6.3.3 Pharmacology of purinergic epscs

The physiological role of synaptic P2X currents in the CNS is not yet understood. The seven homomeric and the four heteromeric P2X receptors which have been described have different kinetic properties (Fiszman *et al.*, 1999;Evans *et al.*, 1995), especially the rate at which they desensitize and subsequently recover; they also vary in their relative Ca^{++}-permeabilites (Evans *et al.*, 1996). NF279, an analogue of suramin, has the following antagonist profile at P2X receptors: $P2X_1 > P2X_2 > P2X_3 > P2X_4$ (Rettinger *et al.*, 2000). NF279 is reported to be inactive at P2Y receptors and relatively ineffective in blocking ectonucleotidases (Damer *et al.*, 1998), all of which properties make it a potentially useful pharmacological tool for investigating P2 receptors. In the experiments in this section NF279 was used to determine whether $P2X_1$ and / or $P2X_2$ receptors are involved in fast purinergic transmission.

6.3.4 NF279: partial inhibition

The Kds of NF279 at the $P2X_1$ and $P2X_2$ receptors are 14nM and 360nM respectively (Rettinger *et al.*, 2000) and these were used as approximations to the IC_{50}s for synaptic $P2X_1$ and $P2X_2$ receptors. This approximation is

justified at fast synapses because the time course of the transmitter in the cleft is estimated to be extremely brief (in the order of 1ms) such that the antagonist effectively behaves non-competitively. The equation for the occupancy of receptors in the presence of a non-competitive antagonist is

$$P_A = ([A] / [A]+K_A)(1-P_B)$$

where P_A is the proportion of the receptors occupied by the agonist, A, P_B is the proportion of the receptors occupied by the antagonist, B, and K_A is the dissociation constant for A. From the equation it can be deduced that when 50% of the receptors are occupied by the antagonist ([B]=Kd), the agonist occupancy is half of its value in the absence of the antagonist and can not be increased by increasing the agonist concentration. This means that the Kd and the IC_{50} are the same. In addition, NF279 dissociates slowly (over the course of 10s of seconds) from the $P2X_2$ (Rettinger *et al.*, 2000) receptor which further justifies the use of the Kd as an approximation to the IC_{50}.

The block of the apscs by NF279 was dose-dependent with a maximum inhibition of 37%. However, NF279 also facilitates apscs as is clear from the data point at 10nM. The resulting dose-response curve is therefore a convolution of a facilitatory and an inhibitory curve, making the interpretation of the inhibitory effect more difficult. The question is whether the inhibitory effect is due to a block of $P2X_1$, $P2X_2$ or both receptors? To try and answer this question, the data were fitted with theoretical curves: these were a sum of a facilitatory sigmoidal curve and either one or two inhibitory sigmoidal curves with the IC_{50}s fixed at 14nM (Kd of $P2X_1$) and 360nM (Kd of $P2X_2$) to simulate inhibition of $P2X_1$ and $P2X_2$ receptors respectively. The best fit was obtained with a combination of three curves (fig 3.4,A, black curve) indicating that the most likely interpretation is one in which both $P2X_1$ and $P2X_2$ subunits are present. The data points were less well fitted with sums of a facilitatory curve and one inhibitory curve whether the IC_{50} was set at 14nM (fig 3.4, A, red curve) or 360nM (fig 3.4, A, blue curve). In fitting the curves, the

simplifying assumption was made that there is just one binding site for NF279. The fit was less good assuming two binding sites.

There is only one data point lying above 100% so it is currently not possible to know either the maximum amount of facilitation or the EC_{50} of this effect; consequently it is not possible to know the actual percentage block of the receptors by NF279. This question is of some interest because immunohistochemistry failed to detect mRNA for $P2X_1$ and $P2X_2$ receptors in the medial habenula but did detect mRNA for $P2X_4$ and $P2X_6$ receptors (Kidd *et al.*, 1995;Collo *et al.*, 1996). (Note however that $P2X_2$ was detected by whole habenula RT-PCR, possibly because it was a more sensitive assay (F. Soto, personal communication)). Assuming that the relative levels of mRNA reflect the relative expression of the proteins and that failure to detect $P2X_1$ and $P2X_2$ subunit mRNA was due to a low signal and not a technical difficulty, $P2X_4$ and $P2X_6$ subunits are more abundant in the medial habenula than $P2X_1$ and $P2X_2$. Nonetheless, the pharmacology suggests that $P2X_1$ and $P2X_2$ receptors underlie at least 37% of the purinergic epscs, and probably more. Presumably, then, the $P2X_4$ and $P2X_6$ subunits are responsible for something other than fast purinergic transmission in medial habenula neurons.

The interpretation of the NF279 block is consistent with the current knowledge of its pharmacology. One caveat is that NF279 has not yet been tested on heteromeric P2X receptors. It cannot be excluded, therefore, that NF279 is a potent antagonist at one of the known P2X heteromers or a heteromer which has yet to be described.

6.3.5 NF279: Facilitation

An unexpected finding was that 10nM NF279 potentiated apscs. This potentiation is presumably also present at concentrations greater than 10nM although this is not a conclusion that can be drawn from these data. Additional data points at concentrations less than 10nM should add constraints to the curve fitting and allow an estimation of the maximum facilitation which can be achieved.

As studying this facilitation was not the purpose of this study, the mechanism underlying it has not been further investigated experimentally, except to count failures in the cells where it was possible to do so. There are four possible mechanisms which could account for the effect:

1. NF279 acts postsynaptically to potentiate currents.
2. NF279 acts at a presynaptic receptor to increase the release probability.
3. NF279 acts at a postsynaptic receptor causing the insertion of P2X receptors into silent synapses.
4. NF279 inhibits ecto-nucleotidases, thus reducing ATP breakdown.

Elucidating this mechanism through future experiments should be an additional pointer towards the role of these apscs as well as determining whether NF279 has properties other than antagonism of some P2X subunits.

Chapter 7

In the previous four chapters the discussions following the results have focused on critically assessing the data and the conclusions which it is valid to draw. This final chapter takes those conclusions and speculates on their meaning for synaptic transmission in the medial habenula. Rather than occupying a separate section, relevant experiments for future work are proposed alongside discussions of candidate mechanisms and hypotheses.

7.1 Purinergic Currents and P2X subunits

In chapter 6 it was shown that purinergic currents in the medial habenula are sensitive to the $P2X_1$ and $P2X_2$ antagonist NF279. The data also revealed an additional facilitation of P2X currents which has not been seen with any of the antagonists previously used to block this current, namely suramin, PPADS and α,β-methyleneATP. Fitting curves to the data indicated that both $P2X_1$ and $P2X_2$ receptors are likely to underlie the fast purinergic currents.

The facilitation is particularly intriguing because it may offer insight into the physiological role of P2X currents. A candidate explanation, consistent with the preliminary data on failures, is that there is a presynaptic increase in ATP release, which raises the possibility that apscs are larger under particular physiological conditions. As the data shows, currents can be facilitated by at least 30%; the maximum facilitation may be greater still. If the mechanisms for this facilitation can be elucidated, it should point towards the conditions under which currents might be potentiated; this, in turn, may give clues to their physiological role. Possible future experiments could include the following:

1. Confirming or rejecting the decrease in failures in 10nM NF279 with data from additional cells.

2. Testing whether NF279 inhibits ectonucleotidases: breakdown of applied ATP could be assayed using the luciferin-luciferase technique.
3. Testing the effect of NF279 at concentrations lower than 10nM: this should add restrictions to the curve fitting and reveal the maximum amount to which apscs can be facilitated.

Initially it was planned to try to fully describe the set of P2X subunits underlying apscs in the medial habenula using a combination of pharmacology, antibody staining and single cell RT-PCR. However, as the dose response curve to NF279 revealed a combination of inhibitory and facilitatory effects and the parallel RT-PCR experiments hit an unexpected technical difficulty in detecting mRNA in these neurons, it was decided not to pursue the question further at the present time. It is interesting, though, to consider the data as it currently stands. Previous experiments in this laboratory have shown that these currents are blocked by PPADS and suramin (Edwards *et al.*, 1992), thus excluding the $P2X_4$ and $P2X_6$ subunits. (Interestingly these subunits were the ones detected in the medial habenula immunohistochemistry (Collo *et al.*, 1996) suggesting they might be prominantly expressed in non-neuronal cells) Apscs are also partially blocked by α,β-methyleneATP (Edwards *et al.*, 1992), which indicates the involvement of some, or all, of the $P2X_1$, $P2X_3$ and $P2X_{2/3}$ subunits. The partial block with NF279, presented in this thesis, positively implicates $P2X_1$ and $P2X_2$ subunits. As the $P2X_1$ subunit rapidly desensitizes and as there was no evidence in these or previous experiments for desensitization of the synaptic currents, it is possible that the $P2X_1$ subunit is present in a heteromeric receptor.

7.2 Presynaptic P2 receptors: short- and long-lasting facilitation

The data presented in chapter 4 is the first description of a presynaptic P2Y receptor which facilitates glutamate release. Perhaps the most interesting result in this thesis is that this facilitation is long-lasting and is therefore a novel method of inducing LTP. Its induction is also dependent on the agonist concentration: it was shown that high concentrations of

UTP activates a second presynaptic P2Y receptor with the result that potentiation does not occur and transmission is actually inhibited. This inhibition was reversible.

It should be noted that uridine nucleotides were used in these experiments because they are inactive at P2X receptors. However, both the rat $P2Y_2$ and $P2Y_4$ receptors are equally sensitive to ATP as to UTP (Chen *et al.*, 1996;Bogdanov *et al.*, 1998). As detailed in the introduction there is currently limited information on the physiological release of uridine nucleotides. The following discussions, therefore, focus on physiological and pathophysiological scenarios involving extracellular ATP.

In chapter 5 it was additionally shown that glutamate transmission at some, or all, glutamate afferents in the medial habenula can be reversibly inhibited by P2 receptor antagonists: this leads to the conclusion that, in addition to inducing LTP, ATP can cause a short-lasting facilitation of glutamate transmission. The effect of the P2X selective agent α,β-methyleneATP argues that a P2X receptor is most likely to underlie this effect.

If a terminal possesses P2 receptors for long and short term facilitation, what determines which effect predominates? Two candidate hypotheses are the concentration of extracellular ATP and its source. A small increase in ATP could be the signal for brief facilitation of glutamate transmission with a larger increase being the trigger for long-lasting facilitation. Alternatively, ATP from, for example, glial cells might induce LTP whilst neuronally released ATP might induce short-lasting facilitation. The appropriate spatial separation of the terminal P2 receptors is a potential mechanism for either of these effects. An alternative, speculative role for P2X receptors, also discussed below, is that they may be necessary for the induction of LTP at some terminals.

7.3 Intraterminal Mechanisms

A future line of investigation to pursue is the intraterminal pathways by which P2Y receptors potentiate and inhibit glutamate transmission. One candidate mechanisms for potentiation is the activation of PKC: all P2Y receptors can activate this enzyme and studies have shown that both glutamate and GABA release is enhanced by exogenous agents which activate PKC (Capogna *et al.*, 1995;Malenka *et al.*, 1986). Experimental approaches could include trying to block the UTP effect with PKC inhibitors and trying to induce a long- lasting facilitation by applying PKC activators. It may be of relevance that in cortical synaptosomes, PKC-activation facilitates glutamate release by uncoupling an inhibitory adenosine receptor (Budd & Nicholls, 1995). As adenosine exerts an inhibitory control on glutamate release in the medial habenula (Robertson & Edwards, 1998), it would be interesting to repeat the LTP experiments with the adenosine receptors blocked.

The adenylyl cyclase (AC) and cAMP signalling cascade is also a candidate mechanism for potentiation: it has a central role in mossy fibre LTP (Weisskopf *et al.*, 1994;Hestrin *et al.*, 1990) although it is not yet clear whether any uridine nucleotide-sensitive P2Y receptors stimulate cAMP accumulation. Similarly to PKC, experiments can be designed to try to block or induce LTP using agents which manipulate the AC / cAMP signalling pathway.

A candidate target for the inhibitory effect of high UTP concentrations is the N-type Ca^{++} channel which can be inhibited by three of the P2Y receptors ($P2Y_{1,2 \text{ and } 6}$) (Filippov *et al.*, 1999;Filippov *et al.*, 1998;Filippov *et al.*, 2000). An initial experimental approach would be to determine whether N-type Ca^{++} channels are involved in the action potential-dependent glutamate release in the medial habenula. A second approach might be to investigate minis. It would first be necessary to establish whether habenula minis are independent of Ca^{++} influx through VDCCs; if

so, it would be interesting to test whether they are inhibited by 200μM UTP.

7.4 LTP

The UTP-induced LTP described in this thesis has some similarities with mossy fibre LTP; establishing which properties it shares with this and other well-studied forms of LTP is a starting point to understanding its physiological role in the medial habenula.

UTP-induced LTP is similar to mossy fibre LTP in that the final effect is a presynaptic increase in glutamate release. In some cells LTP was induced using a minimal stimulating protocol which suggests that cooperativity is not an essential feature of this phenomenon. One would therefore predict that a postsynaptic Ca^{++} rise is unlikely to be necessary to induce LTP. Furthermore, because there is EGTA in the recording electrode, Ca^{++} release from intracellular stores is likely to be adequately buffered; moreover these neurons lack NMDA receptors and the synaptic AMPA receptors are not Ca^{++} permeable (Robertson *et al.*, 1999). It can not be totally discounted that there is a postsynaptic element to LTP induction, nor that the P2Y receptors are postsynaptic and a retrograde messenger signals to the terminals. However, as GTP was not included in the recording electrode, it is probable that postsynaptic P2Y receptors would have been non-functional, at least in the cell being recorded from.

It is tempting to speculate that UTP-induced LTP might be a general mechanism for NMDA receptor-independent long-lasting potentiation. It would be interesting, therefore, to test the effect of UTP at the glutamatergic synapses made by parallel fibres onto Purkinje cells (which also lack functional NMDA receptors in the adult (Farrant & Cull-Candy, 1991)) and at mossy fibre-CA3 synapses, two synapses which express NMDA receptor-independent LTP (Salin *et al.*, 1996;Zalutsky & Nicoll, 1990).

It should help in elucidating the role of habenula LTP if it can be induced by an experimental protocol resembling a physiological or pathophysiological process. Bath application of UTP may be similar to the general accumulation of extracellular ATP resulting from a period of hypoxia. Under such pathological conditions, moderate ATP release could potentiate glutamate transmission, possibly to counteract the inhibition caused by adenosine, whilst extreme accumulation of ATP would act with adenosine to inhibit release. An interesting experimental approach would be to expose the slice to a brief period of hypoxia to try to release ATP and induce subsequent LTP.

Anoxia-induced LTP has been described previously and one of its properties is that synapses are potentiated in a non-specific manner (Hsu & Huang, 1997). This contrasts with LTP in the CA1 region of the hippocampus where only active synapses are potentiated (Andersen *et al.*, 1977). The functional implications of these differences are important as specificity is thought to be an essential property of learning, whilst non-specific potentiation is more likely to underlie a general requirement for upregulation of glutamate transmission. It would be interesting, therefore, to test whether LTP is induced if the synapses are not stimulated during UTP application.

One highly speculative idea related to synaptic specificity revolves around the results in chapter 5 indicating that some, but possibly not all, glutamate afferents possess a presynaptic P2X receptor. Along with VDCCs, this is a pathway for Ca^{++} influx into terminals and it is action potential-independent. If UTP-induced potentiation requires a presynaptic Ca^{++} rise, terminals possessing both P2X and P2Y receptors could be potentiated even if they were inactive; in contrast, terminals lacking the P2X receptor would be potentiated only if they were active.

A second experimental approach to inducing LTP relates to the role of ATP in astrocyte signalling, discussed in the introduction. While there is

currently no evidence that astrocytes use ATP to signal to neurons, it is certainly a candidate molecule for such a role. One hypothesis is that a Ca^{++} wave spreads slowly across habenula astrocytes; as perisynaptic astrocytes become active, they release ATP close to the synaptic cleft and this results in potentiation of synaptic transmission. Such a mechanism could potentiate a large number of synapses but in a more controlled manner than a hypoxia-induced LTP. Nearly all the individual steps of this hypothesis have been demonstrated in one preparation or another (see introduction), so it only remains to test the hypothesis as a whole in the medial habenula. Glial Ca^{++} waves can be triggered simply by touching a glial cell with a microelectrode (Newman & Zahs, 1997). It would be necessary first to demonstrate such waves in the medial habenula; thereafter, it may be possible to combine the triggering of a glial Ca^{++} wave with synaptic electrophysiology to test whether LTP is induced.

Finally, the most common method of inducing previously described forms of LTP is a specific stimulating protocol applied to the presynaptic fibres or neurons. As ATP is released from neurons in the medial habenula following electrical stimulation, it may be that a particular stimulating protocol is effective in accumulating ATP at glutamate synapses and thus inducing LTP. ATP could be released from glutamate afferents themselves and act presynaptically to potentiate transmission: there is some circumstantial evidence supporting co-release (see introduction). If this is the case, P2Y receptors could be located such that only a particular form of synaptic activity releases sufficient ATP to activate them. A long high frequency burst is unlikely to be effective as glutamatergic and purinergic currents fail increasingly as the stimulation frequency exceeds about 5Hz (Robertson & Edwards, 1998); perhaps a pattern of brief high frequency bursts may be sufficent. It is also tempting to speculate that the axo-axonic synapses described in the medial habenula might be preaxonic purinergic fibres synapsing onto glutamate terminals. In this case appropriate coincident firing of the pre and postaxonic neurons would presumably be required to induce LTP and this would be a form of associativity. Future experiments could try to induce LTP using different stimulating protocols.

Ultimately, the synaptic physiology of a nucleus needs to be related to its function. There is some evidence that medial habenula is involved in avoidance learning but its exact role is unclear. If it is a locus where some memories are either formed or stored, one would predict that LTP should be input specific and show associativity and cooperativity. However, as detailed in the introduction, there is also evidence that the medial habenula is involved in controlling aspects of the sleep/wake cycle and levels of arousal. If LTP is involved in this physiology, different properties would probably be required. Transmission between sleep states or states of arousal could require the non-specific increase in transmission at many, or all, excitatory synapses. One can speculate that synapses might be rapidly potentiated at the start of a sleep cycle with the potentiation gradually decaying over the 90 minutes or so that a cycle lasts (Shepherd, 1994), ready for the process to be repeated again.

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