Nicotinic acetylcholine receptor modulation of noradrenaline release in the rodent brain

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Award date:
2011

Awarding institution:
University of Bath

Nicotinic Acetylcholine Receptor Modulation of Noradrenaline Release in the Rodent Brain

Volume 1 of 1

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

September 2011

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Acknowledgements

First and foremost, I am deeply indebted to Sue, without whom this thesis would not exist. From supporting me through the dark times, when data was hard to come by, to sharing my excitement when I found interesting results, and always having a positive way to look at 'unusual' data Sue has been an inspiration and a steadfast support. Many thanks also for the home cooked meals, apples from the garden and chats about all and sundry.

Thanks also to David Heal and all at RenaSci for their help and support whilst I was on my many visits to Nottingham, especially to Mike Prow for advice when I was wrestling the HPLC systems into submission.

Thanks go to all lab members past and present for nights out and support in the lab. Inter-lab collaborations in the form of Cake Wednesdays must also be acknowledged.

I am grateful to all in the 4S annex for their help, advice, support and humour over the years; it wouldn't have been the same without you.

To my family for support throughout my education and for always believing I could achieve whatever I want in life, many thanks.

Last, but not least, my thanks to Gareth for all the visits to Bath and for providing somewhere for me to live while I have been writing my thesis. I promise I'll start doing more housework now this is done!

Abstract

Cognitive function in the brain is controlled by neurotransmitters whose release is tightly controlled. When normal levels are perturbed deficits in function can be observed both in humans and in animal models. The cholinergic system, acting via muscarinic or nicotinic receptors, modulates neurotransmitter release.

The aim of this thesis was to investigate the identity of the nicotinic acetylcholine receptor (nAChR) subtypes involved in modulating noradrenaline (NA) release, in rodent frontal cortex (FC) and hippocampus (HC). Comparisons were made both *in vitro* and *in vivo* using pharmacological tools.

In vitro, acute application of nicotinic agonists evoked release of previously loaded [^{3}H]-NA from prisms of rat FC and HC. There was a 2000-fold more potent response to β2* selective nAChR agonist 5-iodo-A85380 in FC than HC. A greater response to choline in HC than FC, combined with a lack of response to selective α7 ligands supports α3β4* nAChRs as the main mediator of nicotinic stimulated NA release *in vitro* in HC. A proportion of the release in each region was mediated via a potentially excitatory action of GABA. The profile of responses was unchanged after the acute or chronic administration of nicotine *in vivo*.

In vivo microdialysis experiments were designed to test whether the nAChR subtype differences *in vitro* were representative of differences *in vivo*. 5-iodo-A85380 administered by reverse dialysis increased NA levels to a greater extent in FC than HC, supporting differences in the nAChR composition involved in NA regulation between these two regions. Targeted stimulation of these different nAChR subtypes could allow exploitation of this disparity to improve function with novel compounds such as those described in Chapter 2.

Overall the studies described in this thesis show that there are differences in the subtype of nAChRs involved in NA release from terminal fields of FC and HC both *in vitro* and *in vivo*.

Abbreviations

α-Bgt	α-bungarotoxin
[^{3}H]-	Tritiated
3-MT	3- methoxytyramine
5-CSRTT	Five choice serial reaction time task
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin, 5 hydroxy-tyramine
5IA	5-Iodo-[3-(2(s)- azetidinylmethoxy) pyridine] dihydrochloride
Å	Angstrom
ACh	Acetylcholine
AChBP	Acetylcholine binding protein
aCSF	Artificial cerebrospinal fluid
ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of Variance
AP	Anterior-Posterior
CICR	Calcium induced calcium release
CNS	Central nervous system
Cpm	Counts per minute
CPu	Caudate putamen
CSF	Cerebrospinal Fluid
DA	Dopamine
DAT	Dopamine transporter
DHBA	3,4-dihydroxybenzylamine HBr
DHβE	Dihydro-beta-erythroidine
DOPAC	3,4-Dihydroxyphenylacetic acid
DV	Dorsal-Ventral
EC50	Half maximal agonist concentration
ECD	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid
Epi	Epibatidine
EQ	Encephalisation quotient
EtOH	Ethanol
FC	Frontal cortex
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric Acid
HC	Hippocampus
HPLC	High Pressure Liquid Chromatography
HVA	Homovanillic acid
i.p.	Intraperitoneal
KB	Kreb's buffer

KBN	Kreb's buffer + 0.5 uM nomifensine
KDa	Kilo Daltons
LC	Locus coeruleus
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MeOH	Methanol
ML	Medial-Lateral
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	Noradrenaline
NAcc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NET	Noradrenaline transporter
NT	Neurotransmitter
OSA	Octanosulphonic acid
PAM	Positive allosteric modulator
PBS	Phosphate buffered saline
PCA	Perchloric acid
PD	Parkinson's disease
PEI	Polyethyleneimine
PET	Positron emission tomography
PMSF	phenylmethylsulfonyl fluoride
PNU-120596	1-(5-chloro-2,4-dimehtoxy-phenyl)-3-(5-methyl-isoxazol-3-yl) urea
s.c.	Subcutaneous
SEM	Standard error of the mean
SNc	Substantia nigra pars compacta
ST	Striatum
TH	Tyrosine hydroxylase
VOCC	Voltage operated calcium channels
VTA	Ventral tegmental area
WCST	Wisconsin card sorting task

Communications

Posters

Kennett AF., Heal DJ., Wonnacott SJ., (2008). Comparison of the effects of morphine and nicotine on noradrenergic function in the rat frontal cortex. 10th Annual conference of the SRNT Europe, Rome, Italy 23rd-26th September 2008, pp. 147 http://www.srnt.org/conferences/eu/eu_past/08europe.cfm

Kennett AF., Heal DJ., Wonnacott SJ. (2009) Distinct pharmacological profiles for nicotinic AChR-evoked noradrenaline release in rat frontal cortex and hippocampus. Program number B86 34.3, Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online.

Kennett AF., Heal DJ., Wonnacott SJ. (2010) Investigating the subtypes of nicotinic AChR involved in catecholamine release in different regions of the rodent brain British Association of Psychopharmacology Summer Meeting 25th-28th July 2010, Harrogate, UK. Supplement to Journal of Psychophamrmacology 24, pp A32

Oral Communications

Kennett AF., Differences In Nicotinic Acetylcholine Receptor Subtypes Involved In Noradrenaline Release In Frontal Cortex And Hippocampus. Nicotinic Acetylcholine Receptors 2011, 18th-21st May 2011, Hinxton, UK. Abstract number T21 https://registration.hinxton.wellcome.ac.uk/display_info.asp?id=199

Papers

Kennett AF., Heal DJ., Wonnacott SJ. Pharmacological differences between rat frontal cortex and hippocampus in the nicotinic modulation of noradrenaline release implicate distinct receptor subtypes. In submission to Nicotine and Tobacco Research

Chapter One

1. Introduction

1.1. The mammalian brain and its anatomical and functional subdivisions

Throughout the evolution of mammals and primates there has been an increase in brain size relative to body size; however this has been greatest in the human lineage. Deviation from a correlation of the size of the brain in relation to body size however is thought to be an overly simplistic measure (Jerison and Count, 1955). This increase in size has not been uniform across all brain structures. The human brain is unique in its structure, with greatly enlarged cerebral hemispheres. The expansion of the cerebral cortex and the degree of folding are now thought to be important factors in the increased capacity for adaptation our large brain allow us. The degree of encephalisation has been linked to the perceived intelligence across vertebrate species. Reptiles for example have a lower encephalisation quotient (EQ) than mammals. Within mammalian species carnivores have higher EQ than insectivores, with simians and *Homo sapiens* having the highest. Interestingly dolphins have a similar EQ to humans.

The earliest suggestions that specific brain functions could be associated with different brain regions came from the pseudoscience of phrenology, where the shape of different parts of the skull was linked to personality traits. Although this is not now an accepted theory the notion of regional specification remains. Study of patients with brain injuries through trauma, stroke or disease allowed the early mapping of function to specific regions in the brain. Extension of these cases by study of selective lesions of areas of the brain in animal models has allowed a more detailed map to be created, although many functions are so complex that they cannot be mapped to a specific brain region but depend on circuits linking two or more regions. Functional mapping using imaging techniques has also become possible in recent years in both human and animal models. Techniques such as functional magnetic resonance imaging (fMRI) measure the hemodynamic response in relation to activity (Ogawa *et al* 1990). The measurement of uptake of radiolabelled 2-deoxy-glucose by positron emission tomography (PET) scanning allows location of regions with increased glucose utilisation and therefore assumed increase in function (Baron *et al*, 1982). These techniques allow the functional mapping of brain function under physiological conditions and without lesions, giving additional information on the duration and pattern of responses under a range of conditions (Miller *et al*, 1987; Goebel *et al*, 1998). This thesis focuses on a comparison of two regions, frontal cortex and hippocampus, which will be discussed in greater detail below.

1.1.1. Prefrontal cortex

The prefrontal cortex (PFC) is an important region of the brain to study due to its role in executive control of behaviour (Goldman-Rakic, 1996). Executive function involves decision making, and the ability to override instinctual or habitual responses. This region of the brain is proportionally larger in humans in comparison with other species, giving rise to the higher cognitive functions.

All of the major psychiatric disorders have involvement of the PFC, in fact a deficit in executive function in one form or another is the hallmark of a psychiatric disorder (Fogel, 1994). Frontal lobotomy, where connections to and from the PFC are severed, was used as a treatment for psychiatric disorders, and often left patients with little ability to function in normal society. Functions such as decision making were affected, and attentional processes and cognition were also damaged.

Damage to the PFC often causes deficits in executive function, with Phineas Gage a notable example. After an accident fired an iron rod through his brain, damaging his left frontal lobe, Gage became aggressive and impulsive (Harlow, 1868 reprinted 1993; Macmillan, 1986) although reports of the case are likely to have been exaggerated. Brain injuries suffered to the PFC such as from car accidents are known to be linked to behavioural changes, which may take years to recover from (Miller, 2000). Again these changes seem to arise from lack of control over executive function, with aggression and lack of ability to concentrate. These symptoms can persist long after other aspects of recovery (such as relearning motor skills) have been completed.

1.1.1.1. Location and structure of PFC

The PFC is the anterior part of the frontal lobe of the brain, stimulation of which does not lead to motor responses (Figure 1-1). For this reason it is also sometimes referred to as the non-motor cortex. In humans and other primates PFC is highly developed. There is some debate on a precise definition of the PFC, and on if this definition translates between species, especially between primates and rodent models (see Preuss, 1995). Many definitions depend on the arrangement of cellular layers within the cortex, with a granular layer IV, however this is absent in the rat (Preuss, 1995). This definition has been expanded to include some non-granular regions in the frontal cortex, allowing similarities with rodent brain to be identified. Reciprocal connections with other brain regions are now thought to be important for PFC function and therefore specification (Figure 1-1; Arnsten, 2009). In particular, a major projection from the thalamic mediodorsal nucleus is present which reaches both granular and non-granular regions in the primate cortex. These projections are also present in rodent brain, suggesting a similarity of the non-granular region of non-motor cortex across mammalian species (Leonard, 1969).

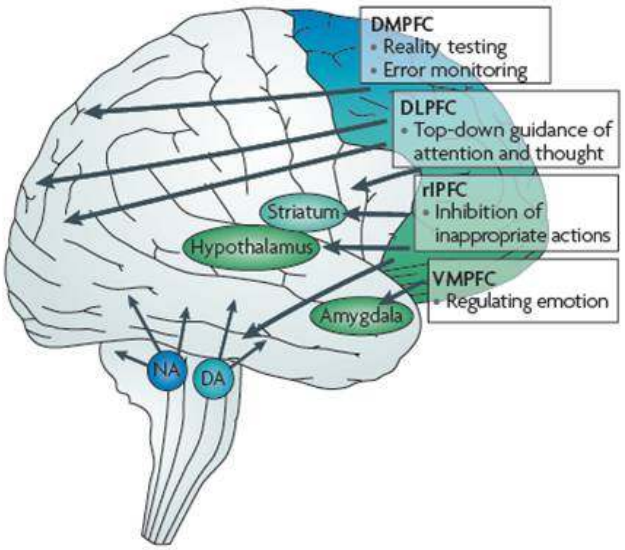


Figure 1-1 Functional subdivisions of the frontal cortex
The frontal cortex has extensive connections with other brain regions and subdivisions are highly connected. This region regulates higher order decision making and planning. From Arnsten, 2009 with permission.

Studies of catecholamine distribution show that primate PFC is richly innervated by the dopaminergic system, with non-primates also showing innervation from dopaminergic nuclei. The distribution of this innervation is broadly similar to the mediodorsal nucleus projection (Divac *et al*, 1978). These projections do not distinguish this region from other parts of the FC (Preuss and Goldman-Rakic, 1991), however connections from PFC to the basal forebrain have been shown in both primate and non-primate species (Figure 1-1; Groenewegen *et al*, 1997).

The PFC is involved in delay-task performance in both primates and non-primates, suggesting a functional similarity of the region across species. Due to the difficulty in achieving a precise definition of the PFC across species the broader term FC will be used within this thesis. Emphasis on functional as opposed to structural homology generally shows more translatability of the definition of FC across species, and therefore allows the use of rodents as a practical and useful model for aspects of FC function (Preuss, 1995).

1.1.1.2. Interrogation of FC function

Examination of executive function and activity of the FC is possible due to development of behavioural tests, however none of these is perfect and can only give a surrogate for aspects of FC functioning. For many of the tasks multiple brain regions are involved, although attempts have been made to separate the roles of these regions. Another issue is that animals do not communicate in the way that we do, inferences must be made from observing behaviour and extrapolating to cognition. With studies in humans, of course, language can be used as well as behavioural measures giving a fuller picture, albeit

with more limited experimental regimens. Some of the common behavioural tests that rely on FC function, at least in part, are outlined below.

Five-choice serial reaction time task

The five-choice serial reaction time task (5CSRTT) requires a nose poke or lever press on one of five locations in response to a cue. Usually the cue is a light above the appropriate response location. This task can be used to assess attention, reaction time, accuracy of responses and impulsivity in the form of prepotent responding. The duration of the light presentation and the time between trials can be varied to increase the power of the test. The 5CSRTT is a useful tool in the investigation of attention deficit hyperactivity disorder (ADHD) as several aspects of attention can be investigated simultaneously. This task is further reviewed in Robbins (2002). This task has been further refined with the addition of a 'No-Go' condition to allow the separation of motor impulsivity from behavioural disinhibition (Young *et al*, 2009; 2011). Performance in go/no-go tasks has been localised to specific FC subregions (Chikazoe *et al*, 2010).

Attentional set shifting/ Wisconsin card sorting task

In humans and primates this task involves the sorting of a series of cue cards into groups. They may be sorted by colour, shape or the number of symbols present. Once the rule for sorting has been learned an interdimensional shift, where, for example, two new colours are the relevant cues, takes place. Reversals then take place, where the previously correct answer is now incorrect. The most cognitively challenging aspect of the task is the extradimensional shift, where the previously relevant dimension (colour/shape) is no longer relevant. The animal version of the task requires some modification, but involves digging in bowls for food rewards, with odour and digging medium as the variables for the task (Birrell and Brown, 2000). Perseveration is the persistent repetition of an action, often in a manner which cannot be controlled. This symptom is in some psychiatric disorders such as autism and obsessive compulsive disorder. Perseveration of responding for the incorrect dimension is common in schizophrenia models, and is separate from learning of the initial task and so not a deficit in ability to learn. McGaughy *et al* (2008) showed that noradrenergic but not cholinergic deafferentation to the FC impaired attentional set shifting, with the extradimensional shift greatly affected.

1.1.1.3. Summary

FC is an important brain region as it is involved in many disorders, with particular impact on attentional tasks and executive function. FC does not act in isolation; it receives inputs from many other brain regions and acts to integrate signals to balance cognition and emotion. Another region of the brain known to be important in learning and memory tasks is the hippocampus.

1.1.2. Hippocampus

The hippocampus (HC) has been widely studied due to its roles in learning and memory. It forms part of the limbic system and has an important role in consolidation from short to long term memory and spatial navigation. Damage to the HC can cause anterograde amnesia, the inability to form new memories. A well known case of this is that of H.M. (named as Henry Gustav Molaison since his death in 2008). After bilateral removal of his HC to relieve severe epilepsy H.M. was unable to form new episodic memories although he was able to learn new skills albeit with no knowledge of having done so (case review in Corkin, 2002). Learning in picture recognition tasks was seen, possibly due to part of the ventral perirhinal cortex being spared. H.M. was impaired in spatial tasks, showing an important association between HC and spatial ability, although he was able to draw a map of the floor plan of his residence, suggesting that repeated exposure can allow this deficit to be partially overcome (Kolb and Wishaw, 1996; Corkin, 2002).

The role of the HC in spatial tasks is well established (Morris *et al*, 1982; Deiana *et al*, 2011). Place cells, which fire when the animal is in a certain location in the environment; have been shown in rodents (O'Keefe, 1976; Moser *et al*, 2008). They interact with head direction cells and grid cells in the entorhinal cortex helping to form cognitive maps. These maps may be egocentric (with self as a reference point in relation to intramaze cues) or allocentric (with self in relation to objects in 3-dimensional space). The HC is involved in the learning of allocentric spatial representations (Deiana *et al*, 2011).

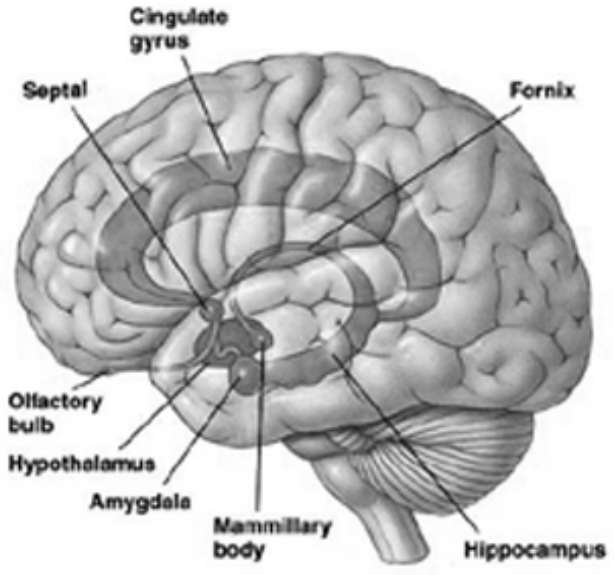


Figure 1-2 The location of the hippocampus
A diagram of the human brain with the hippocampus, amygdala and other areas of the limbic system indicated. There are extensive connections between the HC and other parts of the limbic system leading to roles in emotion and motivation. Reproduced from Joseph, 2000.

1.1.2.1. Location and structure

The hippocampus is located in the medial temporal lobe, beneath the cortical surface (Figure 1-2). The majority of HC cell bodies are glutamatergic, with around 10 % GABAergic interneurons, however much of the innervation in the HC is noradrenergic, originating from the locus coeruleus. Afferent pathways from other regions of the brain contribute innervation by dopamine, serotonin, acetylcholine, GABA and glutamate. Each of these transmitter systems can interact with the others, and autoreceptor modulation of responses also takes place leading to great complexity within the HC (for full review see Vizi and Kiss, 1998). Table 1-1 summarises the afferent and efferent pathways of the main HC neurotransmitter systems. The main interactions that are important in the context of this thesis are those of the noradrenergic and cholinergic systems, specifically the actions of ACh mediated by nicotinic acetylcholine receptors. Nicotinic acetylcholine receptors (nAChRs) have been shown to elicit noradrenaline (NA) release by presynaptic mechanisms (1.3.2).

Table 1-1 Major hippocampal transmitter systems
ND- not determined. Reproduced from Vizi and Kiss (1998) with permission with additional information from Shohamy and Adcock, 2010.

Transmitter system	Afferent pathways	Efferent pathways	Synaptic incidence of varicosities	Intrahippocampal cell body
Glutamate	Entrorhinal, commissural	Hippocampal-subicular, hippocampal-entorhinal, commissural	100 %	Pyramidal cell, Granule cells
GABA	Septohippocampal, commissural	Hippocampal-septal, commissural	100 %	Interneurons
ACh	Septohippocampal	-	7 %	Interneurons
NA	Locus coeruleus-hippocampal	-	15 %	-
5HT	Raphe-hippocampal	-	21 %	-
DA	VTA-hippocampal	-	ND	-

There are large bidirectional connections between the ventral HC and the amygdala (Figure 1-3; Kishi *et al*, 2006), a region linked to stress and emotion. These projections and the knowledge of involvement of noradrenaline in both HC and amygdala suggest a role for ventral HC in emotional responses. As place fields are present across the whole HC this role may be to do with goal directed behaviours and the motivational significance of an area (Fanselow and Dong, 2010). Stress is known to trigger reinstatement of drug seeking; links between noradrenaline, amygdala and HC may therefore be implicated in this behaviour (1.2.2; Belujon and Grace, 2011).

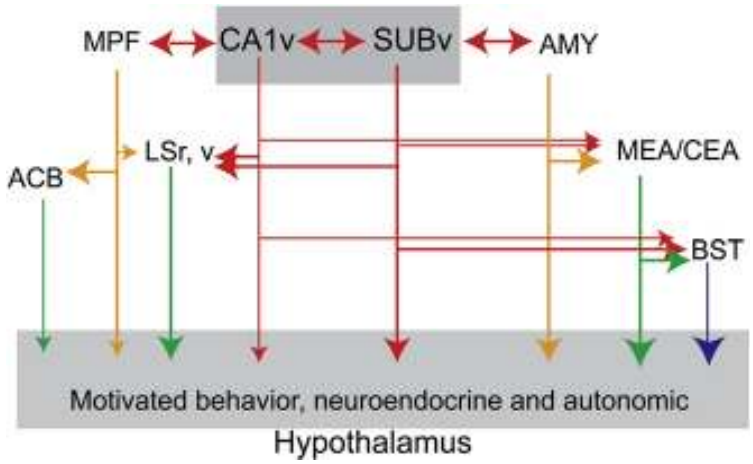


Figure 1-3 Neuronal connectivity of the ventral HC
Abbreviations: ACB, nucleus accumbens; AMY, cortical-like amygdalar areas (nuclei); BST, bed nuclei of the stria terminalis; CEA, central amygdalar nucleus; LSr, v, the rostral and ventral parts of the lateral septal nucleus; MEA, medial amygdalar nucleus; MPF, medial prefrontal cortex; SUBv, the ventral subiculum. From Fanselow and Dong, (2010) with permission.

1.1.2.2. Examining the HC

Separating procedural and episodic memory is relatively simple in humans although, as the case of H.M. shows, there are links between these. In animals these aspects are more difficult to separate and so many of the tasks available make some assumptions about what precisely is measured. Most tasks that are affected by HC manipulation in animal models are spatial in nature.

Morris water maze

This task involves the animal being placed in a tub of opaque water; within which there is a hidden platform which allows escape (Morris, 1981). The criteria measured are the time and distance swum to reach the platform. Both extra-maze (e.g. symbols on walls) and intra-maze cues are used although it was shown that starting position had little effect on the speed to locate the platform (Morris, 1981). Animals with hippocampal lesions do not learn the location of a hidden platform in the Morris water maze, whilst escape to a visible platform is unaffected (Morris *et al*, 1982). The role of the hippocampus in this model includes self-localisation and route replay (Redish and Touretzky, 1998).

Radial arm maze

This is a maze with a central platform surrounded by a variable number of arms, which can be blocked, baited or left open and unbaited. Animals must navigate to food rewards placed at the ends of arms. In the simplest forms of the test the animal must remember which arms have already been visited in the current test session, as this the most efficient way to visit all arms and therefore collect all food rewards. More complex versions of the test involve removing animals from the maze between arm entries to extend the time that previously visited arms must be remembered. Procedural or reference memory

(learning arms that are consistently unbaited) and episodic or working memory (remembering which arms have been visited in the current session) can be separated to a certain extent in this task, with pharmacological interventions shown to affect one component or the other. Bouffard and Jarrard (1988) showed that HC lesions affect both reference and working spatial memory.

Odour span task

This is a non-match to sample task, where increasing numbers of differently scented pots are presented, with a food reward in the novel scented pot. The previously presented pots do not remain in the same locations between trials, to separate spatial working memory from the odour span. Animals with damage to the hippocampus perform this task normally, but with poor performance if a delay is introduced, suggesting that this form of working memory does not rely on HC (Dudchenko *et al*, 2000). Indeed H.M. retained close to normal performance in working memory tasks (1.1.2). This suggests that working memory for lists of previously presented stimuli is slightly different to spatial working memory, and so some rodent tasks may not be ideal for separating these factors.

1.1.2.3. Summary

The HC is involved in learning and memory tasks, in particular the consolidation of memory and in spatial mapping. Ablation of the HC or perturbations of its function lead to anterograde amnesia and dysfunction in spatial tasks. Projections between HC and amygdala suggest a role for HC in emotional responses and stress, with mediation via the catecholaminergic systems likely to be important.

1.2. Catecholamines

1.2.1. Dopamine

Although originally thought to be merely a precursor to noradrenaline (Figure 1-4), following the discovery of its neurotransmitter properties the role of dopamine (DA) in the brain has been widely studied (Carlsson *et al*, 1957; 1958; Benes 2001). DA has strong links to movement and reward in the striatum, attention in the FC and many other processes (Floresco and Magyar 2006; Scultz 2002; Thomas and Beal, 2007).

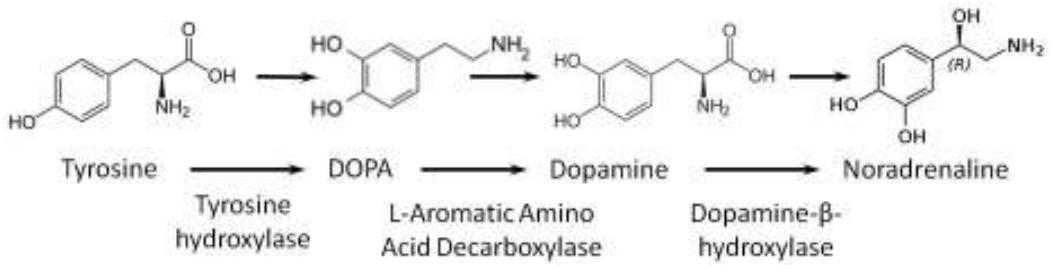


Figure 1-4 Synthesis pathway of dopamine and noradrenaline

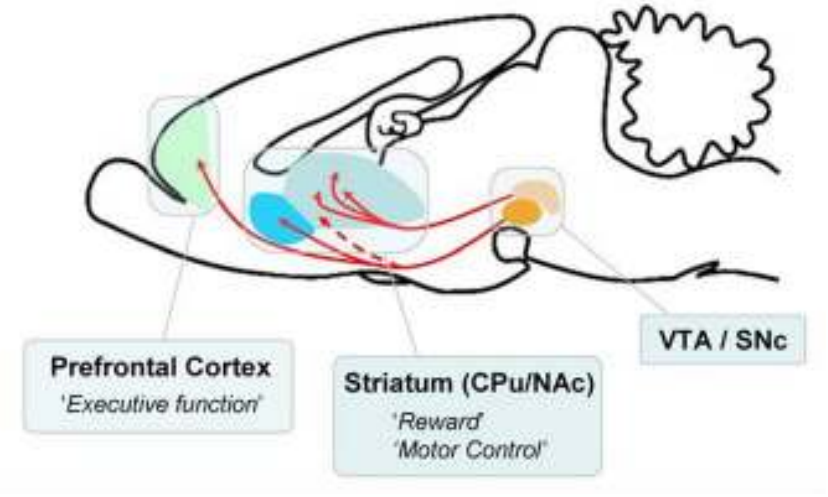


Figure 1-5 Major dopaminergic pathways in the rat brain
The caudate putamen (CPu) or dorsal striatum (light blue) is innervated by dopaminergic neurons from the substantia nigra pars compacta (SNc, light orange) forming the nigrostriatal pathway and, to a lesser extent, from the ventral tegmental area (VTA, orange, dashed line). Two major dopamine pathways originate in the VTA; the mesolimbic pathway that terminates in the nucleus accumbens (NAcc, dark blue) and the mesocortical pathway in the prefrontal cortex (green). From Livingstone and Wonnacott (2009), with permission.

DA acts via well defined pathways that give dense innervation of specific regions of the brain. Neurons from the ventral tegmental area (VTA) project to the ventral striatum (or nucleus accumbens, NAcc) in the mesolimbic pathway and also to the FC forming the mesocortical pathway (Bjorklund and Dunnett, 2007; Figure 1-5). The dorsal striatum (caudate putamen, CPu) is innervated by dopaminergic neurons from the substantia nigra pars compacta (SNc,) forming the nigrostriatal pathway and, to a lesser extent, from the VTA.

DA receptors are metabotrophic G-protein coupled receptors that fall into two main classes, D_1-like (D_1 and D_5) and D_2-like ($D_{2\text{-}4}$). Stimulation of D_1-like receptors activates adenylyl cyclase and thereby increases cAMP production. D_2-like receptors are inhibitory; activation inhibits the formation of cAMP. The distribution of the receptors is distinct but overlapping, D_1 receptors are found in the striatonigral neurons and D_2 in the striatalpallidal and as autoreceptors, inhibiting the further release of DA. Density of D_2 receptor availability as measured by PET has been inversely linked with propensity to abuse drugs both in humans and in animal models. It is seen that lower basal D_2 receptor availability precedes drug taking and increases impulsivity, however decreases in D_2 availability can also be induced after drug administration (reviewed by Nader and Czoty, 2005). This suggests that a lower basal D_2 availability increases impulsivity and therefore propensity to administer drug and the drug administration then causes a decrease in D_2, which feeds back to an increased likelihood of further drug use. Phasic firing of dopaminergic neurons indicates differences between actual and predicted rewards (Scultz, 2002) and may be hijacked by drugs of abuse. Most drugs of abuse increase striatal DA, but by different

mechanisms. Some cause increased firing of dopaminergic neurons but others increase DA by inhibiting or reversing dopamine transporters or by decreasing metabolism. Amphetamine-based drugs that reverse the DA transporter have high abuse liability as there is no dose ceiling effect and their actions on catecholaminergic systems are very powerful (Heal *et al*, 2009a). Other drugs such as nicotine act to increase DA by promoting its release. These different mechanisms of action will be explored in Chapter 3.

In striatum DA is also involved in motor control, but the mechanism varies. Motor processes are enabled by tonic dopaminergic firing as opposed to phasic changes. There is an optimal level of DA for correct functioning. Levels of DA that are too low are found during Parkinson's disease (PD) and cause poverty of movement. Other symptoms include depression, apathy and cognitive problems (Krishnamoorthy and Craufurd, 2011). A common model of PD is treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This potent toxin destroys dopaminergic neurons in the substantia nigra and so causes symptoms of PD. Motor deficits are not seen unless there is an almost complete lesion, so low numbers of neurons can compensate for substantial losses. Noradrenaline has also been suggested to play a role in PD (see 1.2.2). Levels of DA that are too high in striatum cause hyperactivity (Gainetdinov *et al*, 1999). Increased DA levels have also been linked with schizophrenia and mania.

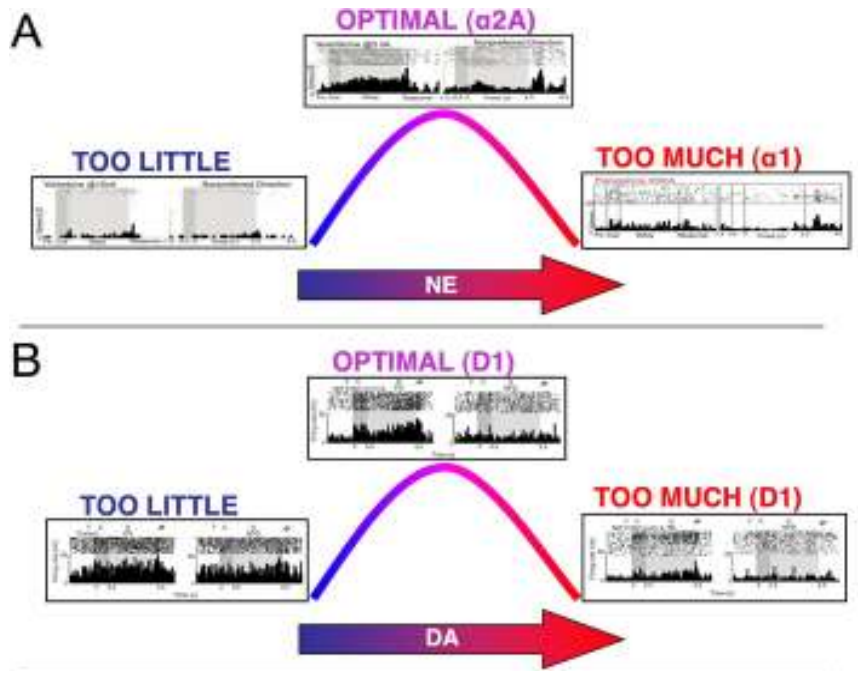


Figure 1-6 Inverted U shaped influences of catecholamines on FC function
Both NA **A)** and DA **B)** must be present at optimal levels for optimal FC function. There are increasing levels of catecholamine release with arousal level with low levels during fatigue, optimal phasic release during alert conditions and high levels during stress. The examples are patterns of FC neuronal firing from monkeys performing a delayed response spatial working memory task with the preferred direction (PD) on the left of each example, and non-preferred direction (NPD) on the right. Figure adapted from Arnsten (2011) with permission.

DA in the FC is involved in attention and working memory tasks, with low levels during fatigue or depletion leading to poor performance (Arnsten, 2011; Figure 1-6). Optimal performance and FC neuron firing is found at moderate DA levels, with levels that are too high (under stress or after drug administration) causing the FC to become disengaged (Cools and D'Esposito, 2011). Disengagement of the FC can cause cognitive inflexibility seen as perseverance in the WCST (1.1.1.2) and often noted in schizophrenics. Schultz (2002) has attributed the modulation of executive function by DA to its roles in FC.

1.2.2. Noradrenaline

Noradrenaline is produced from dopamine by dopamine-β-hydroxylase (Figure 1-4). In contrast with the well studied role of dopamine within the nervous system, less focus has been directed upon noradrenaline (NA) recently. The role of NA has been shown to include attribution of salience to stimuli, attentional control, executive functioning in the FC and modulation of the responses of the DA system. Neurons releasing NA arise from the locus coeruleus (LC; Figure 1-7) and project across the brain. NA release from nerve terminals is exocytotic, and requires Ca^{2+}; termination of signalling is by reuptake.

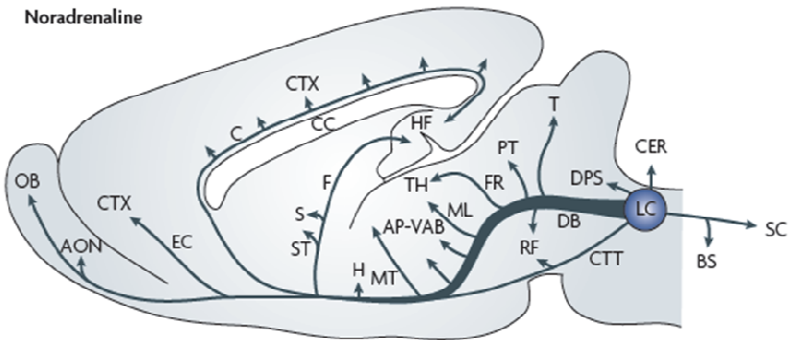


Figure 1-7 Noradrenergic pathways in the brain
The main noradrenergic pathways in the rat brain. Abbreviations A, amygdala; AON, anterior olfactory nucleus; AP-VAB, ansa peduncularis–ventral amygdaloid bundle system; BS, brainstem nuclei; C, cingulum; CC, corpus callosum; CER, cerebellum; CTT, central tegmental tract; CTX, cortex; DB, dorsal bundle; DPS, dorsal periventricular system; F, fornix; FC, frontal cortex; FR, fasiculus retroflexus; H, hypothalamus; HF, hippocampal formation; LC, locus coeruleus; ML, medial lemiscus; MT, mamillothalamic tract; OB, olfactory bulb; PT, pretectal area; RF, reticular formation; S, septum; SC, spinal cord; ST, stria terminalis; T, tectum; TH, thalamus. From Sara, 2009 with permission.

Similarly to DA receptors NA receptors are G protein coupled. The actions of the β adrenergic receptors are mediated by G_s coupled to adenylyl cyclase and GTP leading to the formation of cAMP and then activation of PKA which can phosphorylate a variety of proteins (Figure 1-8). The actions of β1 and β2 receptors oppose one another, which may be due to the different distribution, with β2 on glial and β1 on neuronal cells. The α2 subtype of NA receptor is coupled to G_i instead of G_s, and so inhibits the cAMP/PKA pathway. This is important as it is present presynaptically where it acts as an inhibitory autoreceptor (reviewed by Starke, 2001). The postsynaptic α1 adrenoceptor acts rather differently and is excitatory through activation of intracellular Ca^{2+} stores via IP_3 (Figure 1-8).

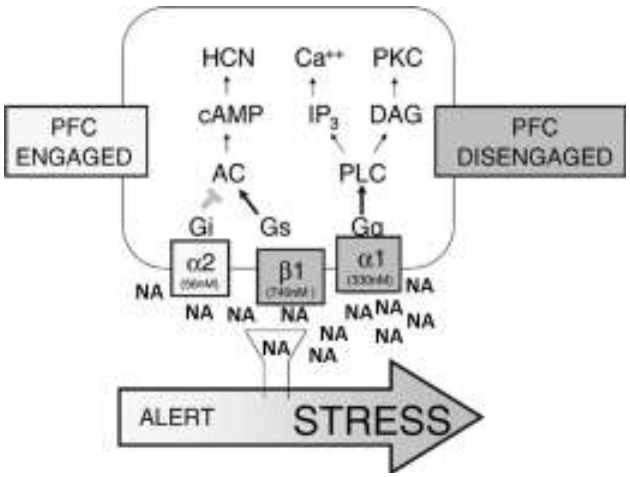


Figure 1-8 NA receptor subtypes
Increasing levels of NA stimulates receptors with different affinities that activate different intracellular pathways. AC, adenylyl cyclase; LC, locus coeruleus; PLC, phospholipase C; IP_3, inositol 1,4,5-triphosphate; DAG, diacylglycerol. Adapted from Ramos and Arnsten (2007) with permission

Regulation of NA is extremely important, as its influence on cognition has an inverted U shape, with optimal functioning at moderate levels of NA, as seen for DA (Arnsten, 2011; Figure 1-6). Low NA levels, through fatigue, depletion or antagonism impair performance and cause poor attention. The subtype with the highest affinity is α2 and so moderate levels of NA enhance FC cognitive function. Higher NA levels (such as during stress) stimulate α1 and β1 receptors, causing disengagement of the FC (Figure 1-6 and Figure 1-8).

Attention deficit hyperactivity disorder (ADHD) is characterised by deficits in attention and impulse control. It has been linked to several genetic variants, including some in catecholaminergic systems; in particular DA receptor genes, the DA transporter (DAT) and the NA transporter (NET; Arime *et al*, 2011). Low FC DA and NA in comparison with control are seen as a result of the dysfunction of the transporters (reviewed in Prince, 2008; Levy, 2009). One animal model of ADHD is the spontaneous hypertensive rat, with the DAT knockout mouse also used by some groups (Meneses *et al*, 2011; Arime *et al*, 2011).

The main classes of drug prescribed for ADHD are stimulants and catecholamine reuptake inhibitors although α_2-adrenoceptor agonists and monoamine oxidase inhibitors are also used. The efficacy and safety of these pharmacological treatments is further discussed in Heal *et al* (2009a). Briefly, these drugs act to increase FC NA and DA to optimal levels, although the administration of high doses can cause levels that are too high and thus suboptimal performance on attentional tasks. Stimulant medications are more efficacious than reuptake inhibitors, but with a higher abuse liability (1.2.1). All of the medications currently available for ADHD influence both DA and NA transmission and improve aspects of FC function. The role of nAChR agonists as possible alternative ADHD treatments has been investigated for several drugs in recent years (1.4).

NA has also been considered in conditions such as Parkinson's disease (PD). Although usually thought to be characterised by loss of nigrostriatal DA neurons (1.2.1) there is also a loss of NA neurons arising from LC in PD (Marien *et al*, 2004; Rommelfanger and Weinshenker, 2007). A neurotoxic lesion of DA neurons with MPTP is the usual PD animal model, but this does not model the motor deficits well in rodent models (Tillerson *et al*, 2002). The dopamine-β-hydroxylase knockout mouse (dbh-/-) which is unable to produce noradrenaline from dopamine has abnormalities in coordinated movement (Rommelfanger *et al*, 2007), and this is not exacerbated by MPTP treatment. The motor impairment is also seen in NA-lesioned animals and could be improved by the restoration of NA or by DA agonist treatment. These data suggest a synergistic effect of DA and NA loss during the early stages of PD, possibly opening up new treatment options.

Noradrenergic transmission also has a role in the positive rewarding effects of drug taking, dbh-/- mice do not show conditioned place preference for morphine, although place preference for food rewards (a natural stimulus) is the same as for wild-type, suggesting that that there is no effect on learning. Place preference was found in these animals for morphine if NA signalling was rescued in the central nervous system (Jasmin *et al*, 2006). Highly salient natural stimuli induce a greater release of FC NA than low salience stimuli. Selective FC NA depletion with DSP-4 has been shown to impair CPP induced by these highly salient stimuli to a greater extent than for stimuli with low salience (Ventura *et al*, 2008). This suggests that NA is particularly important in regulating responses to natural stimuli when they are of high salience, the responses to many drugs of abuse are higher than for natural stimuli and so the FC NA system is likely to be involved in responses.

NA is also increased in withdrawal from addicting substances. Destruction of the LC reduces physical symptoms during withdrawal from opiates (Maldonado and Koob, 1993). Noradrenergic neurons project to the amygdala, causing the release of corticotrophin releasing hormone, further activating the release of NA. This feed forward mechanism during withdrawal is aversive, leading to anxiety and increasing drug seeking in order to relieve craving. The shift from the hedonic reward-seeking to the negative relief-seeking mode is an important factor in the theory of drug addiction (compulsive behaviour) and drug taking (Koob, 2009). The involvement of NA and modulation of emotional centres in the brain goes some way to explain how stress can trigger relapse to drug seeking in a variety of paradigms (reviewed in Steketee and Kalivas, 2011).

Many factors are known to influence the release of NA, and hence influence the conditions mentioned. One of these factors is the administration of nicotine. NA has a number of modulatory effects in various brain regions; a greater understanding of the regulation of NA in various brain regions by nicotinic receptors may contribute towards a greater understanding of nicotine's effects.

1.3. Nicotinic Acetylcholine Receptors

In the Western world tobacco smoking is the leading preventable cause of premature death, contributing to an estimated 5 million deaths per year from smoking related illnesses such as cancers and vascular and chronic lung diseases (WHO, 2009). Only 4-6% of those who attempt to quit are successful in doing so (RCP, 2007) despite a range of therapies being available to assist quit attempts. Nicotine is the main addictive component of tobacco products, and exerts its effects via the nicotinic acetylcholine receptor (nAChR). Aside from the physical symptoms, one of the many reasons given for failure (or unwillingness) to quit smoking is perceived effects on cognition during nicotine withdrawal (Beaver *et al*, 2011). This, along with reports that patients with schizophrenia, depression, ADHD and other disorders affecting cognition smoke more, has led to research into the cognitive enhancing effects of nicotine. Data support an enhancement of cognition by nicotine in abstinent smokers, with varying reports of the effects in non-smokers (Snyder *et al*, 1989; Wignall and de Wit, 2011). Not all studies with human subjects have found improvements in cognitive performance with nicotine, suggesting that improvements are stronger when there is suboptimal function, such as during nicotine withdrawal. Many of the cognitive responses to nicotine have been shown to follow an inverted U-shape curve, as seen for catecholamines (Figure 1-6). This suggests that the cognitive benefits may be due to optimisation of neurotransmitter function. These data suggest that there may be benefits to nicotine treatment in other groups with cognitive impairment, especially as cognitive components are often an unmet treatment need in psychological disorders such as schizophrenia and depression. The addictive nature and side effects of nicotine mean that it is not an ideal drug and so drug design has focussed on nAChR subtype-selective compounds as possible therapeutic agents. This is made possible by knowledge of the structure, function and localisation of nAChRs within the brain.

1.3.1. Structure of nAChRs

The nAChR is found not only at the neuromuscular junction but also in the peripheral and central nervous systems, where it is more important in terms of addiction and withdrawal. The nAChR is a pentameric ligand gated ion channel of the cys-loop family (reviewed in Sine and Engel, 2006). In muscle nAChRs are formed from two *alpha*-1 subunits in combination with one each of *beta*-1, *gamma* and *delta* subunits with α subunits being defined by the presence of a pair of adjacent cysteine residues in the N-terminal domain that contribute to the ligand binding site. In CNS the nAChR consists of *alpha* (α2-10) and *beta* (β2-4) subunits. In the mammalian brain the α2-7 and β2-4 nAChR subunits are found. The pentameric channel can either be homomeric (α7) or heteromeric comprising combinations of α and β subunits with the ligand binding site at the interface

between two subunits. The most common heteromeric nAChR in the mammalian brain is the α4β2*, where * indicates the possibility of other subunits (Lukas *et al*, 1999). The α3β4* subtype is also found, as well as more complex subtypes e.g. α3/α6α4β2* (1.3.2). The α7 subunit can contribute both primary and complementary faces of the binding site and so is found as a homomeric pentamer (Figure 1-9). Although found throughout the brain there was little evidence of nAChRs mediating synaptic communication until their role in modifying transmission via a presynaptic localisation was shown (McGehee *et al*, 1995). The cellular localisation of nAChRs is further discussed below (1.3.2).

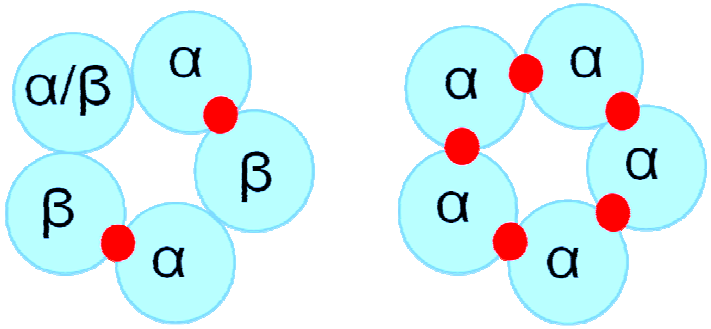


Figure 1-9 Example subunit stoichiometries of neuronal nAChRs
Neuronal nAChRs are either heteromeric (A) or homomeric (B). The agonist binding site (red dot) is between the *alpha* subunit and the adjacent subunit.

The binding site is at the interface between pairs of subunits and is comprised of residues from the primary and complementary faces. A number of important residues have been identified as being involved in ligand binding and these are highly conserved (Figure 1-10). Most of the conserved residues are present on the primary face of the binding site on the *alpha* subunit. The *beta* subunit contributes the complementary face and a number of less well conserved residues have also been identified, which are thought to be involved in conferring specificity to different receptor subtypes. The fifth subunit in a heteromeric receptor can be either *alpha* or *beta* and is usually thought to be structural (Figure 1-9), although functional roles of this accessory subunit have been postulated (1.3.3). The subtypes of nAChR differ in their affinities for ligands and so can be distinguished from one another. For example receptors containing α4 subunits have higher affinity for nicotine than those containing α3 subunits (Fenster *et al*, 1997). The significance of receptor composition will be discussed below (2.6.3.3).The interactions of several ligands (both agonists and antagonists) have been mapped by Parthiban *et al* (2010) and are shown below using the α7 nAChR residues (Table 1-2). Numbering of the conserved residues differs between subunits and from the acetylcholine binding protein (AChBP) hence the differences between Figure 1-3 and Table 1-2 (see Appendix 1for table summarising these differences).

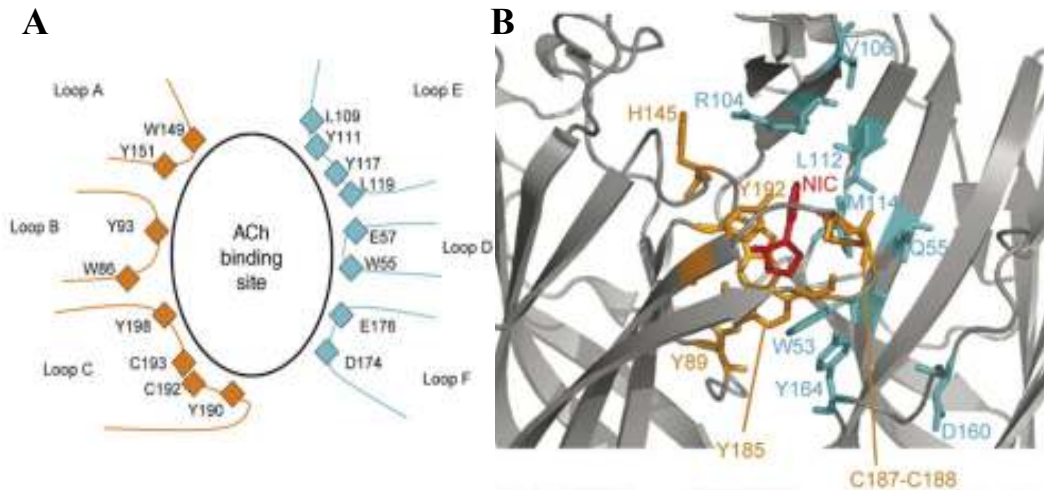


Figure 1-10 Conserved residues in the Ach binding pocket
A number of important residues around the Ach binding site have been identified. **A)** A schematic to show the loops contributing important residues from the primary (orange) and complementary (blue) faces of the binding site. **B)** X-ray crystallography structure of the ligand binding site in AChBP in complex with nicotine. Important residues are in stick structure and coloured according to the same scheme as A. From Changeux and Taly, (2008), with permission.

The difference in the effect of mutations on the binding of different drugs suggests that the residues that ligands interact with can differ. The main interactions for a selection of ligands are shown below (Table 1-2). Alterations to the structure of the ligand can strengthen or disrupt the bonds between ligand and receptor. Exploiting this can lead to the development of new selective ligands, however the interactions must be well modelled, which is only the case for the most highly conserved residues. Modelling selectivity is more difficult as there is variability between species as well as between subunits near the binding pocket in the non-conserved residues, meaning that the validation of selective drugs is hampered if they are to be developed for clinical use.

Table 1-2 Interacting residues from neuronal α7 nAChR
Those residues that are important for binding across all ligands listed are indicated in bold. Adapted from Parthiban *et al* (2010) with permission

Ligand	Interacting residues
Agonists	
Acetylcholine	Tyr91, Ser146, **Trp147**, Tyr186, **Cys188, Cys189**, Tyr193, Trp53, **Leu117**
Nicotine	Tyr91, Ser146, **Trp147**, Ser148, **Cys188, Cys189**, Tyr193, Trp53, Leu107, **Leu117**, Tyr116
Epibatidine	**Trp147**, Tyr186, **Cys188, Cys189**, Tyr166, Gln115, **Leu117**, Leu109
Antagonists	
Mecamylamine	**Cys188, Cys189**, Tyr193, **Trp147**, **Leu117**, Glu115, Leu107
DHβE	**Tyr147**, Tyr186, **Cys188, Cys189**, Tyr166, Gln115, **Leu117**, Leu109

1.3.2. Distribution and localisation

NAChRs are found throughout the mammalian brain, with higher densities in cortex, hippocampus and striatum than cerebellum. Early studies using autoradiographic techniques found little correlation between binding sites for [^{3}H]-nicotine and [^{125}I]-α-bungarotoxin (α-Bgt; Clarke *et al*, 1985). This led to the supposition that these were labelling different populations of nicotinic receptor, which are now known to be the high-affinity heteromeric subtypes and α7 for nicotine and α-Bgt respectively.

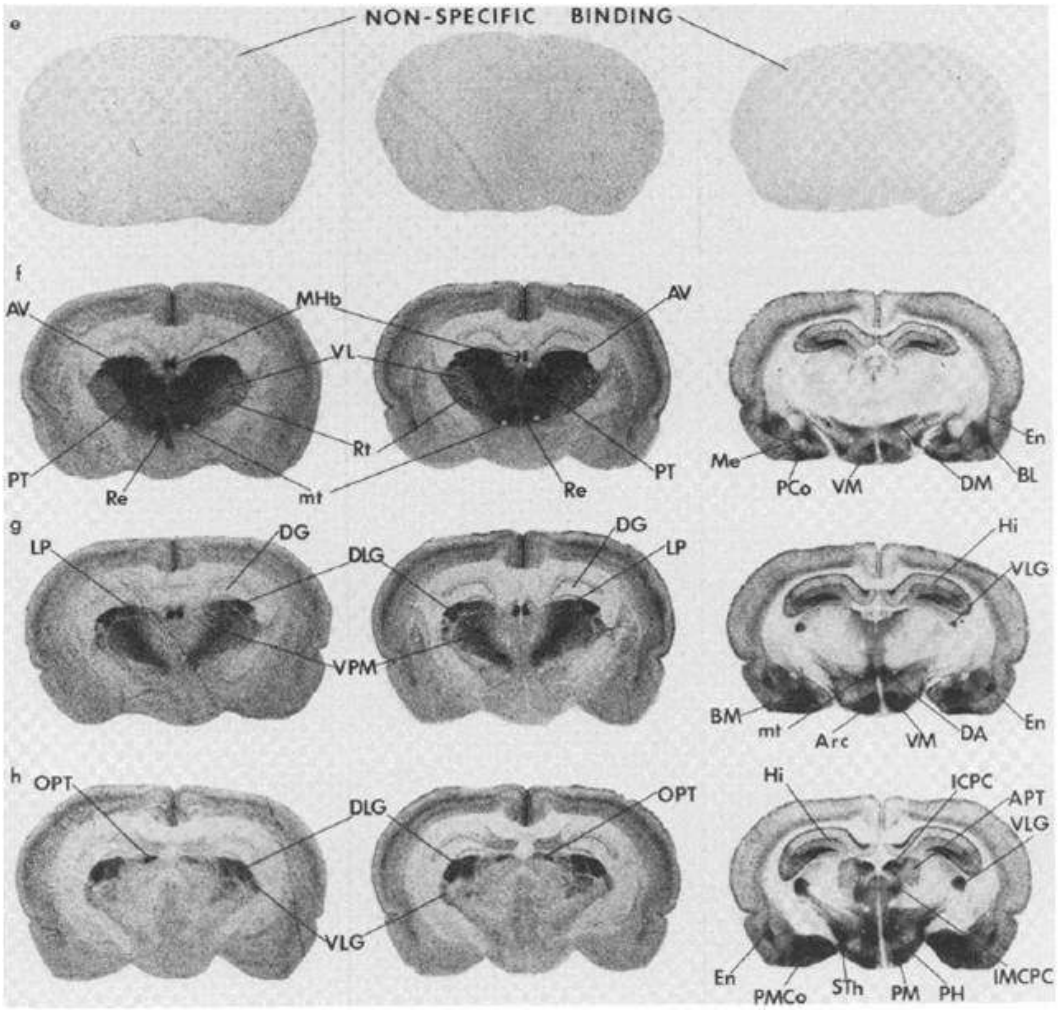


Figure 1-11 Autoradiographic nicotinic binding sites in rat brain
Serial sections were incubated with [^{3}H]-Acetylcholine (**left**), [^{3}H]-Nicotine (**middle**) or [^{125}I]-bungarotoxin (**right**). Non-specific binding was determined in an excess of unlabelled nicotine (10 µM for nicotine, 1 mM for α-Bungarotoxin) or carbachol (100 µM for Ach). Abbreviations; APT, Anterior pretectal area; Arc, arcuate hypothalamic nucleus; AV, anteroventral thalamic nucleus; BL, basolateral amygdaloid nucleus; BM, basomedial amygdaloid nucleus; DA, dorsal hypothalamic area; DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; DM, dorsomedial hypothalamic nucleus; En, endopiriform nucleus; Hi, hippocampus; ICPC, intracommisural nucleus of the posterior commisure; IMCPC, interstitial magnocellular nucleus of the posterior commissure; LP, lateral posterior thalamic nucleus; Me, medial amygdaloid nucleus; MHb, Medial habenular nucleus; mt, mammiliothalamic tract; OPT, olivary pretectal nucleus; PCo, posterior cortical amygdaloid nucleus; PH, posterior hypothalamic nucleus; PM, paramedian lobule; PMCo, posteromedial cortical amygdaloid nucleus; PT, paratenial thalamic nucleus; Re, reuniens thalamic nucleus; Rt, reticular thalamic nucleus; STh, subthalamic nucleus; VL, ventrolateral thalamic nucleus; VLG, ventral lateral geniculate nucleus; VM, ventromedial hypothalamic nucleus; VPM, ventral parabrachial nucleus. From Clarke *et al* (1985), with permission.

Binding patterns for [^{3}H]-ACh, [^{3}H]-Nicotine and [^{125}I]-α-Bgt in sections of rat brain showing hippocampal and cortical regions are shown in Figure 1-11 (Clarke *et al*, 1985). The proportion of different nAChR subtypes varies between different brain regions with the FC containing mostly α4β2* and the HC having a higher proportion of α7 (Clarke *et al*, 1985). More complex subtypes such as the α3/α6α4β2 have been shown to be present in the striatum (Livingstone *et al*, 2009). Epibatidine (Epi) is a nicotinic agonist with high potency at heteromeric nAChRs (Marks *et al*, 1998). A study immunoprecipitating [^{3}H]-Epi bound nAChRs with a variety of subunit specific antibodies has shown that the majority of the nAChRs in a variety of regions of the rat nervous system are comprised of α4 and β2 subunits (Figure 1-12; Gotti and Clementi, 2004). All the regions examined also contained some α5 subunits, and HC, superior cervical ganglia and retina had α3 and β4 subunits present. The α6 and β3 subunits were expressed in striatum and retina with expression of α2 almost exclusively in retina. Immunoprecipitation of α7 was not included due to the absence of a suitable antibody.

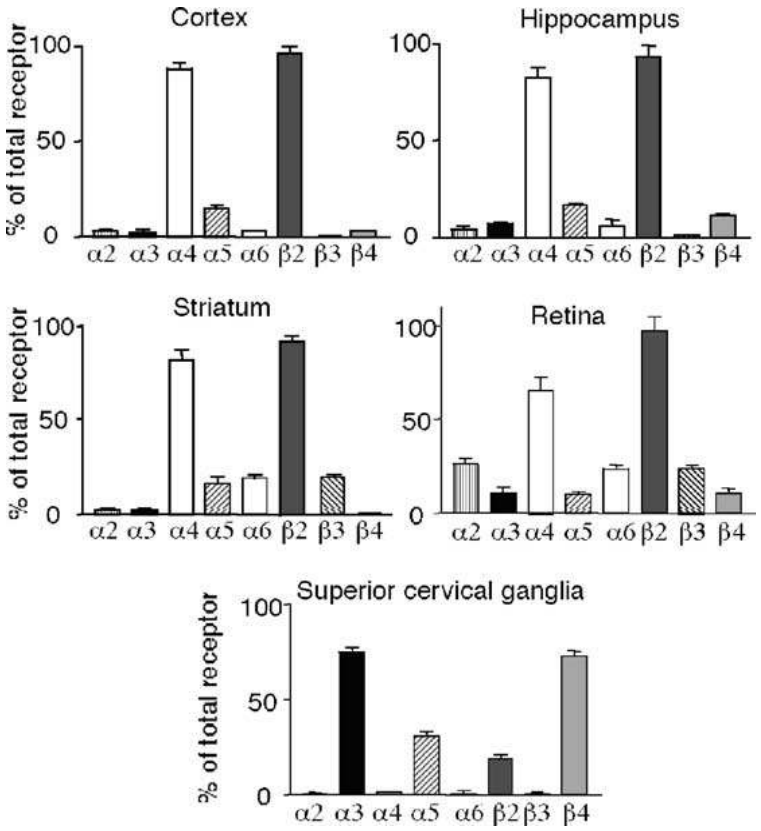


Figure 1-12 Distribution of nAChRs in rat nervous system
Immunoprecipitation analysis of [^{3}H]-Epi labelled nAChRs expressed in rat cortex, hippocampus, striatum, retina and superior cervical ganglia. The level of Ab immunoprecipitation is expressed as the percentage of [^{3}H]-Epi labelled receptors immunoprecipitated by the indicated antibodies, taking the amount of receptors present in the Triton X-100 extract solution before immunoprecipitation as 100%. From Gotti and Clementi (2004) with permission.

As well as brain region distribution, the cell types upon which nAChRs occur are important in determining consequences of their activation. Jones *et al* (2001) showed extensive colocalisation of tyrosine hydroxylase (TH) and β2 subunit antibodies in sections from rat substantia nigra. Within striatal synaptosomes there was some overlap of TH and β2 staining, with additional synaptosomes showing TH staining alone. This shows a presynaptic localisation of β2* nAChRs in dopaminergic neurons in striatal areas, in agreement with functional data discussed below.

The α7 nAChR is found localised to axon-dendritic synapses in association with the neuronal membrane in tissue from HC. In the ventral tegmental area (VTA) most α7 nAChRs were found in somatodendritic and presynaptic locations, with the majority of presynaptic receptors found on glutamatergic axon terminals. Receptors were also associated with dopaminergic neurons but not with astrocytes (Jones and Wonnacott, 2004). The presence of α7 nAChRs on LC noradrenergic neurons was shown by Bitner and Nikkel (2002), with a number of small, possibly GABAergic, α7 positive cells also present. The same work also found a population of α7 positive serotonergic cells in dorsal raphe, again with a population of putatively GABAergic cells also positive for α7 nAChRs (Bitner and Nikkel, 2002). Dehkordi *et al* (2007) showed extensive expression of α7 and α4 subunits in GABAergic neurons in rostral ventral medulla.

1.3.3. Function

Upon binding of the endogenous ligand, acetylcholine, or exogenous ligand such as nicotine, the probability of the ion channel opening increases and allowing Na^+ and Ca^{2+} influx and K^+ efflux, causing a depolarisation within the cell. Both presynaptic and preterminal localisations of nAChRs have been found. Presynaptic nAChRs elicit neurotransmitter release at the synapse by virtue of Ca^{2+} influx through the receptor (McGehee *et al*, 1995). The Ca^{2+} influx through nAChRs can also elicit responses from voltage operated calcium channels (VOCCs) or Ca^{2+} induced Ca^{2+} release (CICR) from intracellular stores (Figure 1-13A), giving larger rises in intracellular Ca^{2+} than would result from receptor opening alone. As well as vesicular neurotransmitter release this increase in calcium can trigger a variety of intracellular signals (Figure 1-13B) leading to both short and long term effects.

Many ACh release sites in the brain are non-synaptic, giving a low concentration of ACh within the extracellular space. This ACh is unlikely to diffuse into synapses and activate presynaptic nAChRs. A large number of nAChRs are located preterminally, that is not directly in the synaptic region. Preterminal nAChRs have been shown to be involved in neurotransmitter release at the synapse, via a tetrodotoxin sensitive mechanism (Lena *et al*, 1993).

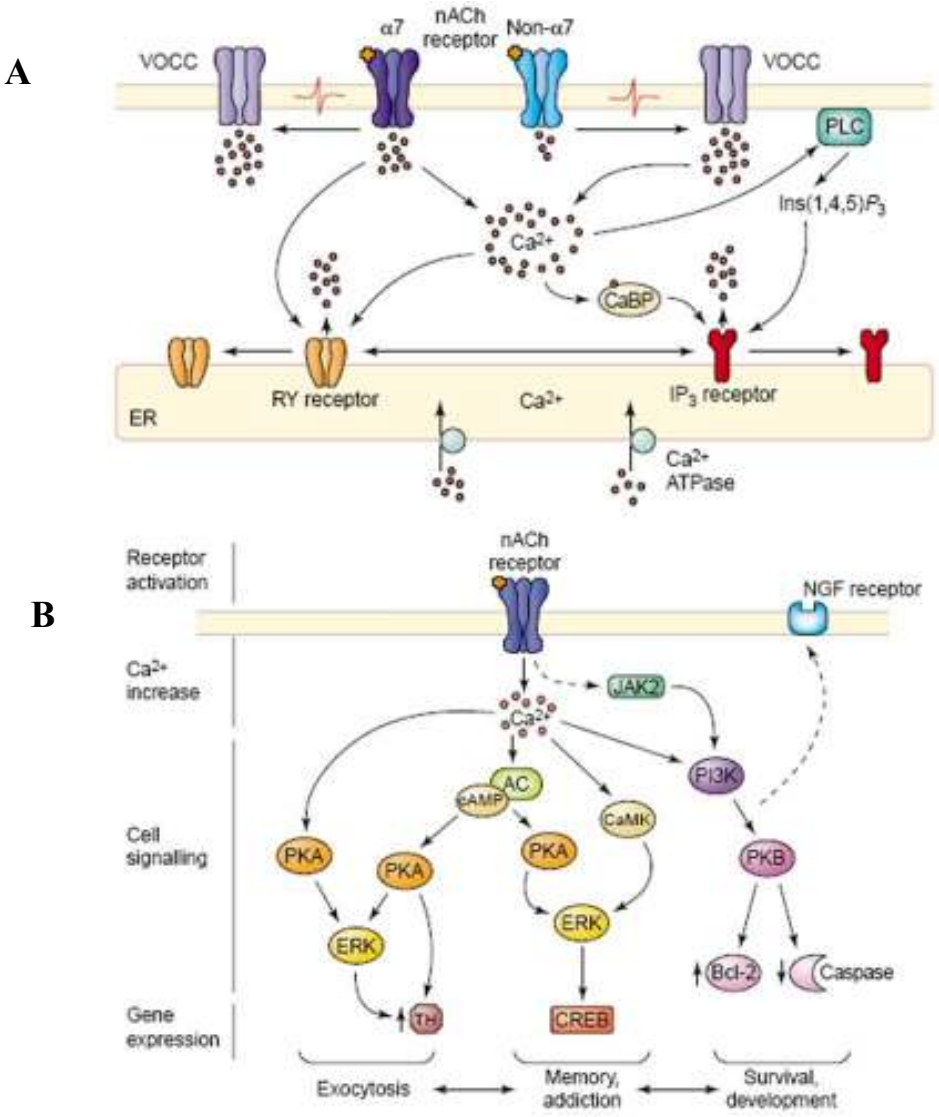


Figure 1-13 Intracellular Ca^{2+} signalling
A) Different mechanisms by which nAChR stimulation increases intracellular calcium. **B)** Intracellular signalling pathways affected by Ca^{2+}. Adapted from Dajas-Bailador and Wonnacott (2004), with permission

Preterminal nAChRs can also elicit neurotransmitter release non-synaptic sites; transmitters must diffuse away from the release site to activate receptors and may be involved in volume transmission. The increase in intracellular Ca^{2+} through preterminal nAChRs is also a mediator of VOCCs and CICR as seen for presynaptic localisations.

1.3.4. Structure and gating

Each nAChR subunit has four membrane spanning domains, termed M1 to M4 and a large N-terminal domain. The M2 region of each subunit lines the central pore and there is an intracellular loop between M3 and M4 that contains phosphorylation sites involved in receptor regulation (Lopez-Hernandez *et al*, 2008). Due to its membrane spanning domains there are no x-ray crystal structures of the nAChR; however a soluble molluscan acetylcholine binding protein (AChBP) from *Lymnaea stagnalis*, comprising regions

homologous to the extracellular binding domains of the nAChR has been crystallised and the structure determined at a 2.7 Å resolution (Brejc *et al*, 2001). This has given insight into the locations of residues critical to ligand binding at the subunit interface. A 4 Å resolution model of the acetylcholine receptor from *Torpedo marmorata* muscle has been produced, using electron microscopy images and has been shown to have similar topology (Unwin 2005). A comparison of the structure of the AChBP and *Torpedo* nAChR is shown below (Figure 1-14). The α subunits in the *Torpedo* structure differ from those in the homopentameric AChBP, likely because they represent the closed unbound state and indeed a 'spray-freezing' technique used by Unwin (2005) has shown that upon ACh binding, the α subunit B and C loops are rearranged to more closely resemble those of the AChBP in bound form, indicating local structural rearrangement upon ligand binding.

The N-terminal cys loop consists of a 15 amino acid sequence with a cysteine-cysteine disulphide bond. This loop is common to a family of ion channel receptors across both anion and cation permeable classes and is critical for channel opening and signal transduction (Sine and Engel, 2006). Hydrophobic residues on neighbouring pore lining M2 helices form a tight girdle that prevents ions passing through in hydrated form. As the hydration shells of Na^+ or K^+ ions are not easily lost without polar surfaces to substitute for water, this hydrophobic ring forms an energetic barrier to ion permeation (Unwin, 2003). Agonist binding leads to a 15 ° rotation of the two muscle α subunits and a concerted movement of all 5 M2 helices, widening the pore by 3 Å and allowing cations to pass through (Unwin, 2003; Figure 1-15). This mechanism of channel opening by a twisting of the subunits has been tested by several models and is further reviewed by Taly (2007).

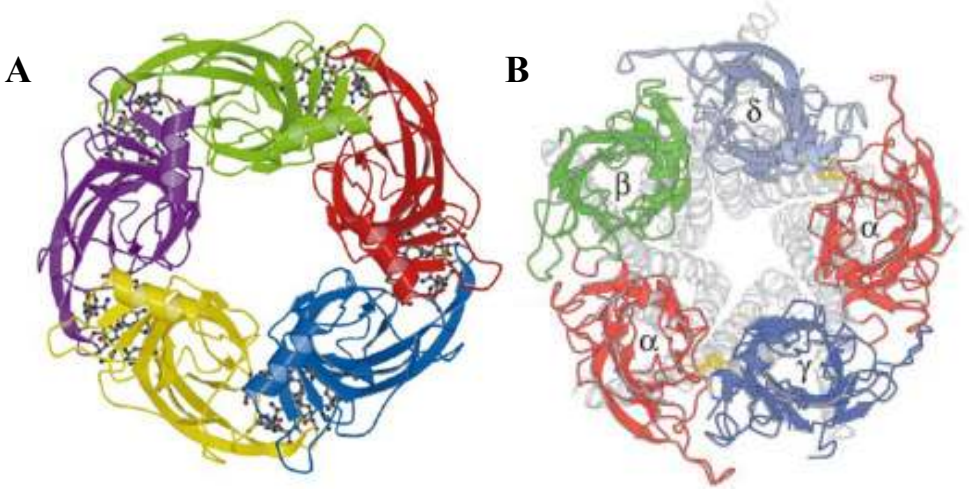


Figure 1-14 Subunit topology of the AChBP and nAChR
Ribbon diagrams of A) AChBP from *Lymnaea* and B) muscle nAChR from *Torpedo*. The receptor has 5-fold rotational symmetry. The α subunits in the *Torpedo* structure are rotated in comparison to the ligand bound AChBP, with an opening of loop C. Adapted from Brejc *et al*, 2001 and Unwin, 2005 with permission.

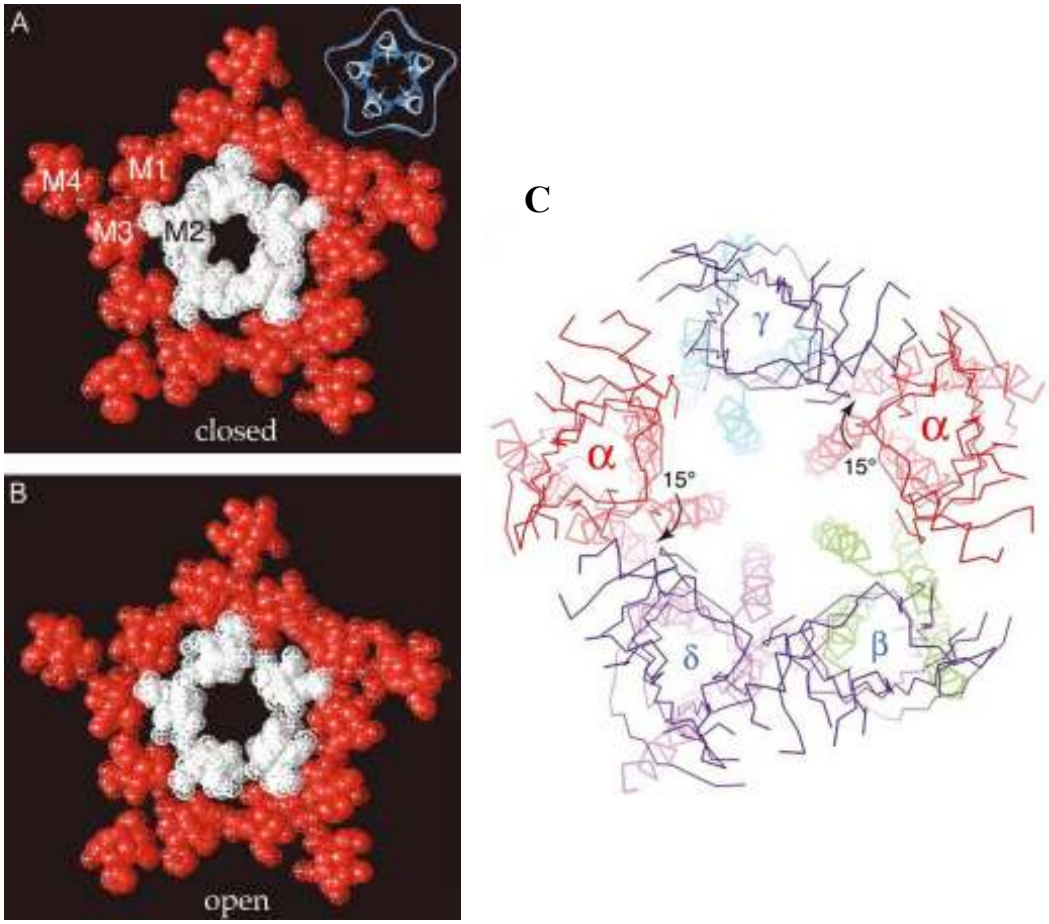


Figure 1-15 Model of nAChR channel opening
Cross section of the gate showing atoms encircling the closed (**A**) and open (**B**) pore. This opening is modelled by applying 15 ° rotations of the M2 helices (**C**), resulting in a 3 Å widening of the pore. Adapted from Unwin, 2003 with permission.

Many of the elements in the extracellular domain and the transmembrane domains are rigid, whereas there is a more flexible linker region between the two. In α subunits short loops joining two β strands dock into a hydrophobic pocket made by the end of the M2 helices (Unwin, 2003). This gives a direct link through which to transmit the rotational movement to the membrane spanning regions. Many mutations implicated in myasthenia gravis and autosomal dominant nocturnal frontal lobe epilepsy have been traced to the rigid transduction regions of the α subunit, suggesting that they have a direct effect on the translation of binding to gating (Taly *et al*, 2007).

It is possible for the channel to open briefly without ligand present; this is best explained by a model where the different receptor conformations are in equilibrium, with ligand binding shifting the equilibrium to favour the open conformation (Figure 1-16). Binding of ligand therefore stabilises the open state, with a second ligand molecule further stabilising the channel opening. Creation of chimeric receptors has proven experimentally that binding at two sites gives optimal channel opening (Andersen *et al*, 2011). This study also showed that each coupling region contributes equally to the open channel stability. A

minimal requirement in a homomeric receptor is for a single binding site flanked by two active coupling regions, or alternately a single coupling region flanked by two active binding sites (Andersen *et al*, 2011). The addition of a third, desensitised, state is important when modelling nAChRs. This is an important mechanism of signal termination. Many authors now suggest the existence of a second non-activable desensitised state, where ligand binding and channel become functionally uncoupled due to alterations in the structure of the coupling domain (daCosta and Baenziger, 2009).

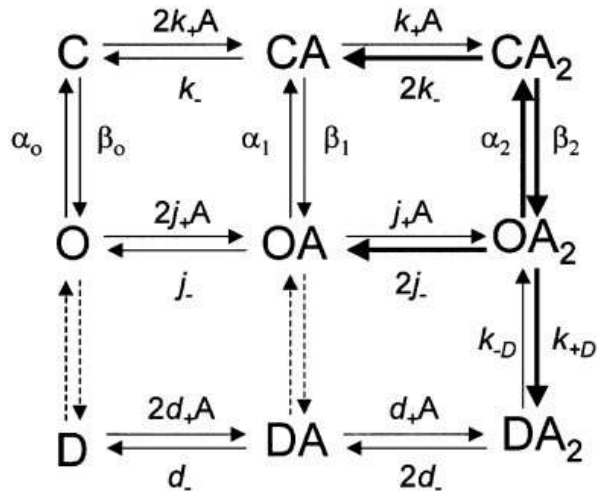


Figure 1-16 A Monod-Wyman-Changeux model for nAChRs
C, O and D are closed, open and desensitised receptor forms respectively. A represents the agonist concentration. The equilibrium constants are represented on the appropriate arrows. From Grosman and Aerbach, 2001 with permission.

As well as differences in ligand sensitivity, nAChR subtypes have differing calcium permeability and gating and desensitisation kinetics; this can be affected by both the alpha and beta components. Receptors containing β2 desensitise more quickly than those containing β4 (Nelson and Lindstrom, 1999). Recovery from desensitisation also varies with α3 containing receptors recover more quickly than those containing α4; similarly β2 recover quicker than β4 (Fenster *et al*, 1997). The homomeric α7 nAChR has the fastest rates of desensitisation and recovery. The α5 subunit does not form a binding site and does not coassemble with *beta* subunits alone; however it will assemble into a pentamer with α3β2 or α3β4 combinations (Wang *et al*, 1996). This role as a structural accessory subunit does not change the binding affinity of receptors although the presence of α5 causes faster desensitisation (Wang *et al*, 1996). There is also an effect of α5 on single channel properties in α3β4 nAChRs, with a larger channel conductance found (Nelson and Lindstrom, 1999).

1.3.5. Summary

The stimulation of neuronal nAChRs causes a rotation of the M2 helices, opening the channel pore and allowing an influx of Ca^{2+} and a depolarisation within the cell. Due to

the subcellular distribution of the receptors this can lead to intracellular signalling (Figure 1-13B) or, more relevantly to the current work, neurotransmitter release. The consequences of this release of neurotransmitter have already been discussed (1.2). The ability to influence neurotransmission by targeting nAChRs leads to the possibility of nicotinic drugs being used as therapies for a wide range of diseases.

1.4. Health and disease

The most obvious involvement of nicotine in disease is its role in addiction to tobacco. Nicotine replacement therapy is widely used in assisting smoking cessation, however as the mode of administration tend to give a slower pharmacokinetic profile than tobacco use, and the prevalence of side effects other treatments have been investigated. Partial nicotinic agonists (e.g. cytisine) are used as smoking cessation therapies with the premise that they will give some stimulation of receptors, lessening withdrawal, whilst also decreasing reinforcement upon tobacco use and so preventing relapse. There are often side effects of these therapies which more selective drugs may help overcome. Work with nAChR knockout mice has shown that the β2 nAChR is involved in nicotine reinforcement and tolerance however withdrawal symptoms are mediated by α7 nAChRs (Besson *et al*, 2006; Grottick *et al*, 2000; Salas *et al*, 2007). Knowing which subtypes are most involved in addiction has helped to suggest α4β2* as a suitable target. Varenicline was developed from cytisine as a smoking cessation therapy due to its partial agonist activity at α4β2* nAChRs; however it is also a full agonist at α7 nAChRs (Coe *et al*, 2005; Mihalak *et al*, 2006). Recent reports have shown that whilst effective in improving quit rates varenicline is associated with side effects such as nausea and suicidal ideation (Cahill *et al*, 2011). Varenicline has also been shown to be a full agonist at 5-HT3 receptors, a subtype known to be highly homologous to the α7 nAChR (Lummis *et al*, 2011), which may be responsible for some of the side effects of varenicline treatment such as nausea. Development of α4β2* selective partial agonists that avoid the α7 receptor may therefore give a drug with a better clinical profile.

Depression is another condition for which nAChRs are a target. Many antidepressants such as monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors, and serotonin-noradrenaline reuptake inhibitors act to increase amine levels. Surprisingly both nAChR agonists and antagonists are effective in models of depression (reviewed in Philip *et al*, 2010), suggesting that their mechanism of action is not via increases in neurotransmitter levels, and that nAChR desensitisation may be involved in the agonist effects in relieving depression. The nAChR antagonist mecamylamine has recently been shown to have antidepressant-like effect in the mouse forced swim and tail suspension tests (Rabenstein *et al*, 2006) and several studies have shown positive effects of

mecamylamine or varenicline in preclinical and clinical trials (reviewed in Philip *et al*, 2010). Mecamylamine exists in two enantiomers, which are not usually separated. Fedorov *et al* (2009) have shown that these have different affinities for the high and low sensitivity stoichiometries of α4β2 nAChRs. Lippiello *et al* (2008) have shown that TC-5214, the + stereoisomer of mecamylamine, is effective in a range of preclinical models of antidepressant activity, and is well tolerated and has a good safety profile in a range of species. Initial clinical findings using TC-5214 as an adjunct therapy in combination with citalopram in the management of depression have been encouraging (Dunbar, 2010). Side effects were mild, suggesting that this treatment may be well tolerated.

Acetylcholine has been shown to have important roles in attention (Klinkerberg *et al*, 2011). Both muscarinic and nicotinic acetylcholine receptors have roles but here the focus will be on the nAChR mediated mechanisms. It has been shown that nAChRs in the FC but not the HC are important for attentional functions (Hahn *et al*, 2003). Antagonism with α7 nAChR antagonists impaired performance in challenging tasks suggesting that FC nicotinic signalling is involved in effortful processing, rather than the psychostimulant effects of systemic nicotine being responsible for the changes (Granon *et al*, 1995). Performance on the radial arm maze is differentially affected by α4β2* and α7 nAChRs, with α7 antagonism in HC causing an increase in working memory errors, whereas DHβE, a β2* selective antagonist impaired both working and reference memory performance. An α7 knock out mouse learns the 5-CSRTT more slowly and has a higher rate of omissions than wild type with impaired attention central to the deficit (Young *et al*, 2004; 2007). Guillem *et al* (2011) showed that β2 subunits are also involved in attention in the FC.

The role of nAChR agonists as possible ADHD treatments has been investigated for several drugs in recent years. Both α4β2 and α7 nAChRs are being investigated as potential targets. Although these drugs show positive effects in animal models of cognitive performance this does not always translate to clinical efficacy. ABT-418, an agonist at α4β2 nAChRs was shown to be effective in improving symptoms on several ADHD scales in adolescents, but that effects were not as great in more severe cases or in adult ADHD (Wilens *et al*, 1999; 2007). Various α7 nAChR agonists have been found to be effective at increasing attention in animal trials and are currently being investigated in clinical trials. Another avenue that could be explored in future is the use of positive allosteric modulators (PAMs) of nAChRs. These act to increase receptor signalling upon activation by slowing or reversing desensitisation of the receptor. These may be a useful tool to enhance cognition as they do not give a constant, non context related, stimulation of receptors, but rather act to increase the signal to noise ratio. As α7 nAChRs have not been shown to be greatly involved in the hedonic properties of nicotine consumption PAMs targeting this subtype should not increase abuse of nicotinic agonists.

Other conditions for which the cognitive enhancing properties of nAChR ligands may be useful are schizophrenia, where cognition is a major unmet treatment need, and Alzheimer's disease.

1.5. Summary of Aims

The work undertaken in this thesis aims to elucidate the subtypes of nAChR involved in noradrenaline release in the frontal cortex in comparison to the hippocampus. This was done using an *in vitro* system, in order to define key aspects to examine *in vivo*. Modulation of NA release *in vivo* was then examined using microdialysis, and further comparisons made between frontal cortex and hippocampus.

Some novel compounds were also examined for their effects *in vitro*. The catecholamine metabolite profile in response to nicotine was also assayed in a preliminary attempt to examine regional differences in catecholamine regulation.

Chapter Two

2. NAChR modulation of monoamine release *in vitro*

2.1. Introduction

2.1.1. NAChR pharmacology of neurotransmitter release

The release of neurotransmitters elicited by nicotine has been seen by many groups. Arqueros *et al* (1978) found efflux of catecholamines from preloaded slices in a superfusion system that was quantitatively and qualitatively different from that induced by KCl challenge. This study however used high concentrations of nicotine (>1 mM) and did not find that the responses were Ca^{2+} sensitive. Westfall (1974) had earlier shown that [^{3}H]-DA release could be stimulated from striatal tissue but concentrations of nicotine were again in the mM range. Giorguieff-Chesselet *et al* (1979) were the first to show that μM concentrations of nicotine could induce [^{3}H]-DA release from striatal slices. By 1988 it was shown that μM concentrations of nicotine were effective in releasing DA from striatal synaptosomes, with EC_{50} 3.8 μM (Rapier *et al*, 1988). Sacaan *et al* (1995) showed nAChR stimulated release of [^{3}H]-NA from HC slices was different to [^{3}H]-DA release from striatal slices. There were different sensitivities to nicotine and cytisine, and unlike striatum HC was not sensitive to DHβE, a β2* selective antagonist. No inhibition of [^{3}H]-NA release from HC slices with α7 antagonists methyllycaconitine (MLA) or α-Bgt was seen, suggesting little contribution of α7 nAChRs (Sacaan *et al*, 1995). Clarke and Reuben (1996) showed that in synaptosomes nAChR-induced release of hippocampal [^{3}H]-NA differed from that of striatal [^{3}H]-DA. The HC was less sensitive than striatum to nicotine, DHβE and MLA.

Using a novel 96-well format for release assays (see 2.1.2 for comparison) Anderson *et al* (2000) showed that nicotine was more potent in releasing [^{3}H]-NA from FC than HC, and that DHβE caused a small non-competitive inhibition of responses. This is in contrast to the competitive inhibition observed for [^{3}H]-DA release from striatum reported in the same paper. These results agreed with previous findings from superfusion studies in both slices and synaptosomes. Using the 96-well plate assay Barik and Wonnacott (2006) found that the α7 nAChR may play an indirect role in NA release in HC, although several other papers (Sershen *et al* 1997; Clarke and Reuben, 1996; Sacaan 1995) have shown no reduction in [^{3}H]-NA release in response to agonist stimulation in the presence of α7 antagonists.

Together these studies show a lack of β2* mediated responses in HC [^{3}H]-NA release with questions over the involvement of α7 and suggest the involvement of α3β4* nAChRs. In FC NA release has been less studied, with the work done so far suggesting a higher sensitivity to nicotine and partial as opposed to full agonism of cytisine (Sacaan *et al*, 1995). DA release from the FC has been somewhat better studied, with responses

broadly similar to striatum with a major contribution of β2* nAChRs. There is no sensitivity to α-conotoxin MII, which is a selective antagonist for α6/α3β2* nAChRs and partially inhibits striatal [^{3}H]-DA release (Livingstone *et al*, 2009). A role for α7 nAChRs in DA release in FC has also been shown, with release elicited by choline and compound A, potentiated by PNU-120596 and blocked with selective antagonists.

2.1.2. 96 well release method vs. superfusion

In this study use has been made of a relatively novel 96-well neurotransmitter release assay. This method was developed and reported to expand throughput in comparison with the better established superfusion technique (Anderson *et al*, 2000). Results have been shown to be largely comparable to superfusion where the two techniques have been compared (2.1.1). The assay is a static release assay with collections of filtrate for baseline and stimulated responses and so does not allow the assessment of desensitisation of receptors or time course of responses as is possible with superfusion. Superfusion often makes use of synaptosomes (isolated nerve terminals), which allows the separation of purely presynaptic effects. In contrast we have used 150 μm prisms of tissue, which maintain some synaptic connectivity giving a better picture of the *in vivo* regulation of neurotransmitter release via indirect as well as direct mechanisms. This is important as there is some evidence of interneuronal contributions to neurotransmitter release, for example a glutamatergic contribution to DA release (Kaiser and Wonnacott, 2000; Barik and Wonnacott, 2006) and a GABAergic effect on NA in HC (Leslie *et al*, 2002).

The relatively high throughput of this assay, with multiple replicates possible for a number of conditions in parallel means that concentration response curves can be easily constructed. *In vivo* microdialysis studies have previously been designed to test the findings from 96-well *in vitro* release assays and have found nAChR subtypes and interactions to be largely translatable, although concentrations of drugs required vary between the techniques (Livingstone *et al*, 2009). We therefore feel this assay is reliable, translatable and a useful tool for this project.

2.1.3. Upregulation and chronic functional changes

It is well established that repeated or chronic systemic nicotine administration leads to upregulation in nAChR binding in animal models (Jacobs *et al*, 2002). Upregulation in cell lines/neuronal cultures exposed to nicotine is also seen (Bullock *et al*, 1994; Lomazzo *et al*, 2010). High affinity receptors are thought to be upregulated to a greater extent than low affinity, with evidence that α4β2 nAChRs are particularly sensitive (Flores *et al*, 1997). Different subtypes of nAChR are upregulated to different extents, and as these are often expressed in differing brain regions this can have differential effects. The α4β2α5 subtype is resistant to upregulation (Mao *et al*, 2008) and a larger proportion of the α4β2* nAChRs

in the HC are associated with α5 than in the striatum and cerebral cortex. After 14 days of nicotine treatment binding sites increased by 50 % in cortex and hippocampus, whereas striatum was unaffected, suggesting a shift in the proportion of the different subtypes (Sanderson *et al*, 1993). Nuutinen *et al* (2005) also found that there was no increase in [^{3}H]-Epi binding in striatum after chronic nicotine treatment, with increases in hippocampus and cortex. This paper also examined low-affinity MLA binding sites (α7 and α3/α6β2β3*), finding upregulation in striatum and cortex after 4 weeks and hippocampus after 7 weeks treatment (Nuutinen *et al*, 2005). As well as changes in the number of agonist binding sites, changes to the stoichiometry of receptors have also been suggested, with an increase in the proportion of high affinity receptors.

The implications of this upregulation are difficult to ascertain as there is evidence that there is not always a concomitant change in functional responses to nicotinic stimulation. Animals in fact develop tolerance to repeated nicotine administration as with many other drugs of abuse (Vann *et al*, 2006; Collins *et al*, 1988); however behavioural sensitisation to nicotine has also been reported (Kita *et al*, 1992; Kirshenbaum *et al*, 2011).

The causes of upregulation are thought to be desensitisation and internalisation of receptors (Fenster *et al*, 1999), especially entry into the slowly recovering deeply desensitised or inactivated state. The mechanism of upregulation is debated, with increased receptor subunit assembly and trafficking being postulated, changes in degradation of receptors could also be involved in increasing numbers. It has been shown that increases in nAChR binding after chronic nicotine treatment are due to post-translational mechanisms, as mRNA levels remain the same (Marks *et al*, 1992; Mugnaini *et al*, 2002) and inhibition of protein synthesis does not inhibit the increase in binding (Wang *et al*, 1998; Darsow *et al*, 2005). It has also been shown by correlation of radioligand binding and antibody labelling that the upregulation measured is due to an increase in protein levels, not simply the creation of additional binding sites from subunits already present (Marks *et al*, 2011), supporting a theory of reduced turnover. Work from the Lester lab (Lester *et al*, 2009) has suggested that nicotine itself can enter cells and act as a pharmacological chaperone, stabilising the assembly of subunits and enhancing their trafficking (see 2.6.5.3).

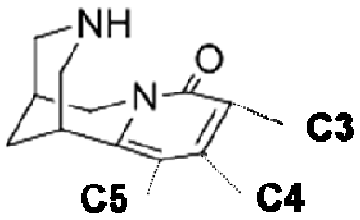
2.1.4. Production of novel compounds and rationale for design

Partial agonists as smoking cessation therapies are well established. They are preferred to full agonists as potential therapies as there is a lesser side effect profile meaning they are better tolerated and a lower abuse liability as well as a more effective mechanism of action. Partial agonists act to relieve cravings by low level stimulation of nAChRs, but they also block the rewarding effects of nicotine administration by receptor

occupancy. There still some side effects of these therapies due to their actions at other nAChR subtypes centrally and peripherally, as well as interactions with other receptors.

2.1.4.1. Cytisine analogues

Varenicline is an analog of cytisine, developed in an attempt to create drugs with a better side effect profile for smoking cessation. As more prescriptions have been issued side effects including nausea, commonly associated with nicotinic drugs, as well as more serious side effects such as depression and suicidal thoughts have emerged, leading to a 'black box' safety warning to be issued. These side effects may be due to off target effects on $5HT_3$ receptors (Lummis *et al*, 2011). Due to this there is a need for additional, more selective, partial agonists to enter development.



Cytisine

Figure 2-1 The structure of cytisine
Cytisine has a bridged bicyclic framework. Substitutions can be made at several of the carbon atoms (C3, C4 and C5, labelled above). C3 and C5 substitutions have been reported previously to have effects on cytisine binding and function.

Substitutions (often halogenations) have previously been reported at C3 and C5 of cytisine (Figure 2-1). C3-halogenation increases binding affinity at nAChRs and also increases the functional potency of these compounds in comparison with cytisine (Abin-Carriquiry *et al*, 2006), with no changes determined in subtype selectivity. In particular 3-Br-Cytisine has been shown to have increased binding affinity compared with cytisine, and to have good functional potency both *in vitro* and *in vivo* (Abin-Carriquiry *et al*, 2006, 2010a). C5 halogenation on the other hand is detrimental to binding and functional efficacy (Abin-Carriquiry *et al*, 2006).

The development of an *in silico* docking model of the acetylcholine binding protein (AChBP), a soluble molluscan homologue of the nAChR, has allowed the interactions with cytisine analogs to be calculated (Abin-Carriquiry *et al*, 2010b). This model has been tested by comparing the structure of the AChBP co-crystallised with nicotine to the predicted docking, showing high accuracy of reproduction of the docking pose. The model gives a good correlation between docking energy and experimentally measured affinity for a number of cytisine analogs with various substitutions including the C3 and C5 halogenations mentioned above.

Modelling by Tasso *et al* (2009) in rat and human $(\alpha4\beta2)_2\beta2$ homology models has suggested that in general substitution in the aromatic moiety weakens binding by interfering with hydrogen bonding to a conserved tryptophan in the receptor binding site. The amino acids involved in cytisinoid binding are highly conserved between the AChBP and mammalian receptors, with 7 conserved amino acids in the α subunit and one in the complementary face shown to be particularly important. Interactions with other amino acids in the more variable complementary face of the binding site are more difficult to model and may have some effect on selectivity, however the main interactions with these is through the backbone atoms and so mutations will have less effect on the interactions (1.3.3; Abin-Carriquiry *et al*, 2010b).

2.1.5. Aims & hypotheses

- To investigate the nicotinic stimulation of NA release in FC in comparison with HC as a better studied model. The hypothesis is that HC will be modulated by α3β4* nAChRs as previously shown, and that as this subtype is not highly expressed in cortical regions responses in FC may be regulated by other nAChRs notably α4β2*, as expression is high throughout the brain.
- To investigate the responses *in vitro* after acute or chronic *in vivo* nicotine administration. Previously it has been shown that receptor upregulation after chronic nicotine treatment does not correlate with a functional change. The hypothesis is that there will not be significant changes in function after *in vivo* nicotine treatment.
- To investigate novel compounds to assess binding and functional potency. These compounds are analogs of cytisine and so are hypothesised to cause displacement of [^{3}H]-Epi binding, although the changes to the molecules are expected to alter the potency of this binding.

2.2. Materials and Methods

2.2.1. Chemicals and Reagents

[7,8-^{3}H]-Noradrenaline ([^{3}H]-NA; 34-50 Ci mmol^{-1}), [^{3}H]-dopamine ([^{3}H]-DA; 38-45 Ci/mmol) and [^{3}H]-epibatidine (55.8 Ci/mmol) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK) or Perkin Elmer (N.V. /S.A., Zaventem, Belgium). (-)-Nicotine hydrogen tartrate, pargyline hydrochloride, choline tartrate, ascorbic acid, mecamylamine hydrochloride, morphine, DIOA, dihydro-beta-erythroidine (DHβE) and nomifensine maleate were obtained from Sigma-Aldrich Co. (Poole, Dorset, UK). Nisoxetine hydrochloride, CGP54626, 5-Iodo-[3-(2(s)-azetidinylmethoxy) pyridine] dihydrochloride (5-Iodo-A85380, 5IA), bicuculline, bumetanide and GBR12909 dihydrochloride were purchased from Tocris Cookson (Avonmouth, UK). α-bungarotoxin (α-Bgt) was acquired from Molecular Probes (Pourt Gebaow, Netherlands). PNU-120596

was synthesised at, and provided by GlaxoSmithKline (Harlow, Essex, UK). Optiphase Supermix was obtained from Perkin Elmer (N.V. /S.A., Zaventem, Belgium) and Optiphase 'Safe' acquired from Fisher Chemicals (Loughborough, Leicestershire, UK). Naloxone hydrochloride was purchased from Ascent Scientific (Weston-super-Mare, UK). 3-Br-Cyt and novel cytisine analogs were obtained from the laboratory of Dr Timothy Gallagher. Other standard chemicals of analytical grade were obtained from Sigma-Aldrich Co.

2.2.2. Animals

Male Sprague-Dawley rats were purchased from Charles River Laboratories (Margate, Kent, UK). They were housed in groups (4-6 per cage) with *ad libitum* access to food and water. Animals weighed 200-320g at time of experiments. All procedures were in accordance with the Animals (Scientific Procedures) Act of 1986.

2.2.3. [^{3}H]-Noradrenaline Release

A 96 well plate method was adapted from previous studies of neurotransmitter release (Anderson *et al*, 2000; Barik and Wonnacott, 2006). Krebs buffer (KB; 118 mM NaCl, 2.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 10 mM D-glucose and 1 mM ascorbic acid) was gassed for 1 h at 37 °C with a 95% O_2, 5% CO_2 mix and pH adjusted to 7.4. Rats were killed by cervical dislocation, decapitated, brains removed and placed on an ice-cold platform. FC (the 2mm anterior to corpus callosum), striatum and/or hippocampus (as stated for each experiment) were rapidly dissected and immediately immersed in KB containing 10 μM pargyline (a monoamine oxidase B inhibitor, to prevent degradation of the catecholamine) at 4 °C. A monoamine oxidase A (MAOA) inhibitor such as tranylcypromine could also be used as MAOA is involved in the metabolism of NA. Tissue was sliced using a McIlwain tissue chopper three times with 60° rotations to produce 150 μm prisms and transferred to 5 ml KB at 37 °C and triturated to suspend. Tissue was washed twice in warm KB and then incubated with radiolabelled catecholamine (70 nM [^{3}H]-NA, 50 nM [^{3}H]-DA) in 5 ml KB containing 10 μM pargyline, for 30 min with regular agitation. Tissue was washed 4 times with 5 ml KB supplemented with 10 μM pargyline and 0.5 μM nomifensine (KBN) to inhibit catecholamine reuptake. The tissue was resuspended in 5 or 10 ml KBN (5 ml per half plate) then transferred (100 μl per well) to a 96 well filter plate (Millipore Corporation, Hertfordshire, UK) with a multichannel pipette ensuring even distribution. Tissue in each well was washed twice with 100 μl buffer before addition of 70 μl buffer (with or without antagonists) and incubation at 37 °C for 5 min (except for studies including α-Bgt which was included during all post catecholamine uptake washes to give an additional 20 min incubation). Wells were filtered and buffer collected into a 96 well Optiplate (Perkin Elmer N.V. /S.A., Zaventem, Belgium). Tissue was then exposed to 70 μl buffer containing

agonists and/or antagonists for a further 5 min at 37 °C. Following a second filtration into a separate collection plate, filters were dried in an incubator at 37 °C, removed from plate and frozen overnight in a 96 well Optiplate after addition of 70 µl water per well. Optiplates containing collected buffer and release fractions were agitated for 45 min with 170 µl Optiphase Supermix and radioactivity determined using a MicroBeta liquid scintillation counter (Wallac 1450 MicroBeta Trilux; Perkin Elmer Wallac, Turku, Finland) at 30% counting efficiency. Percent release was determined as a fraction of the [^{3}H]-NA in the tissue at the start of the stimulation period (defined by the amount released + that remaining in the tissue). Every condition used was performed in 6 replicates and each assay was repeated a minimum of three times with tissue from different animals. Buffer or antagonist only incubations were carried out before stimulation to confirm they had no effect on basal release for each assay.

2.2.4. [^{3}H]-Noradrenaline Uptake

FC minces were prepared as for release assays (from one animal per experiment) and washed for 5 min with KB at 37 °C. Aliquots (100 µl) were pre-incubated for 10 min in 0.5 ml Eppendorf tubes with buffer alone, nomifensine (0.5 or 5 µM), GBR12909 (50 nM or 0.5 µM), morphine (10, 30 or 100 µM) or nisoxetine (1 µM or 10 µM), at 37 °C or 4 °C. All tubes then received 100 µl 140 nM [^{3}H]-NA (70 nM final concentration) and were incubated with intermittent shaking for 30 min. Tissue was collected by filtration through a pre-moistened 96 well filter plate (Millipore Corporation, Hertfordshire, UK) and washed three times with 100 µM ice-cold KB before transfer to liquid scintillation vials. Optiphase 'Safe' (4 ml) was dispensed into each vial before counting for 1 min in a 1600 Tri-carb liquid scintillation counter with 46 % counting efficiency (Perkin Elmer Life and Analytical Sciences) to determine radioactivity. Each condition was examined in four replicates and each experiment was repeated at least 3 times.

2.2.5. Radioligand binding

2.2.5.1. Membrane preparation

Rat brain P2 membranes were prepared by the method outlined in Davies *et al*, (1999). Rat brains (minus cerebellum) were homogenised in ice cold 0.32 M sucrose containing 1 mM EDTA, 0.1 mM PMSF and 0.01 % NaN_3 (pH 7.4, 10 % w/v). The homogenate was centrifuged at 1000 g for 10 min and the supernatant retained on ice. The pellet was then resuspended in sucrose and the spin repeated. The supernatants were combined (S1) and centrifuged at 15 000 g for 30 min. This pellet (P2) was resuspended in 5 ml/g original weight of 50 mM phosphate buffer (40 mM K_2HPO_4, 10 mM KH_2PO_4, 1 mM EDTA, 1mM PMSF and 0.01 % NaN_3, pH 7.4) and centrifuged at 15 000 g for 30 min. The wash step was repeated and the final pellet resuspended in 2.5 ml/g original

weight in ice cold 50 mM phosphate buffer and frozen until use. Protein concentration was estimated by use of a BCA protein assay microplate procedure (Pierce Biotechnology, IL USA), a colorimetric protein dye reagent.

2.2.5.2. [^{3}H]-Epibatidine competition binding assay

[^{3}H]-epibatidine ([^{3}H]-Epi) binding was carried out with a final concentration of 200 pM [^{3}H]-Epi (Houghtling *et al*, 1995). Rat brain membranes were diluted 1 in 40 in 50 mM phosphate buffer (2.2.5.1) to a final volume of 2 ml, with 20 µl of serial dilutions of drugs to be tested (10^{-4} to 10^{-10} M final concentration) added to each tube. Nonspecific binding was determined in the presence of 1 mM nicotine. Samples were incubated for 2 h at room temperature followed by 30 min at 4 °C. Samples were then filtered through glass fibre filters (FB59431) pre-soaked in 0.3 % polyethyleneimine (PEI). Filters were washed three times using ice cold PBS (150 mM NaCl, 8 mM K_2HPO_4, 10 mM KH_2PO_4, 0.01 % NaN_3, pH 7.4), and counted for radioactivity using a Packard 1600 Tricarb scintillation counter (efficiency 45 %). Each assay was conducted in triplicate.

2.2.6. *In vivo* nicotine administration

2.2.6.1. Nicotine administration by injection

Rats were handled and weighed at the same time daily for at least seven days, with saline (0.9 %, 1 ml/kg) injected subcutaneously for the final three days. Rats were then injected once daily s.c. with either 0.9 % saline or nicotine (0.4 mg/kg free base, adjusted to pH 7.4) for 14 days. Thirty minutes after the final injection rats were sacrificed by cervical dislocation and brains rapidly removed and dissected. To study withdrawal effects rats were killed 3 days after the final injection. An additional group of rats received a single injection of nicotine (0.4 mg/kg s.c.) or saline and were sacrificed 30 min later to study the effects of acute nicotine administration on *in vitro* neurotransmitter release. No effect of nicotine administration or withdrawal was observed on growth rates.

2.2.6.2. Chronic nicotine administration by osmotic minipump

Rats were anaesthetised with isoflurane (3% isoflurane to induce; 1.5-2% to maintain: 1L/min O_2) and the area between the scapulae shaved and swabbed with a mixture of 70% ethanol and Hibicet (a skin antiseptic). A small incision (~1 cm) was made and a subcutaneous pocket opened by blunt dissection to fit the minipumps. Alzet® osmotic minipumps (model 2002) were swabbed with 70 % ethanol and saline and implanted subcutaneously with the outlet of the flow moderator away from the incision. Osmotic minipumps were filled with nicotine bitartrate, dissolved in 0.9 % saline and pH adjusted to 7.4, to deliver nicotine (4 mg/kg/day freebase) at a rate of 0.5 µl/h for 14 days. Control animals received a 'blank' implant formed from inert PTFE of similar size and weight to

the minipump. The concentration of nicotine in the pumps was calculated by the following formula;

$$\text{Nicotine (mg/ml)} = \frac{\text{(X mg/kg/day) * (estimated animal weight (g) on day 7)}}{\text{24 * (Pumping rate of minipump (µl/h))}}$$

Following the insertion of the pump, the incision was closed with two wound clips and checked daily throughout the course of the experiment. Fourteen days later animals were sacrificed by cervical dislocation and brains rapidly removed and dissected. Pumps were removed and inspected to ensure correct delivery of nicotine. To study withdrawal effects pumps were surgically removed under isoflurane anaesthesia after 14 days and animals killed 2 or 3 days after pump removal. Nicotine and saline animals were paired for each condition, with samples run in parallel.

The brain tissue remaining after the removal of FC and HC for release assays was frozen until use in binding assays (see 2.2.8). No effect of nicotine administration or withdrawal was observed on the weight of animals.

2.2.7. [^{3}H]-Noradrenaline release from nicotine treated animals

Release experiments were carried out as previously described (2.2.3) with minor modifications. Release was assessed from tissue from each animal individually. The incubation with [^{3}H]-NA was in a volume of 2.5 ml and the final resuspension in a volume of 5 ml with prisms distributed across one half of a 96 well plate. On each plate tissue taken from nicotine- and saline-treated animals was assessed in parallel.

2.2.8. Radioligand binding after chronic treatment

2.2.8.1. Membrane preparation

Membrane preparation was carried out as above (2.2.5.1) with minor modifications. Membranes from individual brains (minus cerebellum, frontal cortex and hippocampus) were prepared. Protein concentration was estimated by use of a BCA protein assay microplate procedure (Pierce Biotechnology, IL USA), a colorimetric protein dye reagent.

2.2.8.2. [^{3}H]-Epibatidine binding

[^{3}H]-Epi binding was performed on 150 µg of brain membranes in a final volume of 1 ml in Tris-HEPES buffer (NaCl 118 mM, KCl 4.8 mM, $CaCl_2.2H_2O$ 2.5 mM, HEPES 20 mM, Tris 20 mM, PMSF 0.1 mM, NaN_3 0.01 %; pH 7.4). The final concentration of [^{3}H]-Epi was 500 pM. Non specific binding was determined in the presence of 1 mM nicotine. Samples were incubated for 1.5 h at room temperature, followed by 30 min at 4 °C and filtered and counted as above (2.2.5.2).

2.2.9. Data analysis

Data were plotted using SigmaPlot version 10 (Jandel Corp., San Rafael, USA). Points or bars typically represent mean $\pm$ SEM of at least 3 separate samples, each being the mean of 6 replicates performed in parallel.

Concentration response curves were fit to a non-linear regression by fitting to a single site Hill equation:

$$y = a / [1 + (k / x)^{n}]$$

where 'a' is the asymptotic maximum, 'k' represents the EC_{50} or IC_{50} value, 'n' is the Hill number and 'y' is the amount of neurotransmitter released, or [^{3}H]-epibatidine bound, in the presence of concentration 'x 'of drug. Data were analysed by Students *t* test, or one or two way ANOVA followed by an appropriate post-hoc test such as Holm-Sidak (with adjustment for multiple comparisons) for pairwise comparisons or Dunnett's to compare each value to control as appropriate using SigmaPlot or GraphPad Prism v5. Competition binding curves were analysed by non-linear regression with extra sum of squares F test to compare pIC50 values.

2.3. Results – [³H]-Noradrenaline release in FC and HC

2.3.1. Neurotransmitter uptake

2.3.1.1. [³H]-NA uptake specificity

Before performing experiments examining [^{3}H]-NA release from rat brain prisms, it was important to ascertain that the uptake of [^{3}H]-NA was specific to noradrenergic cells. The uptake of amines, especially dopamine, is not always mediated by the correct transporter alone (Carboni *et al*, 2001). To assess the specificity of NA uptake, FC prisms were incubated with [^{3}H]-NA (70 nM) at 37 °C for 30 min (uptake period for release assays) with a variety of amine transporter inhibitors; nisoxetine to inhibit the noradrenaline transporter (NET), GBR-12909 to inhibit the dopamine transporter (DAT) and nomifensine, to inhibit both NET and DAT. Samples were also incubated at 4 °C, to prevent transporter mediated uptake and so serve as a control for non-specific NA interactions with the tissue. Uptake in the presence and absence of inhibitors was determined by filtration and scintillation counting (2.2.4).

Rat FC prisms accumulated 9267 ± 542.9 cpm [^{3}H]-NA/mg wet weight (244.1 ± 14.3 fmol/mg) over 30 minutes when incubated in Krebs buffer alone at 37 °C. At 4 °C very little [^{3}H]-NA was taken up (3.6 ± 0.4 fmol/mg) and there was no difference in [^{3}H]-NA taken up in buffer alone or with inhibitors. These values were not different from a control with no tissue (data not shown).

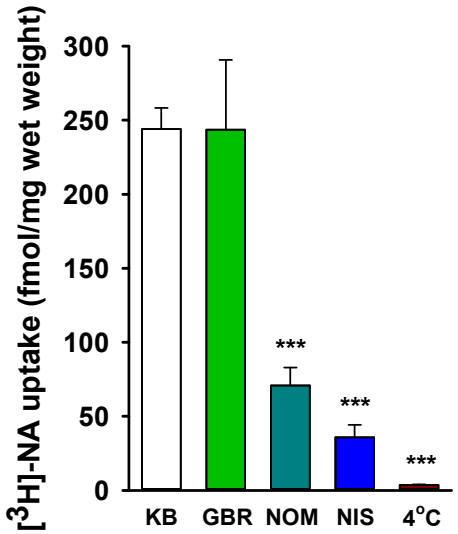


Figure 2-2 [^{3}H]-NA uptake in the presence of catecholamine uptake blockers.
Rat FC tissue was dissected, chopped into 150 µm prisms and preincubated with Krebs buffer (KB) alone, or catecholamine uptake inhibitors, GBR-12909 (GBR: DAT; 50 nM), nomifensine (NOM: general; 0.5 µM) or nisoxetine (NIS: NET; 1 µM) for 10 min, then incubated for 30 min with 70 nM [^{3}H]-NA for 30 min at 37 °C. Prisms were also incubated at 4 °C as a control. Prisms were washed by filtration with ice cold KB. [^{3}H]NA uptake was assessed by scintillation counting with each condition examined in four replicates from each experiment. Bars represent mean ± SEM from 4 separate experiments. *** $p<0.001$ significantly different from KB, one way ANOVA with *post hoc* Holm-Sidak test. $F(4,15)=25.54$, $p<0.001$

The selective DAT inhibitor, GBR-12909 (50 nM), did not reduce NA uptake (Figure 2-2). In contrast, the catecholamine uptake inhibitors nomifensine (NET and DAT; 0.5 μM) and nisoxetine (NET; 1 μM) reduced uptake by 71 ± 6.6 % and 85 ± 3.6 % respectively (Figure 2-2). At higher concentrations (0.5 μM GBR-12909, 5 μM nomifensine or 10 μM nisoxetine) there were no further reductions in uptake in comparison with the lower concentration (data not shown) showing an optimal block of uptake with the lower concentrations.

Together these results show that the [^{3}H]-NA uptake under these conditions is highly specific to the NET and therefore to noradrenergic cells within the FC, and so no DA transporter inhibitor was included during [^{3}H]-NA incubation steps during subsequent release assays.

2.3.1.2. Effect of morphine on [^{3}H]-NA uptake

Many drug addicts use multiple drugs, so the interactions of an opiate (morphine) and NA uptake in FC were also explored with the intention of investigations in other aspects of the project. To assess the effect of morphine on [^{3}H]-NA uptake FC prisms were preincubated for 10 min with morphine (10-100 μM) prior to addition of [^{3}H]-NA for 30 min before determination of [^{3}H]-NA uptake by filtration and scintillation counting. The opioid receptor antagonist naloxone (10 μM) was also used to determine if any responses seen were receptor mediated.

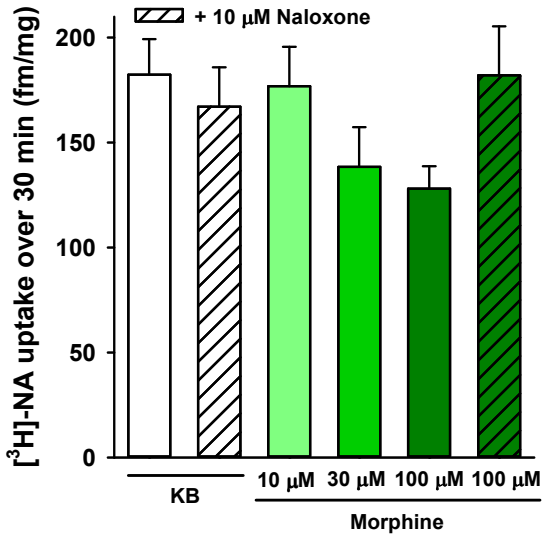


Figure 2-3 [^{3}H]-NA uptake in the presence of morphine
Rat FC tissue was dissected, chopped into 150 μm prisms and preincubated with Krebs buffer (KB) alone or morphine (10 -100 μM; green) in the presence (hatched bars) or absence of naloxone (10 μM), before incubation with 70 nM [^{3}H]-NA for 30 min at 37 °C. Prisms were then washed by filtration with ice cold KB. [^{3}H]-NA uptake was assessed by scintillation counting with each condition examined in four replicates from each experiment. Bars represent the mean ± SEM from 4 separate experiments. Data analysed by One way ANOVA, Holm-Sidak *post hoc* test. $F(5, 18)=1.645$, $p=0.199$

Naloxone alone had no effect on [^{3}H]-NA accumulation into FC prisms. No difference in [^{3}H]-NA uptake was seen at 10 μM morphine and a small, non-significant difference at 30 μM morphine. At 100 μM morphine caused a noticeable but non-significant reduction of NA uptake compared to KB alone, reducing uptake from 182.4 ± 17 to 128.2 ± 10.7 fmol/mg (Figure 2-3) which was reversed with the mu opioid selective antagonist naloxone (10μM) to 182.1 ± 23.4 fmol/mg, showing that the effect of morphine was mediated via opioid receptors, and not a direct effect on the NET itself.

The effect of morphine on [^{3}H]-NA release was also examined and there was no significant difference in basal, KCl or nicotine stimulated release (data not shown) in the presence or absence of morphine. This shows that the reduction in uptake is due to uptake changes not a concurrent release of [^{3}H]-NA during the uptake period.

2.3.2. [^{3}H]-NA release in naïve animals

[^{3}H]-NA release from rat brain prisms was assessed as described (2.2.3). Briefly, prisms were incubated with 70 nM [^{3}H]-NA, distributed onto a 96 well filter plate and incubated with buffer/antagonist. Filtrate was collected (basal) and prisms then incubated with drug ± antagonist. Filtrate was again collected into a separate plate (stimulated). Basal, stimulated and tissue plates were each counted for radioactivity and results calculated as a percentage of the radioactivity in the sample at the start of the collection period. Basal release of [^{3}H]-NA in buffer alone were around 15-20 % fractional [^{3}H]-NA content, and data are expressed as % basal for each experiment except as stated.

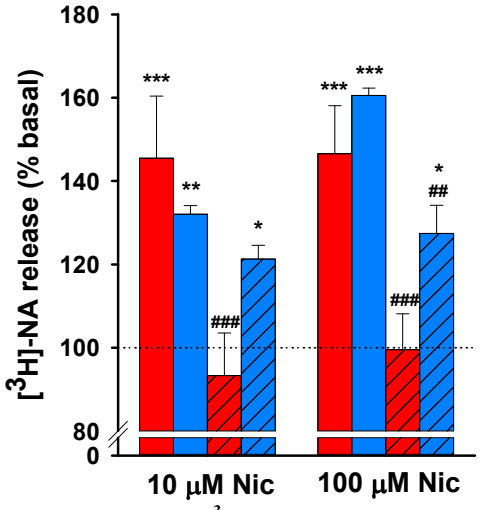


Figure 2-4 Antagonism of nicotine induced [^{3}H]-NA release
Prisms (150 μm) from rat FC or HC were loaded with [^{3}H]-NA (70 nM) and stimulated for 5 min with nicotine. FC (Red) and HC (Blue) prisms were incubated with 10 or 100 μM nicotine to stimulate [^{3}H]-NA release ± 10 μM mecamylamine (hatched bars), a non-selective nAChR antagonist. Bars represent the mean ± SEM from 3 separate experiments as % basal. * $p<0.05$, ** $p<0.01$, *** $p<0.005$ compared to basal efflux. ## $p<0.01$, ### $p<0.001$ compared to agonist alone, one way ANOVA, Holm-Sidak *post hoc* test for each region. FC $F(4,12)=22.57$, $p<0.001$, HC $F(4, 8)=23.16$, $p<0.001$

2.3.2.1. Nicotine stimulated [^{3}H]-NA release

To determine if nAChRs are involved in eliciting [^{3}H]-NA release in the rat brain [^{3}H]-NA release from rat FC or HC prisms was determined in response to nicotine (10 or 100 μM), in the presence or absence of general nicotinic antagonist mecamylamine. *Post hoc* analysis revealed that both concentrations of nicotine tested induced significant [^{3}H]-NA release in both FC and HC ($p<0.01$; Figure 2-4). This nicotine induced [^{3}H]-NA release in FC and HC was shown to be mediated by nAChRs by use of mecamylamine. Mecamylamine (10 μM) blocked the response to 10 and 100 μM nicotine in FC ($p<0.001$), and to 100 μM nicotine in HC ($p<0.01$; Figure 2-4); the block in HC was not complete ($p<0.05$ compared to basal). This may be due to the involvement of nAChR subtypes less sensitive to mecamylamine suggesting a difference between the two regions or a proportion of non-nAChR mediated release. To further investigate the specificity of nicotine mediated [^{3}H]-NA release, [^{3}H]-NA release from rat FC or HC prisms was examined in response to nicotine (0.1 μM – 1 mM), in the presence or absence of β2* selective competitive antagonist dihydro-β-erythroidine (DHβE; 10 μM).

Nicotine concentration-dependently elicited [^{3}H]-NA release from FC prisms (Figure 2-5A) with EC_{50} 4.9 μM, reaching a maximum of 165 $\pm$ 1.1 % basal at 300 μM ($F(8,52)=31.1$, $p<0.001$). This response was competitively inhibited with DHβE with a rightward shift of nicotine dose response curve (EC_{50} 28.6 μM; $F(1,52)=18.21$; $p<0.001$) with *post hoc* Bonferroni test showing a significant difference at 1 and 10 μM nicotine. There was no interaction between nicotine dose and DHβE administration ($F(8,52)=1.06$ $p=0.4074$). These results suggest that β2* nAChRs are involved in [^{3}H]-NA release in FC prisms in this assay.

In the HC nicotine also elicited significant release ($F(3,24)=33.28$, $p<0.001$) maximal release of [^{3}H]-NA was higher (191.5 $\pm$ 14.7 %) measured at 100 μM nicotine (Figure 2-5B). There was no shift of the curve with DHβE in HC ($F(1, 24)=0.41$, $p=0.53$) and no interaction between nicotine concentration and DHβE ($F(1,24)=0.1$, $p=0.96$) suggesting that β2* nAChRs are minimally involved in nicotine elicited [^{3}H]-NA release under the conditions used in these experiments.

2.3.2.2. Cytisine stimulated [^{3}H]-NA release

The differences between FC and HC responses to nicotine are suggestive of differing nAChR subtypes in the two regions, which was investigated with agonists with greater selectivity. Cytisine is a full agonist at α7 and a partial agonist at β2* and β4* nAChRs (Luetje and Patrick, 1991; Papke and Heinemann, 1994) with higher efficacy at β4* combinations.

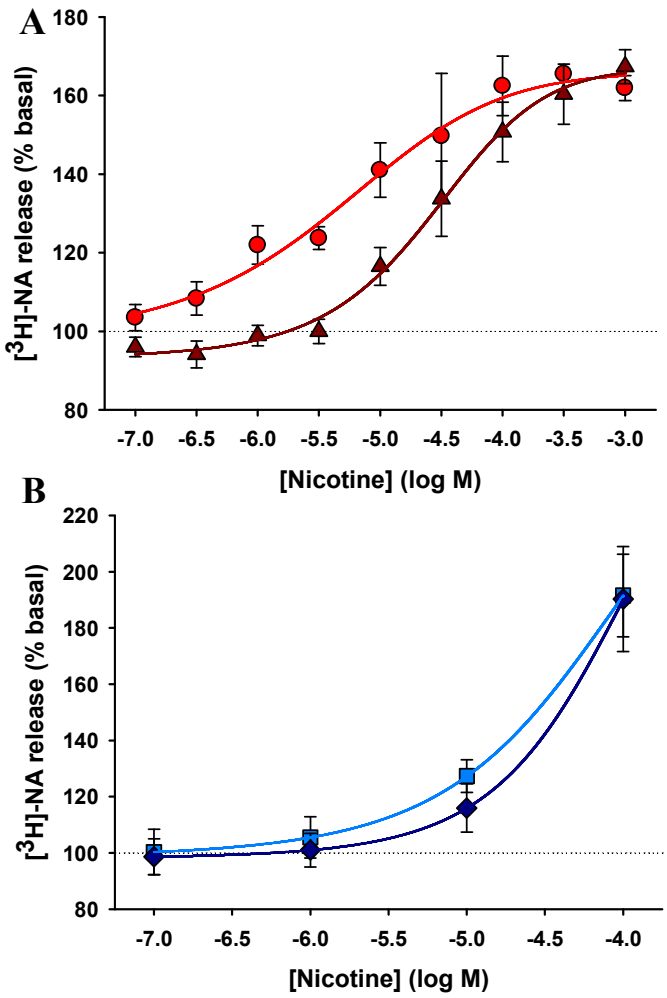


Figure 2-5 Effect of nicotine on [^{3}H]-NA release in FC and HC
Prisms (150 µm) from rat FC (**A**) or HC (**B**) were loaded with [^{3}H]-NA (70 nM) and stimulated for 5 min with nicotine (0.1 µM-1 mM). **A)** Nicotine evoked [^{3}H]-NA release from FC prisms (Red; pEC_{50} -5.3 ± 0.34; 4.9 µM), and this was competitively inhibited by DHβE (dark red; pEC_{50} -4.5 ± 0.16; 28.6 µM) with a rightward shift of nicotine dose response curve. Data analysed by two way ANOVA with *post hoc* Bonferroni ($F(1,52)=18.21$; $p<0.001$). **B)** Nicotine evoked [^{3}H]-NA release from HC prisms (Blue) showed no significant rightward shift with DHβE (Dark blue; $F(1, 24)=0.41$; $p=0.53$) Data analysed by two way ANOVA. Points represent the mean ± SEM from 4-6 separate experiments as % basal.

Cytisine elicited significant release of [^{3}H]-NA in both regions ($F(6,28)=17.07$, $p<0.0001$). In FC cytisine elicited release to a maximal of 133.9 ± 3.8 % basal with EC_{50} 4.2 µM (Figure 2-6). In HC cytisine was less potent (EC_{50} 25.5 µM) but reached a maximal of 140 ± 8.8 % basal, comparable of that in FC. This difference in response between the regions was significant ($F(1,28)=9.45$; $p=0.0047$) but there was no interaction between region and cytisine concentration ($F(6,28)=1.47$, $p=0.2237$) and post hoc testing showed no individual concentrations with significantly different responses between the two regions. The maximal release elicited by cytisine was less than that elicited by nicotine in each region, possibly showing partial agonism, as previously seen for cytisine.

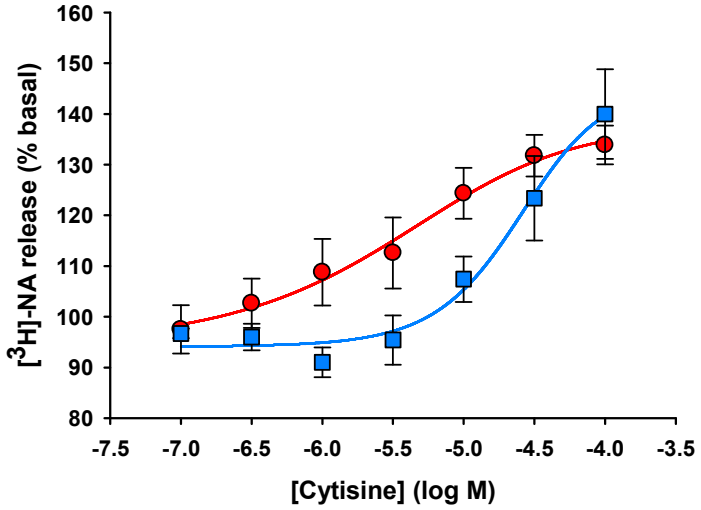


Figure 2-6 Effect of cytisine on [^{3}H]-NA release in FC and HC
Prisms (150 µm) from rat FC or HC were loaded with [^{3}H]-NA (70 nM) and stimulated for 5 min with cytisine. [^{3}H]-NA release was stimulated with cytisine, a partial α4β2 and α3β4 agonist. Cytisine was equally efficacious in releasing [^{3}H]-NA in FC (red) and HC (blue). In FC pEC_{50} -5.4 ± 0.44 (4.1 µM) and in HC pEC_{50} -4.6 ± 0.26 (25.5 µM) and the curves are significantly different between the two regions Data analysed by two way ANOVA with Bonferroni *post hoc* test (F(1,28)=9.45;p0.0047). Points represent the mean ± SEM from 4 separate experiments as % basal.

2.3.2.3. 3-Bromo-Cytisine

3-Bromo-Cytisine is a full agonist at α7 and partial agonist at α4β2* (with similar efficacy to cytisine), with slightly higher activity at α4β4* compared with 30 µM acetylcholine (Houlihan *et al* 2001). 3-Bromo-cytisine elicited significant release ($F(4,50)=7.90$, $p<0.001$). *Post hoc* tests showed that in FC concentration-dependent release of [^{3}H]-NA was seen, which reached significance at 100 µM (128.7 ± 10 % basal; Figure 2-7). In the HC [^{3}H]-NA release was higher, reaching a maximum of 162.1 ± 13.1 % basal at 10 µM 3-Bromo-cytisine. Region had a significant effect on the responses ($F(1,50)=9.635$, $p<0.01$) but there was no interaction between region and treatment ($F(4,50)=2.173$, $p=0.0855$). Post hoc Bonferroni tests showed that the responses were significantly different at 10 µM 3-Bromo-cytisine. In HC EC_{50} was 0.6 µM, in FC the EC_{50} could not be determined due to lack of significant responses and the linear nature of the response in the concentration range tested. The responses to 10 µM 3-Bromo-cytisine were fully inhibited with 10 µM mecamylamine in both regions.

2.3.2.4. 5-Iodo-A-85380 stimulated [^{3}H]-NA release

As covered earlier (2.1.1) NA release in HC is thought to be principally mediated by α3β4* nAChRs, with no sensitivity to β2* antagonists. The preceding results suggest differences between HC and FC in nAChR subtypes involved in [^{3}H]-NA release, and a probable involvement of β2* nAChRs in FC, as shown by DHβE inhibition of nicotine-mediated responses. 5-iodo-A-85380 (5IA), an agonist with a distinct profile for β2* versus β4* nAChRs was therefore utilised.

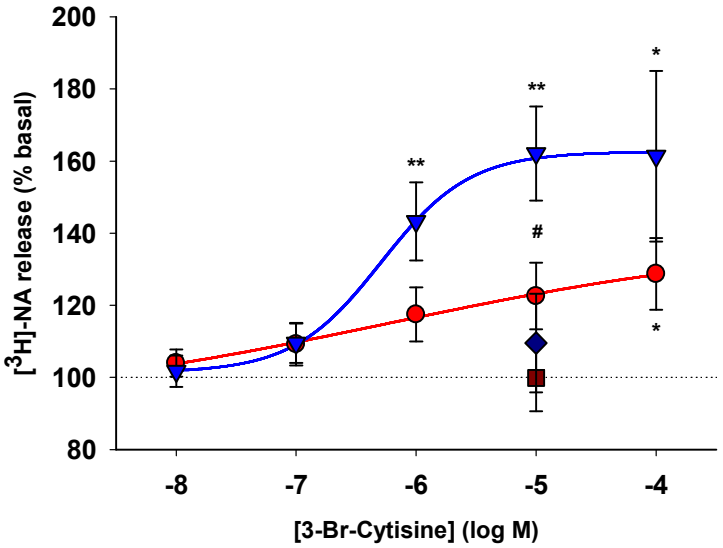


Figure 2-7 Effect of 3-Bromo-Cytisine on [^{3}H]-NA release in FC and HC
Prisms (150 µm) from rat FC or HC were loaded with [^{3}H]-NA (70 nM) and stimulated for 5 min with 3-Bromo-Cytisine. [^{3}H]-NA release was stimulated from FC (red) or HC (blue) prisms with 3-Bromo-Cytisine (10 nM-100 µM) in the presence (darker symbols) or absence of 10 µM mecamylamine. Points represent mean ± SEM from 4 separate experiments as % basal. * $p<0.05$, ** $p<0.01$ compared to basal $F(4, 50)=7.90$, $p<0.0001$. # $p<0.05$ between FC and HC $F(1, 50)=9.63$, $p=0.0031$. Analysed by two way ANOVA with Bonferroni *post hoc* multiple comparisons.

5IA is a very potent selective β2* nAChR agonist with much lower affinity at β4* nAChRs (Mukhin *et al*, 2000). In FC 5IA elicited [^{3}H]-NA release with EC_{50} 1.6 nM reaching a maximum of 143.4 ± 9.9 % at 30 nM ($F(6,46)=5.732$, $p<0.001$; Figure 2-8A). This release was sensitive to DHβE ($F(1,46)=5.69$; $p=0.0213$) although *post hoc* Bonferroni showed no individual concentrations were significantly different with or without DHβE. A change in the EC_{50} was seen (EC_{50} 43.0 nM; $F(1,54)=5.87$; $p=0.0188$ by non-linear regression) confirming the involvement of β2* nAChRs. There was no interaction between 5IA concentration and DHβE administration ($F(6,46)=0.965$, $p=0.4593$).

HC was much less sensitive to 5IA and although release was elicited by higher concentrations this did not quite reach significance ($F(5,24)=2.513$, $p=0.0577$), [^{3}H]-NA release reached a maximal of 131.6 + 12.6 % at 30 µM (EC_{50} 5.9 µM) and the response was not sensitive to DHβE ($F(1,24)=0.00$, $p=0.99$; Figure 2-8B; EC_{50} 6.9 µM). As DHβE did not account for any of the variance in the responses there was no interaction between 5IA concentration and DHβE ($F(5,24)=0.05698$, $p=0.998$). The large difference in EC_{50} between these two regions agrees with studies on the binding potency and functional responses of 5IA at α4β2* and α3β4* nAChRs (Mukhin *et al*, 2000; Mogg *et al*, 2004) that have found comparable differences, suggesting these as possible nAChR types involved in FC and HC respectively.

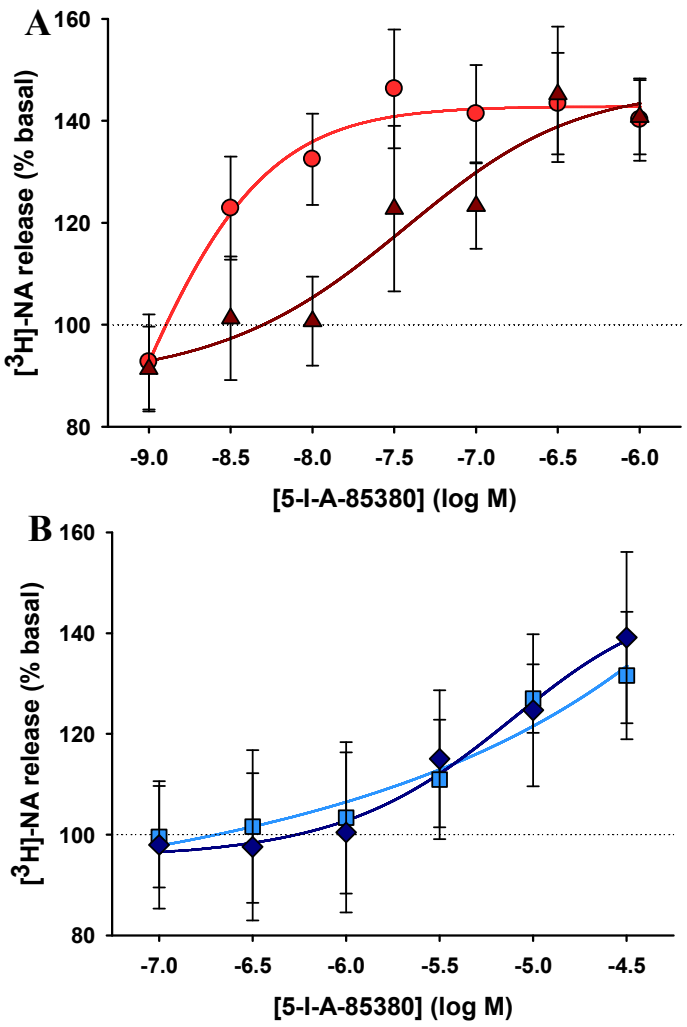


Figure 2-8 Effect of 5-iodo-A-85380 on [^{3}H]-NA release in FC and HC
Prisms (150 µm) from rat FC or HC were loaded with [^{3}H]-NA (70 nM) and release was stimulated from FC (A) and HC (B) prisms with various concentrations of 5IA. 10 µM DHβE was used as antagonist. **A)** 5IA evoked [^{3}H]-NA release in the FC (red; pEC_{50} -8.8± 0.6; 1.6 nM), and this was competitively inhibited by DHβE (dark red; pEC_{50} -7.4± 0.4; 43.0 nM; F(1,46)=5.69; p=0.0213). **B)** 5IA evoked [^{3}H]-NA release in HC (blue; pEC_{50} -5.23 ± 0.76; 5.9 µM) showed no shift in EC_{50} with DHβE (dark blue; pEC_{50} -5.16 ± 0.75; 6.9 µM F(1,24)=0.99; p>0.95) Points represent mean ± SEM from 4-6 experiments as % basal. Note differing x axis scales. Analysed by two way ANOVA.

2.3.2.5. Choline stimulated [^{3}H]-NA release

A component of NA release mediated by α7 nAChR in the HC has previously been shown in several systems (Barik and Wonnacott, 2006; Fu *et al* 1999). To test this in FC, prisms were incubated with choline, a full agonist at α7 and a partial agonist at α3β4 nAChRs. Choline elicited significant release of [^{3}H]-NA at 3 and 5 mM in the HC ($F(3,15)=6.806$, $p<0.01$; 165.5 ± 13.7 %; Figure 2-9A). *Post hoc* tests revealed that mecamylamine (30 µM) inhibited this response ($p<0.05$). In FC there was a small but non-significant release over basal ($F(3,18)=1.269$, $p=0.315$), which was not reduced by mecamylamine showing this was not a nAChR mediated effect. Choline mediated release in HC was not inhibited by α-Bgt (data not shown) or MLA (Figure 2-9B) suggesting that α7 nAChRs are not a major component of the NA release under these conditions.

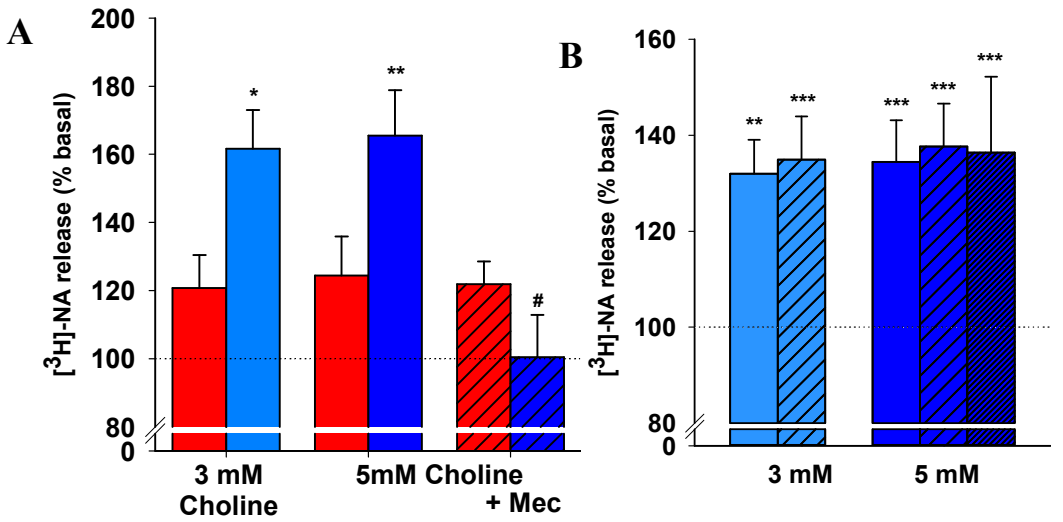


Figure 2-9 Effect of choline on [^{3}H]-NA release in FC and HC
Prisms (150 µm) from rat FC or HC were loaded with [^{3}H]-NA (70 nM) and stimulated for 5 min. **A)** Rat FC (Red) or HC (Blue) prisms were incubated with 3 or 5 mM choline in the presence (hatched bars) or absence of 30 µM mecamylamine. Bars represent mean ± SEM from 4 experiments as % basal. * $p<0.05$ ** $p<0.01$ compared to basal # $p<0.05$ compared to agonist. Analysed by one way ANOVA for each region with Holm-Sidak *post-hoc* test FC $F(3,18)=1.269$, $p=0.315$ HC $F(3, 15)=6.806$, $p=0.004$. **B)** Rat HC prisms were incubated with 3 or 5 mM choline in the presence or absence of 10 nM (light hatched) or 100 nM (close hatched) MLA and [^{3}H]-NA release measured. Bars represent mean ± SEM from 3 experiments as % basal. ** $p<0.01$, *** $p\leq0.001$ compared to basal, analysed by one way ANOVA, Holm-Sidak *post-hoc* test. $F(5, 10)=13.288$, $p<0.001$

To further assess any role of α7 nAChRs in mediating [^{3}H]-NA release in FC or HC the effect of ARR-17779 (α7 nAChR agonist; Levin *et al*, 1999) was tested (Figure 2-10). ARR-17779 (with or without PNU-120596) does not elicit significant [^{3}H]-NA release in either region (FC $F(6,21)=0.680$, $p=0.668$, HC $F(6, 14)=0.385$, $p=0.876$).

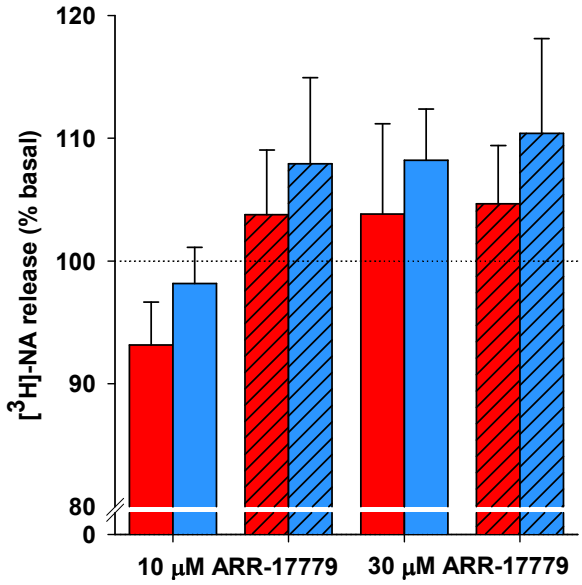


Figure 2-10 Effect of ARR-17779 on [^{3}H]-NA release in FC and HC
Prisms (150 µm) from rat FC (red) or HC (blue) were loaded with [^{3}H]-NA (70 nM) and release stimulated with ARR-17779 (10 or 30 µM) in the presence (hatched bars) or absence of 10 µM PNU120596. Bars represent mean ± SEM from 3-4 experiments as % basal. Analysed by one way ANOVA for each region FC $F(6,21)=0.680$, $p=0.668$, HC $F(6, 14)=0.385$, $p=0.876$.

2.3.2.6. GABA

Previous work in this and other labs has suggested an involvement of GABAergic interneurons, and $GABA_A$ receptors in particular, in NA release (Leslie *et al*, 2002; Barik and Wonnacott, 2006). To test this, GABA (100 µM) was examined to see if it would stimulate [^{3}H]-NA release, with choline (1 mM) and nicotine (100 µM) used as positive controls. This panel of drugs gave significant results in both regions (FC $F(7,24)=10.87$, $p<0.001$, HC $F(7,21)=20.04$, $p<0.001$).

Post hoc Holm-Sidak multiple comparisons showed that GABA (100 µM) elicited significant release of [^{3}H]-NA over basal in both FC (118.9 ± 4.3 % basal) and HC (121.8 ± 6.2 % basal; Figure 2-11), and this release was partially inhibited by bicuculline (100 µM) reducing release to 111.8 ± 6.2 and 108.5 ± 6.8 % basal in FC and HC respectively. These reductions were not significant, but evoked release was no longer significantly different to basal release. Release elicited by nicotine was partially attenuated by bicuculline but not significantly. Release was reduced from 146.2 ± 7.8 to 132.4 ± 7.4 % basal and 136.9 ± 8 to 121.8 ± 8.7 % basal in FC and HC respectively. Interestingly, release elicited by choline in HC was completely blocked by bicuculline (from 121.6 ± 3.9 to 101.5 ± 4.9 % basal) and this was a significant effect.

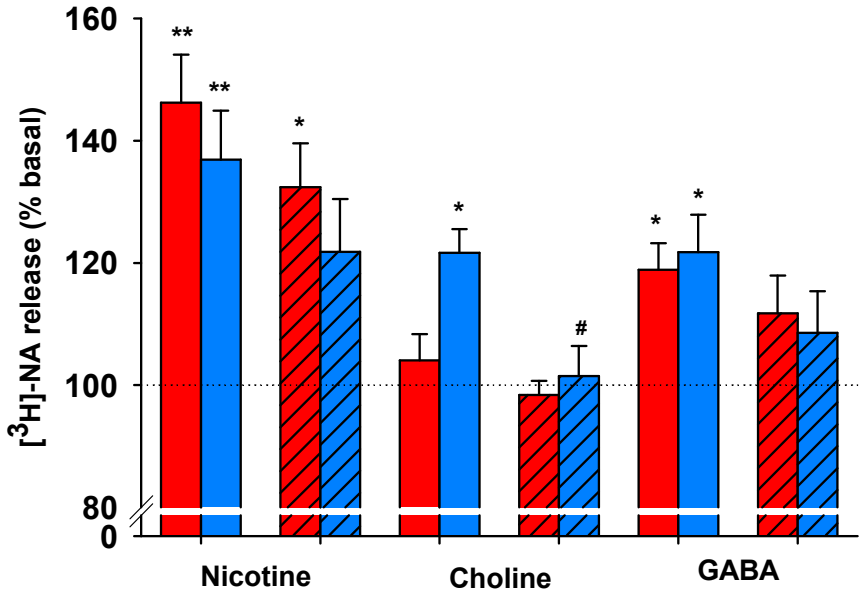


Figure 2-11 Effect of GABA on [^{3}H]-NA release in FC and HC
Prisms (150 µm) from rat FC (red) or HC (blue) were loaded with [^{3}H]-NA (70 nM) and stimulated for 5 min with agonists. [^{3}H]-NA release was stimulated from prisms by incubation with nicotine (10 µM), choline (1 mM) or GABA (100 µM) in the presence (hatched bars) or absence of 100 µM bicuculline. Bars represent the mean ± SEM from 4 separate experiments as % basal. * $p<0.05$, ** $p<0.01$ compared to basal. # $p<0.05$ compared to agonist alone analysed by one way ANOVA, Holm-Sidak *post-hoc* tests for pairwise comparisons for each region. FC $F(7,24)=10.87$, $p<0.001$, HC $F(7,21)=20.04$, $p<0.001$

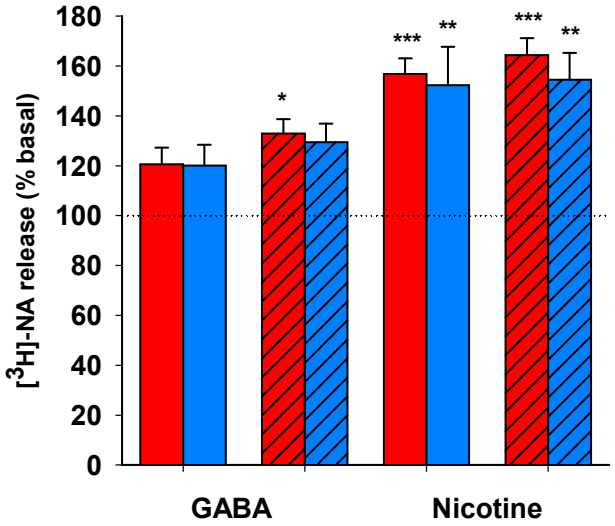


Figure 2-12 Effect of CGP54626 on GABA and nicotine induced [^{3}H]-NA release
Prisms (150 µm) from rat FC (red) or HC (blue) were loaded with [^{3}H]-NA (70 nM) and stimulated for 5 min with agonists. [^{3}H]-NA release was stimulated with 100 µM GABA or nicotine in the presence (hatched bars) or absence of 1 µM CGP54626, a $GABA_B$ antagonist. Bars represent the mean ± SEM from 4 separate experiments as % basal. *p<0.05, ** p<0.01, *** p<0.001 compared with basal. Analysed by one way RM ANOVA, Holm-Sidak *post-hoc* tests for each region FC $F(4,15)=16.466$, $p<0.001$, HC $F(4,15)=4.779$, $p=0.011$

To further investigate the role of GABA in [^{3}H]-NA release the effect of CGP54626 ($GABA_B$ antagonist, 1 µM) on GABA- and nicotine-mediated release in FC and HC was investigated. CGP54626 had no effect on [^{3}H]-NA release elicited by either agonist (Figure 2-12), although the response to GABA in FC became slightly larger (and therefore significant in comparison to basal).

GABA is usually regarded as an inhibitory signal, and so the increased release of [^{3}H]-NA in response to GABA is intriguing but could be due to inhibition of tonically active GABAergic interneurons, or an excitatory effect of GABA, usually thought to be a developmental process. In adult brain stimulation of GABAergic receptor is usually inhibitory due to the existence of a gradient that promotes the influx of Cl^- upon receptor activation, causing a hyperpolarisation of the cell. Excitatory GABA, with an altered Cl^- gradient has been postulated in adult born neurons and also in dendrites (Stein and Nicoll, 2003; see 2.6.4). $GABA_A$ receptor mediated release of [^{3}H]-NA from superfused synaptosomes has been seen (Fassio *et al*, 1999), consistent with a direct excitatory effect. To test which mechanism is involved in [^{3}H]-NA release in this assay, inhibitors of the NKCC and KCC cotransporters were used.

DIOA (a KCC2 inhibitor) provoked a large amount of [^{3}H]-NA release at 100 µM (data not shown) indicative of a disruption of a gradient required for tonic inhibition. At lower concentrations which do not affect basal release (1 µM) no effect of DIOA on GABA or choline mediated release were seen. There is a possible potentiation of some responses but none of these are significant (Incubation $F(4,30)=1.71$, $p=0.1745$; Figure 2-13A). There

was no difference in the responses between the regions (F(1,30)=0.1438, p=0.7072) or interaction between treatment and region (F(4,30)=0.2272, p=0.921).

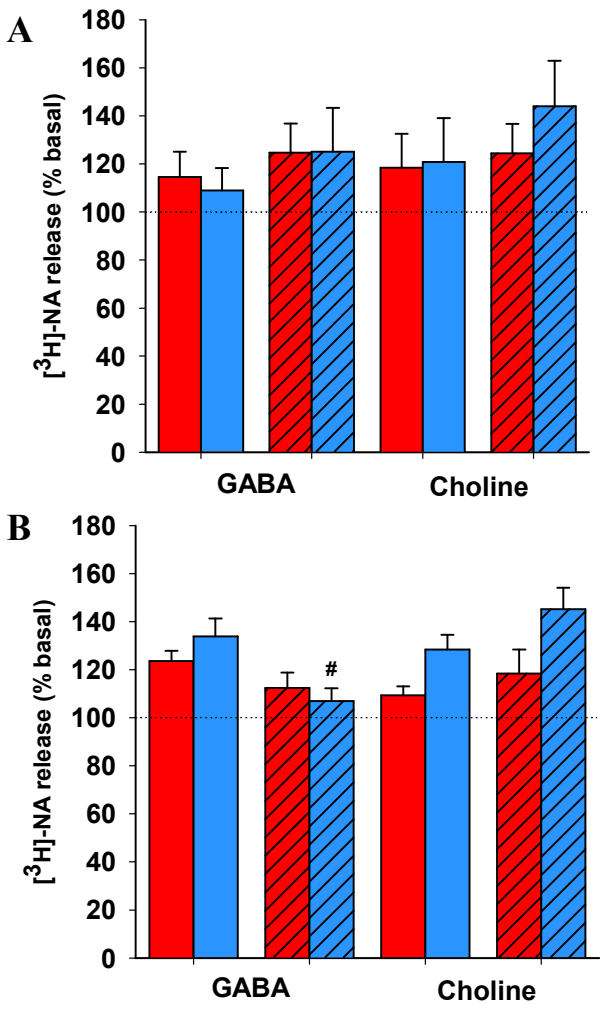


Figure 2-13 Effect of DIOA and bumetanide on GABA and choline induced [^{3}H]-NA release
Prisms (150 µm) from rat FC (red) or HC (blue) were loaded with [^{3}H]-NA (70 nM) release was stimulated with GABA (100 µM) or choline (1 mM) in the presence (hatched bars) or absence of A) 1 µM DIOA or B) 100 µM bumetanide F(4,30)=1.71, p=0.1745 Analysed by two way ANOVA. Bars represent the mean ± SEM from 4 separate experiments as % basal. # p<0.05 compared to agonist alone. Analysed by two way ANOVA with *post hoc* Bonferroni. Treatment F(4, 46)=8.17, p<0.0001, Region F(1,46)=5.98, p<0.05

Bumetanide (100 µM), an NKCC1 cotransporter inhibitor, reduced release of [^{3}H]-NA elicited by GABA in both FC and HC (F(4,46)=8.17, p<0.001), although *post hoc* tests showed that this was only significant in HC (p<0.05; Figure 2-13B). There was no effect of bumetanide on basal [^{3}H]-NA release or on choline elicited release, in contrast with a bicuculline mediated reduction, suggesting different mechanisms of $GABA_A$ mediated release between GABA and choline stimulation. Lower concentrations of bumetanide did not reduce GABA elicited release in either region (data not shown). There was a significant effect of region (F(1,46)=5.98, p=0.0184) *post hoc* Bonferroni showed that only the choline plus bumetanide condition differed significantly between the regions but there was no interaction between region and treatment (F(4,46)=2.043, p=0.1039).

2.4. Results - [³H]-Noradrenaline release and [³H]-Epibatidine binding in nicotine treated animals

After chronic nicotine treatment both *in vivo* and *in vitro* heteromeric nAChRs are known to be upregulated, although the functional consequences of this are less clear. For α7 nAChRs Barik and Wonnacott (2006) found no increase in ^{125}I-αBgt binding, but a change in functional α7 activity when stimulated by choline during nicotine withdrawal.

2.4.1.1. Single injection

In order to be confident that when investigating chronic treatment differences seen in [^{3}H]-NA release are due to chronic changes and not acute receptor occupancy or desensitisation [^{3}H]-NA release was assessed after a single nicotine injection. The animals were given a single injection of either nicotine (0.4 mg/kg s.c.) or saline (see 2.2.6.1). The nicotine dose was chosen as it is a near optimal psychostimulant dose which gives reliable responses across rat strains (See Matta et al, 2007 for a review of nicotine dose selection). Animals were killed by cervical dislocation 30 min after injection, with nicotine and saline animals paired. FC was rapidly dissected and transferred to ice cold KB. Tissue from each animal was kept separate and loaded with [^{3}H]-NA as previously described (2.2.7). Tissue was resuspended in KBN then transferred 100 µl/well to one half of a 96 well filter plate.

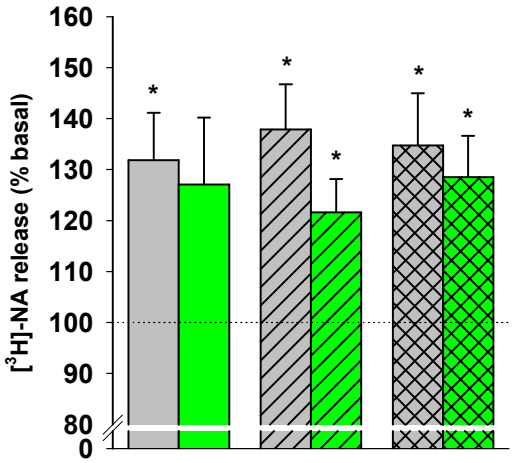


Figure 2-14 [^{3}H]-NA release after single nicotine injection
Saline (grey) or nicotine (0.4 mg/kg, green) was administered s.c. 30 min before tissue collection. Tissue was incubated with 10 µM nicotine with or without PNU-120596 (10 µM; hatched bars) or PNU-120596 + α-Bgt (40 nM; cross hatched bars). Bars represent the mean ± SEM from 7 experiments as % basal * $p<0.05$ compared to basal analysed by two way ANOVA with Dunnett's *post hoc* test for comparisonstocontrol. Incubation $F(4,58)=4.56$, $p=0.0028$ Injection $F(1,58)=2.03$, $p=0.1592$ Interaction $F(4,58)=0.2928$, $p=0.8815$

A single nicotine injection 30 min prior to tissue collection did not alter basal [^{3}H]-NA release from FC tissue (data not shown), or the response elicited by nicotine in comparison to saline treated animals ($F(1,58)=2.03$, $p=0.1592$; Figure 2-14). As previous studies (Barik and Wonnacott, 2006) have found functional differences in α7 nAChR mediated [^{3}H]-NA release after chronic nicotine treatment, the responses to positive

allosteric modulation and antagonism of the α7 nAChR were investigated. No effect of PNU-120596 or α-Bgt (α7 selective antagonist) was seen on nicotine mediated [^{3}H]-NA release in agreement with results for tissue from on naïve animals (2.3.2) and responses did not differ between saline and nicotine treated animals. As there were no changes caused in [^{3}H]-NA release after acute nicotine treatment, any acute receptor occupancy or desensitisation is reversed during the wash stages in this type of experiment.

2.4.1.2. 14 days injection

Nicotine administration or withdrawal did not affect the weight gain of the animals over the time period studied, in comparison with saline treated animals (final weight 339±20g in nicotine and 321±4.6g in saline treated animals and 331±4.4g in nicotine and 341±13.6g in saline withdrawn animals). The basal release elicited by buffer alone was not different between the groups (13.6 ± 1 and 12.7 ± 0.8 % in nicotine and saline groups respectively and 13.6 ± 1.1 and 15.2 ± 2.0 % respectively during withdrawal).

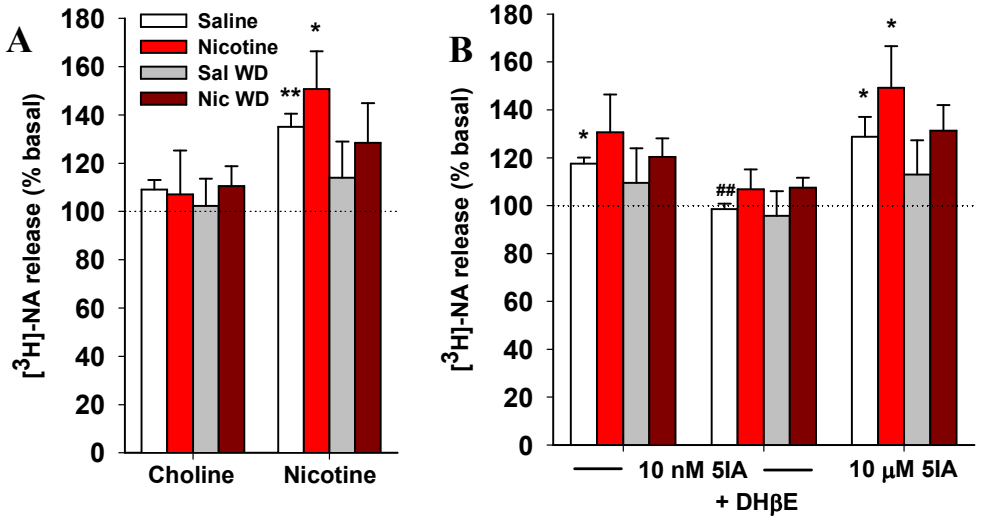


Figure 2-15 Effect of 14 days of nicotine injection on [^{3}H]-NA release in FC
Animals were injected once daily for 14 days with 0.4 mg/kg nicotine, or 0.9 % saline. Animals were sacrificed 30 min or 3 days after final injection. Prisms (150 µm) of rat FC were loaded with [^{3}H]-NA (70 nM) and release was stimulated by incubation for 5 min with agonists. Bars represent the mean ± SEM from 4 experiments as % basal * $p<0.05$, ** $p<0.01$ compared to basal. ## $p< 0.01$ compared to agonist alone. Analysed by two way ANOVA with 'Injection' and 'Incubation' as factors. Incubation $F(5, 70)=6.44$, $p<0.0001$, Injection $F(3, 70)=2.68$, $p=0.0537$

The *in vitro* incubation conditions had significant effects ($F(5,70)=6.44$, $p<0.0001$). *Post hoc* Dunnett's test showed that there was no response to 3 mM choline in FC (Figure 2-15A), in agreement with results from naïve animals (Figure 2-9). The response to a maximally effective concentration of nicotine (100 µM) was no different between the four groups (Figure 2-15A). The response to 5IA at 10 nM was around 120 % of basal in all treatment groups, and this response was blocked with DHβE. In the FC the response to 5IA (10 µM) was around 140 % of the basal response (Figure 2-15B). These responses did not differ between the two treatments or during withdrawal, although responses were in general

slightly smaller (and therefore non-significant) in both nicotine and saline animals on withdrawal days. Overall the injection conditions had no significant effect on [^{3}H]-NA release ((F3,70)=2.68, p=0.0537) and there was no interaction between injection and *in vitro* incubation conditions (F(15,70)=0.3725, p=0.982).

Baseline release values of [^{3}H]-NA in the HC were 16.6 + 0.5 and 16.1 +0.5 % in nicotine and saline animals and 16.0 + 1.9 and 14.5 + 1.4 % respectively during withdrawal. These values were not significantly different from one another. In the HC there was again no difference between nicotine and saline treated or withdrawn animals (F(3,70)=0.2645, p=0.85) however the profile of significant responses (F(5,70)=29.42, p<0.0001) is slightly different to the FC. *Post hoc* Dunnett's reveals a response to 3 mM choline in the HC of around 130% baseline and nicotine (100 µM) elicits a response of around 160% of basal (Figure 2-16A). There is no response to the lower concentration of 5IA (10 nM) in HC consistent with Figure 2-8 and the response to 10 µM 5IA is around 130 % of basal in all treatment groups (Figure 2-16B), showing that chronic nicotine treatment by injection does not alter the sensitivity to 5IA. Again there was no interaction between injection and *in vitro* incubation conditions (F(15,70)=0.1764, p=0.9997).

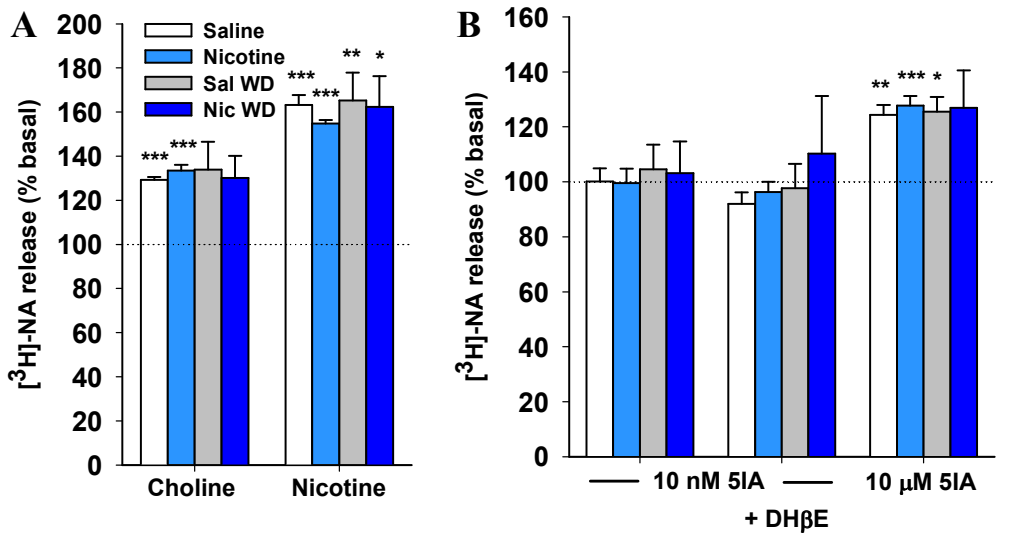


Figure 2-16 Effect of 14 days of nicotine injection on [^{3}H]-NA release in HC
Animals were injected once daily for 14 days with 0.4 mg/kg nicotine, or 0.9 % saline. Animals were sacrificed 30 min or 3 days after final injection, nicotine and saline animals were paired. Prisms (150 µm) from rat HC were loaded with [^{3}H]-NA (70 nM) and release was stimulated by incubation for 5 min with agonists. Bars represent the mean ± SEM from 4 experiments as % basal * p<0.05, ** p<0.01, *** p<0.001 compared to basal. Analysed by two way ANOVA with 'Injection' and 'Incubation' as factors. Injection F(3, 70)=0.26, p=0.8508, Incubation F(5, 70)=29.42, p<0.0001

These profiles of [^{3}H]-NA release after chronic treatment and withdrawal fit with the pharmacology already outlined for the two regions in naïve animals (2.3.2). The same patterns are observed at 14 days of nicotine treatment and after 3 days withdrawal, with no change in any of the responses; however the overall variability increases during withdrawal suggesting that subtle changes may have taken place in some animals.

2.4.1.3. 14 day minipumps

Single daily injections give large peaks in plasma levels of nicotine, which are cleared from the bloodstream by the time of the next injection in rats. In contrast, smokers maintain a plasma concentration of nicotine, and each cigarette gives a smaller peak. To mirror sustained plasma levels of nicotine, rats were implanted with osmotic minipumps as described in methods (2.2.6.2). Briefly, rats were anaesthetised with isoflurane and Alzet® osmotic minipumps to deliver nicotine (4 mg/kg/day) implanted subcutaneously. Fourteen days after pump insertion animals were sacrificed by cervical dislocation and brains rapidly removed and dissected. To study withdrawal effects pumps were surgically removed after 14 days in separate groups of animals and animals killed 2 or 3 days after pump removal. Nicotine and saline animals were paired for each timepoint, with samples used in parallel.

[^{3}H]-Epibatidine binding

To ascertain if receptor binding was upregulated after chronic nicotine as expected (Wonnacott, 1990) brain tissue remaining after the removal of FC and HC for release experiments (2.4.1.3) was used to determine changes in [^{3}H]-Epi binding (2.2.8). Chronic minipump treatment with nicotine (4 mg/kg/day) induced an increase in [^{3}H]-Epi binding ($F(1,37)=14.65$, $p<0.001$) that Bonferroni *post hoc* tests showed was significant at the end of treatment and following two days of withdrawal (Figure 2-17). After three days of withdrawal the [^{3}H]-Epi binding was no longer significantly different from control, although a numerical difference could be observed.

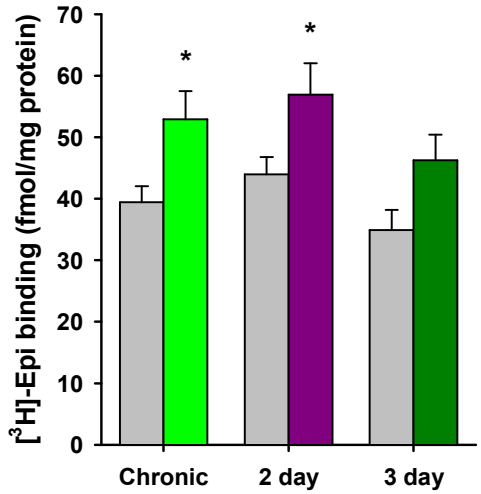


Figure 2-17 [^{3}H]-Epibatidine binding in chronically treated and withdrawn animals
Nicotine was administered at a rate of 4 mg/kg/day for 14 days via Alzet® osmotic minipump. After 14 days animals were sacrificed (light green), or pumps removed and animals sacrificed 2 (dark pink) or 3 (dark green) days later. P2 membranes were prepared and 150 µg incubated with 500 pM [^{3}H]-Epi in a final volume of 1 ml. [^{3}H]-Epi bound was quantified by scintillation counting. Bars represent the mean ± SEM from 4-6 animals as % basal * $p<0.05$ compared to paired control group (grey). Analysed by two way ANOVA with corrections for multiple comparisons in Bonferroni post tests. Injection $F(1,37)=14.65$, $p=0.0005$, Timepoint $F(2,37)=0.0244$, $p=0.976$ Interaction $F(2,37)=0.0244$, $p=0.976$

[^{3}H]-NA release

FC and HC prisms were taken for use in release assays. Basal fractional release is presented in Figure 2-18. In FC (panel A) no differences are found in fractional release after any of the treatments ($F(3,34)=1.483$, $p=0.237$). In HC (panel B) a difference in basal release is seen ($F(3,32)=6.472$, $p<0.01$) with *post hoc* Tukey tests revealing significantly higher basal release of [^{3}H]-NA at 2 days of withdrawal, this returned to baseline levels by the 3rd day of withdrawal.

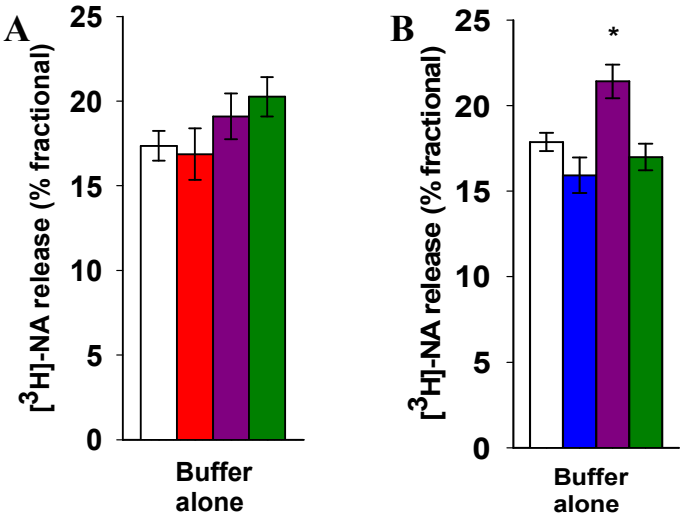


Figure 2-18 [^{3}H]-NA basal release after chronic nicotine treatment
Nicotine was administered at 4 mg/kg/day for 14 days via Alzet® osmotic minipump. After 14 days animals were sacrificed, or pumps removed and animals sacrificed 2 or 3 days later. Responses from control animals did not differ in any treatment regimen and responses were pooled. **A)** Basal [^{3}H]-NA release as % fractional from FC. Control (white), nicotine (red), 2 day WD (dark pink), and 3 day WD (green). **B)** Basal [^{3}H]-NA release as % fractional from HC. Control (white), nicotine (blue), 2 day WD (dark pink), and 3 day WD (green). Bars represent mean ± SEM from 6-7 experiments (18 for control) * $p<0.05$ compared to control. Analysed by one way ANOVA with Tukey *post hoc* test for pairwise comparisons. FC $F(3,34)=1.483$, $p=0.237$, HC $F(3, 32)=6.472$, $p=0.002$

Tissue taken from animals treated chronically via minipump was incubated with the same drugs as used for injections. As previously seen in FC there was significant effect of *in vitro* incubation conditions ($F(5,202)=9.133$, $p<0.0001$). *Post hoc* Dunnett's tests showed that as before choline had no effect on [^{3}H]-NA release (Figure 2-19A). Nicotine induced release in each of the treatment groups. The response to 5IA and block by DHβE was also similar in magnitude between groups. In the frontal cortex chronic nicotine treatment or withdrawal did not have any significant effect on response to a variety of agonists ($F(3,202)=0.1815$, $p=0.9089$) and there was no interaction between *in vivo* and *in vitro* treatments ($F(15,202)=0.08549$, $p=1$).

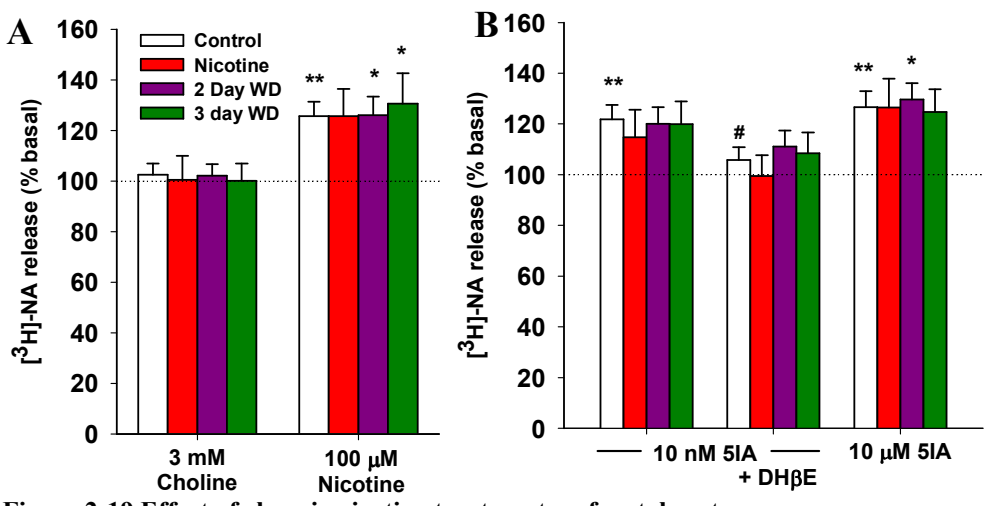


Figure 2-19 Effect of chronic nicotine treatment on frontal cortex responses
Nicotine was administered at a rate of 4 mg/kg/day for 14 days via Alzet® osmotic minipump. After 14 days animals were sacrificed, or pumps removed and animals sacrificed 2 or 3 days later. [^{3}H]-NA release was stimulated from FC with a variety of agonists. Responses from control animals did not differ in any treatment and responses were pooled. In both panels control (white), 14 day nic (red), 2 day WD (dark pink), 3 day WD (green) **A)** Response to choline or nicotine. **B)** Response to 5IA and DHβE. Bars represent mean ± SEM from 6-7 experiments (18 for basal) * $p<0.05$, ** $p<0.01$ compared to basal, # $p<0.05$ compared to agonist alone. Analysed by two way ANOVA with *in vivo* treatment and *in vitro* treatment as factors. *In vivo* $F(3, 202)=0.18$, $p=0.9089$, *In vitro* $F(5,202)=9.13$, $p<0.0001$

Tissue taken from animals treated chronically via minipump was incubated with the same panel of drugs as used for chronic injections ($F(5,192)=31.07$, $p<0.0001$; Figure 2-16). *Post hoc* Dunnett's test showed that as previously seen (Figure 2-9) in HC choline had a significant effect on [^{3}H]-NA release (Figure 2-20A). Nicotine induced release in each of the treatment groups; however this was only significant in control animals due to a smaller SEM. The responses to 5IA were also similar in magnitude and sensitivity to tissue from naive animals (Figure 2-8). Although there were differences in basal [^{3}H]-NA efflux in the HC during withdrawal, chronic nicotine treatment or withdrawal did not have any significant effect on response to a variety of agonists expressed as % basal ($F(3,192)=2.627$, $p=0.0516$) and there was no interaction between *in vivo* and *in vitro* treatments ($F(15,192)=0.4022$, $p=0.9773$).

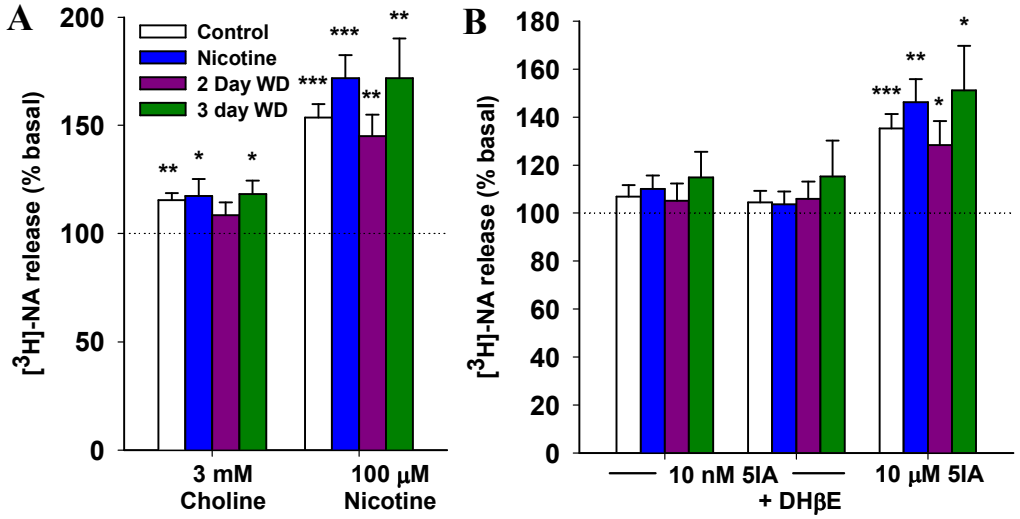


Figure 2-20 Effect of chronic nicotine treatment of hippocampal responses
Nicotine was administered at 4 mg/kg/day for 14 days via Alzet® osmotic minipump. After 14 days animals were sacrificed, or pumps removed and animals sacrificed 2 or 3 days later. [^{3}H]-NA release was stimulated from HC with a variety of agonists. Responses from control animals did not differ in any treatment regimen and so responses were pooled. In both panels control (white), 14 day nicotine (blue), 2 day nicotine withdrawal (dark pink), 3 day nicotine withdrawal (dark green) **A)** Responses to choline and nicotine **B)** Responses to 5IA. Bars represent the mean $\pm$ SEM from 6-7 experiments * $p<0.05$, ** $p<0.01$ compared to basal, # $P<0.05$ compared to agonist alone. Analysed by two way ANOVA with *in vivo* treatment and *in vitro* treatment as factors. In vivo $F(3, 192)=2.63$, $p=0.0516$, In vitro $F(5, 192)=31.07$, $p<0.0001$.

2.5. Results - Novel compound binding and release assays

2.5.1. PM and FA compounds

A number of novel compounds have been developed at the University of Bristol in the laboratory of Dr Tim Gallagher and are investigated here for ability to displace [^{3}H]-Epi binding at heteromeric nAChRs and also their ability to elicit [^{3}H]-NA or [^{3}H]-DA release from rat brain prisms. The PM and FA compounds presented herein (Figure 2-21) have side group substitutions at the C4 position of cytisine (Figure 2-1).

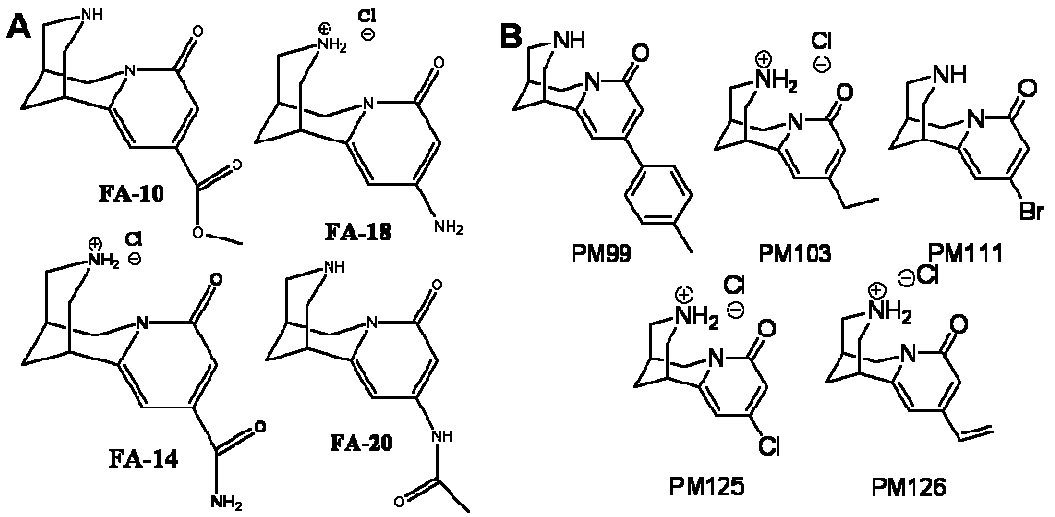


Figure 2-21 FA and PM compounds
Analogs of cytisine with various side group substitutions at C4 were synthesised A) FA series of compounds B) PM series of compounds.

2.5.1.1. [^{3}H]-Epibatidine Binding

P2 rat brain membrane homogenates were prepared by centrifugation and incubated with 200 pM [^{3}H]-Epi for 120 min with various concentrations of drug to be tested (see 2.2.5). Non-specific binding was determined in the presence of nicotine (1 mM).

All of the compounds tested were able to compete for nAChR sites labelled with [^{3}H]-Epi (Figure 2-22). Table 2-1 summarises the IC_{50} values for the FA and PM compounds as well as cytisine. From this it can be seen that all of the IC_{50} values for the FA series are higher than that of cytisine. This shows that substitutions at C4 reduce the ability of cytisine to compete with [^{3}H]-Epi for nAChR binding sites. For the PM series the IC_{50} values are again higher than for cytisine; however the most efficacious compounds exhibit similar binding to that of cytisine, suggesting that they may be of use as nicotinic ligands.

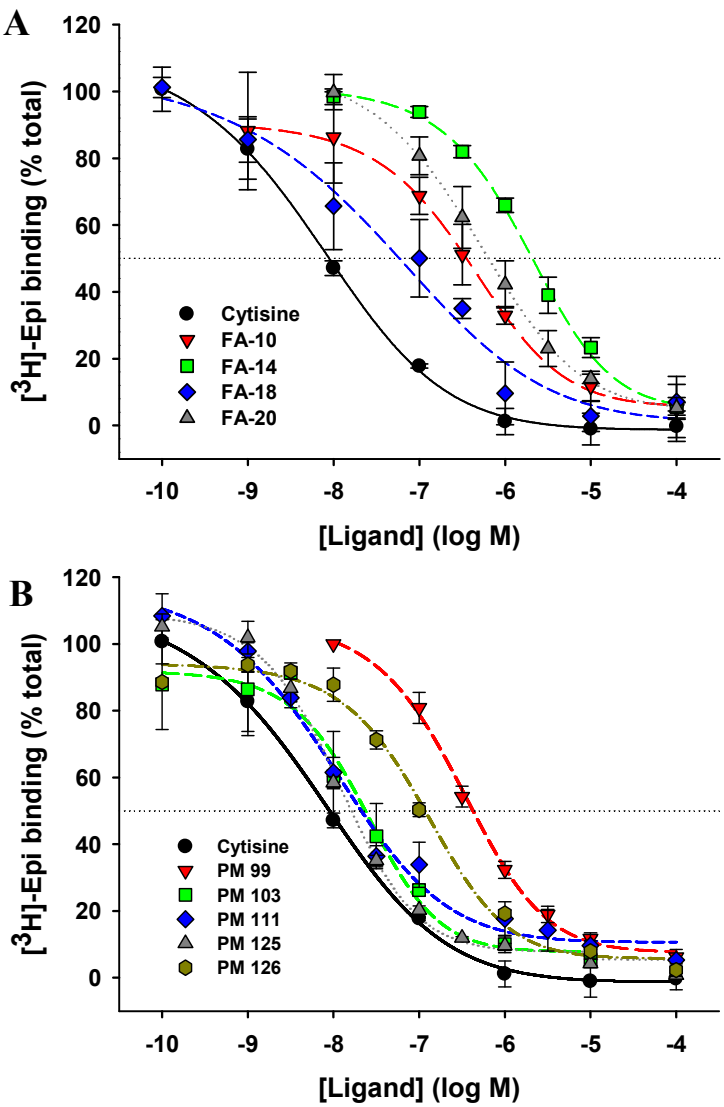


Figure 2-22 FA and PM series competition binding against [^{3}H]-Epibatidine
Rat brain P2 membranes were incubated with serial dilutions (10^{-4} to 10^{-10} M) of drug and 200 pM [^{3}H]-epibatidine. Nonspecific binding was determined in the presence of 1 mM nicotine. **A)** FA series of compounds. **B)** PM series of compounds. The same cytisine data set is shown on each panel.

Table 2-1 Summary of binding potencies of FA and PM compounds.
Rat brain P2 membranes were incubated with serial dilutions (10^{-4} to 10^{-10} M) of drug and 200 pM [^{3}H]-epibatidine. *** p< 0.001, n.s. not significant compared to cytisine. Analysed by non-linear regression with extra sum of squares F test to compare pIC50 values.

Compound	pIC_{50}	S.E.	IC_{50} (µM)	Sig
Cytisine	8.0	0.11	0.008	/
FA-10	6.4	0.19	0.040	***
FA-14	5.7	0.05	1.995	***
FA-18	6.9	0.16	0.398	***
FA-20	6.3	0.12	0.316	***

Compound	pIC_{50}	S.E.	IC_{50} (µM)	Sig
PM99	6.5	0.05	0.316	***
PM103	7.6	0.17	0.025	n.s.
PM111	8.0	0.08	0.01	n.s.
PM125	7.9	0.05	0.013	n.s.
PM126	7.0	0.05	0.126	***

2.5.1.2. Functional assays

Using the results from the binding assays a number of the FA and PM series were selected for functional testing. The [^{3}H]-NA release assay was used, to determine release from rat HC, in addition [^{3}H]-DA release from striatum was assessed for FA compounds. The FA compounds elicited relatively low release of [^{3}H]-NA from the HC (Figure 2-23A), with FA-18, FA-20 and PM111 eliciting significant release above basal at 100 µM. PM111 elicited a similar amount of release to cytisine, and so is likely to be a partial agonist of similar efficacy as well as similar potency. When tested over a larger concentration range, PM111 was the only compound causing significant [^{3}H]-NA release over basal at concentrations below 100 µM (Figure 2-23B EC_{50} 12.6 µM).

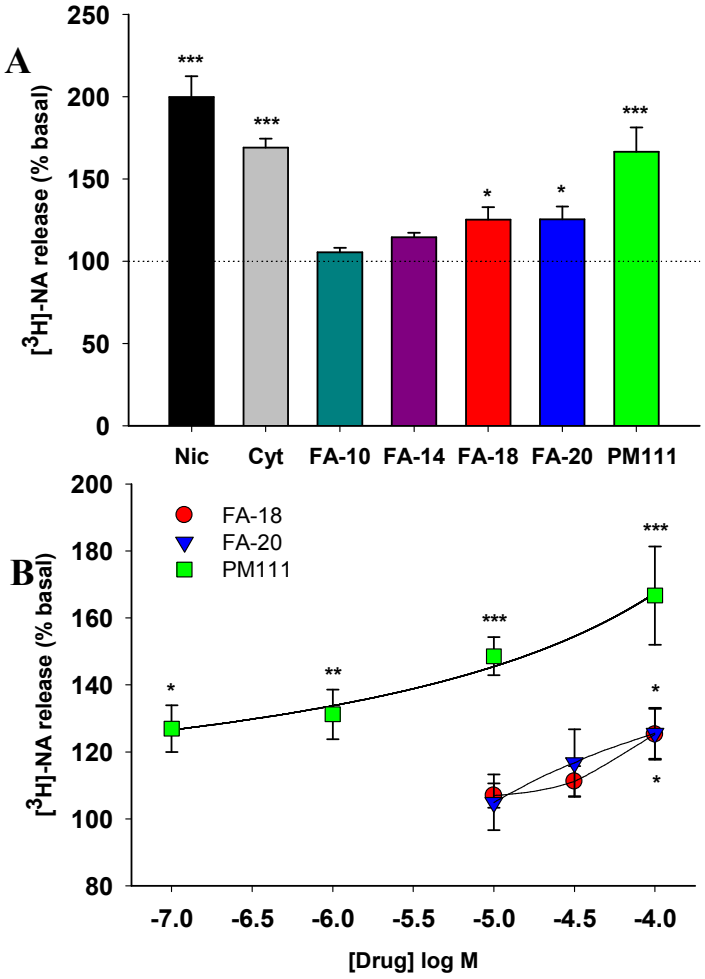


Figure 2-23 Effect of FA and PM compounds on [^{3}H]-NA release
Rat HC prisms (150 µm) were loaded with [^{3}H]-NA (70 nM) and release was stimulated by incubation for 5 min with selected compounds from the FA and PM series. **A)** FA and PM compounds (100 µM), with cytisine and nicotine to compare. Bars represent mean ± SEM of 4 experiments. * $p<0.05$, *** $p<0.001$ compared to basal. Analysed by one way ANOVA with Holm-Sidak *post hoc* test for multiple comparisons to control $F(7,27)=20.886$, $p<0.001$ **B)** Compounds that elicited significant release at 100 µM were incubated with rat HC prisms at a range of concentrations and [^{3}H]-NA release assessed. Points represent mean ± SEM of 3-4 independent experiments. * $p<0.05$, ** $p< 0.01$, *** $p<0.001$ compared to basal. Analysed by one way ANOVA with Holm-Sidak *post hoc* test for multiple comparisons to control for each compound. PM111 $F(4,18)=12.557$, $p<0.001$. FA-18 $F(3,15)=4.193$, $p=0.024$. FA-20 $F(3,15)=3.098$, $p=0.059$

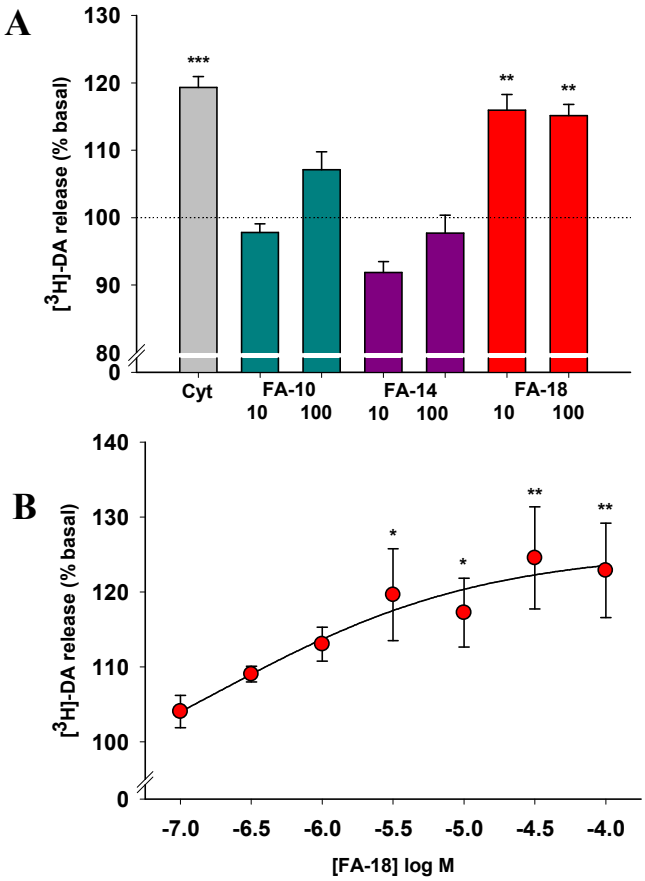


Figure 2-24 Effect of FA compounds on [^{3}H]-DA release
Rat striatum prisms (150 µm) were loaded with [^{3}H]-DA (50 nM) and release was elicited by incubation for 5 min with compounds from the FA series. **A)** FA compounds (10 and 100 µM), with cytisine (100 µM) as comparator. Bars represent the mean ± SEM of 6 replicates from one pilot experiment * $p<0.05$, *** $p<0.001$ compared to basal. Analysed by one way ANOVA with Holm-Sidak *post hoc* test for multiple comparisons to control $F(7,39)=19.957$, $p<0.001$ **B)** Release elicited by FA-18 (0.1 – 100 µM). Points represent the mean ± SEM of 3-5 independent experiments. * $p<0.05$, ** $p< 0.01$, *** $p<0.001$ from basal release. * $p<0.05$, ** $p< 0.01$, *** $p<0.001$ compared to basal. Analysed by one way ANOVA with Holm-Sidak *post hoc* test for multiple comparisons to control. $F(7,23)=3.791$, $p=0.007$ Experiments performed in our lab by Claire Thomas.

[^{3}H]-DA release from rat striatal prisms was also assessed for the FA compounds. In a pilot high concentrations (10 and 100 µM) of FA-10 and FA-14 failed to elicit any release of [^{3}H]-DA (Figure 2-24A). FA-18 elicited significant release at both of these concentrations, and so a wider concentration range was tested. FA-18 elicited [^{3}H]-DA release in a concentration dependent manner from striatal prisms reaching a maximal of 124.5 + 6.8 % basal at 300 µM (EC_{50} 1 µM; Figure 2-24).

2.5.2. Cytisine analogues

Aryl-cytisine, cyfusine and 4-F-cyfusine as well as a further C4 analogue of cytisine, 4-F-Cytisine are also reported (Figure 2-25). Cyfusine is formed when the bridged bicyclic framework of cytisine is fused into a tricyclic form, and 4-F-cyfusine is the subsequent halogenation at the C4 position. The addition of an aryl group, linked to the C3 and C4 of cytisine forms aryl-cytisine.

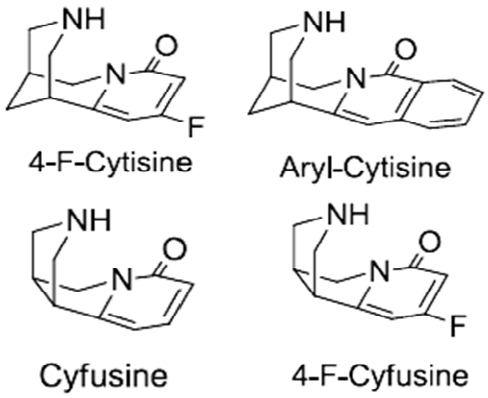


Figure 2-25 Cytisine analogs
Cyfusine and 4-F-Cyfusine have the bridged bicyclic structure of cytisine fused into a tricyclic structure, with a C4 substitution also in the case of 4-F-cyfusine. Aryl-cytisine is an arylation of the cytisine molecule linked to C3 and C4. 4-F-cytisine is a further C4 substitution of cytisine.

2.5.2.1. [^{3}H]-Epibatidine Binding

P2 rat brain membrane homogenates were incubated with 200 pM [^{3}H]-Epi for 120 min with various concentrations of the drug to be tested (see 2.2.5). Non-specific binding was determined in the presence of nicotine (1 mM). Cyfusine gave extremely poor displacement of [^{3}H]-Epi binding to rat brain membranes (Figure 2-26), and 4-F-Cyfusine did not displace binding within the concentration range tested suggesting that alterations to the ring structure of the molecule severely disrupts the ability to bind to nAChRs. 4-F-Cytisine and Aryl-cytisine however show 3 and 10 fold lower binding potency than cytisine respectively. Changes at the C4 position of the molecule have been suggested to play a role in selectivity. While this cannot be determined in these binding assays it can be seen that 4-F-Cytisine and Aryl-cytisine bind to the nAChR with potencies similar to that of cytisine, and so may be active at the receptor.

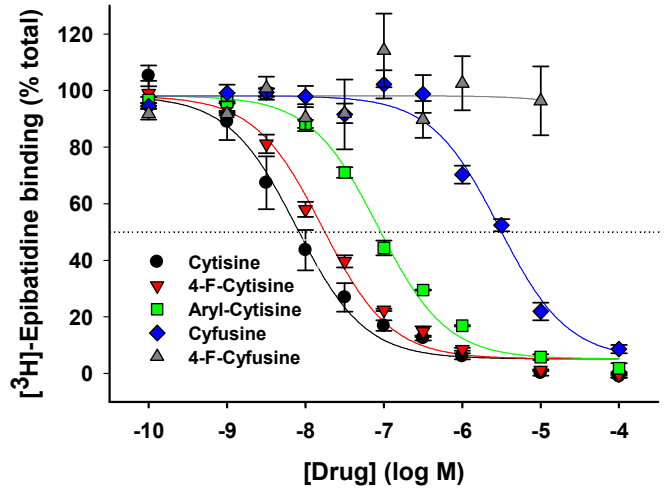


Figure 2-26 Competition binding against [^{3}H]-Epi for cytisine analogs
Rat brain P2 membranes were incubated with serial dilutions (10^{-4} to 10^{-10} M) of drugs to be tested and 200 pM [^{3}H]-epibatidine. Nonspecific binding was determined in the presence of 1 mM nicotine. Cytisine was also assayed as a comparison.

Table 2-2 IC_{50} values for cytisine analogs
Rat brain P2 membranes were incubated with serial dilutions (10^{-4} to 10^{-10} M) of drug and 200 pM [^{3}H]-epibatidine. *** p< 0.001. Analysed by non-linear regression with extra sum of squares F test to compare pIC50 values.

Compound	pIC_{50}	S.E.	IC_{50} (μM)	Sig
Cytisine	8.2	0.08	0.006	
4-F-Cytisine	7.8	0.05	0.017	***
Aryl-Cytisine	7.1	0.04	0.085	***
Cyfusine	5.6	0.12	2.8	***
4-F-Cyfusine	N.D.			

2.6. Discussion

In this chapter the subtypes of nAChRs involved in modulating noradrenaline release in FC and HC were compared *in vitro* by use of a [^{3}H]-NA release assay. The responses to a range of ligands varied between the two regions, suggesting the involvement of different nAChR subtypes. In particular there was much greater sensitivity to β2* nAChR selective compounds in FC than HC, which was found to be predominantly modulated by α3β4* nAChRs in agreement with previous reports. The interaction of GABA and cholinergic mechanisms on NA release was also investigated, showing that there is a potential $GABA_A$ mediated excitatory component to the nicotinic response. Several series of novel structural analogs of cytisine were also assessed for their ability to compete with [^{3}H]-Epibatidine for binding to nAChRs in rat P2 membranes showing a range of affinities. Selected compounds from these series were also assessed for their ability to release [^{3}H]-NA or DA and showed variable functional potency, with most behaving as partial agonists.

2.6.1. 96-well method critique

The 96-well method used in this study was first developed by Anderson *et al* (2000) to study catecholamine release from brain prisms. This assay has both advantages and disadvantages in comparison with other *in vitro* methods. The main advantage is the relatively high throughput of the technique, which allows 16 conditions to be assessed in 6 replicates during each experiment. This means that concentration-response curves can easily be constructed within a single experiment, often from two regions or ± antagonist, reducing animal usage and variability. In comparison superfusion systems usually have 12 chambers, meaning that fewer replicates and conditions can be examined simultaneously. The main disadvantage in comparison to superfusion is that this is a static assay, with only one drug-stimulated sample collected. This means that the timecourse of drug response or desensitisation is not determined. It has previously been shown that Ca^{2+} levels have

returned to baseline after 5 min of stimulation with nicotine (Gueorguiev *et al*, 2000), supporting the use of a 5 min sampling period.

The prism preparation also has advantages and disadvantages. Prisms have additional synaptic connectivity in comparison with synaptosomes, meaning that some interactions and indirect drug affects can be assessed. This is both an advantage and a limitation depending on the questions asked. In general synaptosomes are used when isolation of the presynaptic effects is required to determine, for example, the localisation of certain receptors. The results in section 2.3.2.6 show the importance of retaining some connectivity to examine the interactions between different neurotransmitter systems within brain tissue. A prism or slice preparation with an additional degree of complexity is a closer representation of *in vivo* tissue, although there will not be the full connectivity and intrinsic stimulations that would exist *in vivo*. As with any *in vitro* assay there will be unavoidable differences from the whole animal, but results from this assay have been found to be largely translatable (Livingstone *et al*, 2009).

Fractional release is calculated for each well, i.e. the neurotransmitter released is calculated as a percentage of the neurotransmitter in the well at the start of the release period. This eliminates variability that is an artefact of tissue loading. Data has then been presented as a percentage of fractional release in buffer alone (except where stated). This allows data to be normalised between animals as basal efflux varies but relative responses are usually similar.

2.6.2. Uptake specificity

Previous work (Carboni *et al*, 2001; Moron *et al* 2002) has shown that monoamine uptake is somewhat promiscuous, for example DA uptake occurs in part via the NET. This has been shown to be especially relevant in the FC (Williams and Steketee, 2004) where uptake via NET modulates FC DA levels substantially. Moron *et al* (2002) showed that in wild type mice DA uptake in FC synaptosomes was inhibited by nisoxetine but not GBR-12909, and that in NET knockout mice there was no effect of nisoxetine on DA uptake in FC synaptosomes. The importance of this difference in uptake will be further explored in section 3.7. In order to validate the [^{3}H]-NA release assay used in these experiments it was essential to ensure that the uptake of [^{3}H]-NA was specifically into noradrenergic cells via the NET. This is important in order to interpret the results accurately in a physiological context. Nomifensine and nisoxetine were both effective inhibitors of NA uptake (Figure 2-2). The selective DAT inhibitor, GBR-12909 (Andersen, 1989), did not inhibit uptake of [^{3}H]-NA, showing that uptake is specific to noradrenergic neurons. This result showed that there was no need to include a DAT inhibitor in [^{3}H]-NA uptake steps in release assays. GBR-12909 has also been shown to be an antagonist of nAChRs (Szasz *et al*, 2007), and

although there is some evidence that nomifensine can reduce release in superfused brain slices, it has been shown that this reduction is in carrier mediated, and not exocytotic, release at the concentration used in the current study (Kiss *et al*, 1997). Carrier mediated release is likely to be a non-specific process that would not be triggered at the agonist concentrations used within these studies and so the action of nomifensine on this is not likely to affect results. The inclusion of nomifensine to block reuptake of stimulated release is important in a static assay, as the neurotransmitter released from the tissue is not immediately moved away as is the case with superfusion. Nomifensine increases recovery and reduces variability (own work, data not shown).

2.6.2.1. Interaction with opiates

Many drug users abuse multiple drugs with around three times as many smokers in substance abuse populations as normal subjects (Stark and Campbell, 1993). To determine if there is a direct interaction between nicotine and morphine's actions in the brain the effect of morphine on [^{3}H]-NA uptake was assessed. The reversal by naloxone of the partial inhibition of [^{3}H]-NA uptake by morphine shows that this effect was mediated by opioid receptors, not by a direct interaction of morphine with the NET. When morphine was tested in release assays there was no change in [^{3}H]-NA release in buffer alone in the presence of nomifensine, showing that the reduction in [^{3}H]-NA accumulation is due to a change in uptake, not a concurrent weak releasing action. Responses to nicotine (10 or 100 μM), or KCl (15 mM) were also unchanged in the presence of morphine suggesting minimal direct interaction between the opiate and nicotinic systems in these conditions. *In vivo* it has been shown that NA reduces in response to acute morphine administration in naïve animals (Rossetti *et al*, 1993), in morphine pretreated animals tolerance to this developed, however naloxone caused an increase in NA levels that was not seen in naïve animals. The reduction in [^{3}H]-NA uptake seen here would be expected to translate to an increase in NA measured *in vivo*, so the reduction seen suggests that wider compensatory mechanisms are active *in vivo*. As there were no significant interactions with nicotinic responses in release assays opiates were examined no further.

2.6.3. Pharmacology in naïve animals

2.6.3.1. Nicotinic pharmacology

As nicotine is known to elicit [^{3}H]-NA release it was decided to further investigate these responses and the subtypes involved by the use of a range of selective nAChR agonists in both FC and HC. The responses are summarised in Table 2-3. [^{3}H]-NA release in response to many nicotinic drugs differed between the two brain regions.

Table 2-3 Responses in FC and HC to a range of nAChR ligands

	FC		HC	
Agonist	**EC_{50} (μM)**	**Antagonist**	**EC_{50} (μM)**	**Antagonist**
Nicotine	0.8	DHβE shift Mec block	12.6	No DHβE shift Mec block
Cytisine	3.9		21.4	
3-Br-Cyt	n.d.			
5IA	2.6 nM	DHβE shift to 33.9 nM	4.5	No DHβE shift 5.1
Choline	No response		Significant responses	Mec block No MLA block
Other α7	No response		No response	

HC responds as previously shown, with a significant release elicited by choline and no significant β2* nAChR component in responses elicited by nicotine. In contrast FC showed no significant choline response and a strong β2* nAChR mediated component to [^{3}H]-NA release. HC NA release has previously been shown to be modulated in large part by α3β4* nAChRs, with a contribution of α7 found in some studies but not in others (See 2.1.1). As there is no antagonism with MLA of choline mediated responses and no [^{3}H]-NA release elicited by the more selective α7 agonist ARR-17779 it is likely that the response seen here to choline is due to actions at α3β4* nAChRs. No α7 nAChR responses were seen in FC, which contrasts with the indirect effect of α7 nAChRs seen in FC for [^{3}H]-DA release (Livingstone *et al*, 2009). The presence of the α7 nAChR positive allosteric modulator PNU-120596 made no difference to responses with a range of agonists in either brain region, further confirming that α7 nAChRs are not involved in modulating [^{3}H]-NA release under the current conditions (data not shown). There is little or no response to choline in FC, suggesting a lack of α3β4* involvement in this region which is in contrast with the HC.

Dopaminergic responses in FC have previously been shown to be predominantly mediated by β2* nAChRs with a contribution from α7 (Livingstone *et al*, 2009) and so, given the lack of α3β4* or α7 responses, the role of β2* nAChRs in [^{3}H]-NA release in this region was investigated. In agreement with previous results (Sacaan *et al*, 1995) DHβE, a selective β2* nAChR antagonist, did not affect the response to nicotine in HC but was here found to have a significant effect on FC [^{3}H]-NA release, a further indication that the nAChR subtypes differ in FC from HC. Unlike Anderson *et al* (2000) DHβE was seen to give a rightwards shift of the concentration-response curve in FC, consistent with a competitive antagonism as seen for striatal DA responses.

5-Iodo-A85380 (5IA) was originally developed as a radioiodinated PET ligand derived from A-85380, but assays showed that its selectivity profile differed. 5IA has similar binding potency to epibatidine at α4β2*, however it has much lower binding potency at α3β4* sites. The binding is around 5,000 fold less potent at α3β4* than α4β2*,

and potency at α7 and muscle type nAChRs is even lower. The functional potencies follow a similar pattern with much greater potency at α4β2* than α3β4* nAChRs (Mogg *et al*, 2004). These large differences in potency of binding to the different nAChR subtypes allows 5IA to be used as a selective agonist for β2* nAChRs. FC had much more sensitive responses than HC to 5IA and these responses were competitively antagonised with DHβE (Table 2-3). The difference in potency in the two regions is nearly 2000-fold, suggesting that the subtypes involved are likely to be α3β4* in HC, as previously postulated, and a β2* containing subtype in FC. Although 5IA is highly selective for β2* nAChRs no further information about the subunit combination can be elucidated, although it is likely β2 is the sole *beta* subunit (2.6.3.2).

Responses to the other agonists tested here are consistent with the results already discussed giving strong evidence for differences in the subtypes involved in the two brain regions. If these differences are also found *in vivo* it may be possible to exploit them in order to target NA responses in selected brain regions.

2.6.3.2. Regional differences in nAChRs

The differences in nAChR subtypes involved in [^{3}H]-NA release between the two regions of the brain studied in these experiments may be due to differences in the expression of nAChRs. The noradrenergic neurons mainly arise from the locus coeruleus (LC) and there is evidence of two distinct populations as defined by mRNA expression levels (Lena *et al*, 1999). One population contain predominantly α3 and β4 (and also α6, β3, α5 and α4) mRNAs and the other α6 and β3 (and sometimes also α4 and α7) mRNAs. All cells tested contained β2 mRNA.

Although both of these neuronal populations contain β2 nAChR mRNA it may be that the subcellular distribution of receptors varies between the different cell populations. The β2* nAChRs on projections to HC arising from the LC may be somatic, and so not contribute to terminal field responses. This could lead to β2* responses in the terminal field (the responses measured by this assay) in FC but not HC.

High and low affinity subtypes of heteromeric receptors have been found, with the differing accessory subunit altering the functional profile (Nelson *et al*, 2003). The $(\alpha4\beta2)_2\alpha4$ stoichiometry has previously been shown to have lower agonist sensitivity but a higher efficacy than $(\alpha4\beta2)_2\beta2$. Although the channel of the nAChR is permeable to K^+, Ca^{2+} and Na^+ the differences in CA^{2+} permeability are the most important distinction. The high affinity receptor has a smaller total Ca^{2+} flux, and so a large proportion of cellular responses are mediated by the low affinity stoichiometries. It has recently been demonstrated that a homomeric receptor will open with just one binding site occupied, but that this opening is brief (1.3.4). If two binding sites are occupied (as for heteromeric

receptors) channel opening is stabilised. Occupation of 3 non-consecutive binding sites is required to give maximal channel open time (Andersen *et al*, 2011). Extrapolating this to heteromeric receptors suggests that the accessory subunit may contribute to function, and indeed an α4-α4 interface has been shown to contribute to channel function (Mazzaferro *et al*, 2011). The knowledge of an agonist binding site at the α4-α4 interface explains the ability of the receptor to display different responses with the two α-β interfaces remaining identical which is not well explained with a model of two agonist binding sites. The increased conductance found with α5 as the accessory subunit (1.3.4) would indicate that this subunit may also contribute to a third binding site, and so stabilise the open conformation of the receptor allowing increased Ca^{2+} influx. The necessity for two (or three) binding sites to be occupied to stabilise channel opening suggests that the perceived affinity to agonist of a channel is dependent on the lowest affinity binding site. In terms of the data presented in this chapter this suggests the possibility that the receptor subtype in HC could be complex, containing α3β4β2* combinations, but supports FC nAChRs involved in [^{3}H]-NA release containing β2 as the sole *beta* subunit. This would explain the lower affinity to 5IA in HC while not dismissing the fact that β2 nAChR mRNA has been detected in all cells tested in the LC, the source of the NAdrenergic stimulation of HC and FC. The lack of antagonism in HC by DHβE may suggest the lack of β2* expressed terminally on HC NAdrenergic neurons, however as DHβE is a competitive antagonist the inhibition of the putative β2* interface may be overcome by concentrations of agonist sufficient to activate the non-β2* interface and so the presence of complex receptor subtypes cannot be ruled out.

Overall FC NA release appears to be more similar to FC DA release than to HC NA release with β2* nAChRs involved in FC and α3β4* in HC. The roles of these different brain regions could therefore be targeted independently by use of selective agonists.

2.6.3.3. Importance of regional differences

As examined in the introduction the roles of the FC and HC are different, with FC involved in executive tasks, and HC in spatial tasks (see 1.1.1 and 1.1.2). Hahn *et al* (2003) found attention enhancing effects of nicotine that were mediated via PFC and not HC. This means that drugs that could selectively target one or other of these regions could have specific cognitive benefits. As many of the side effects of nicotinic drugs that have been trialled are thought to be regulated by the α3-containing nAChRs, this leaves β2* or α7 as suitable targets, suggesting FC tasks may be improved most by nicotinic stimulation. In fact several studies targeting these subtypes have found enhancement in FC regulated tasks. Both subtypes seem to improve performance on certain cognitive tasks, although the improvements are in different cognitive domains. β2* nAChRs improve attentional

performance (Guillem *et al*, 2011) whilst α7 is involved in procedural learning (Young *et al*, 2011). Mice with either the β2 or the α7 nAChR knocked out show cognitive deficits (see 1.4). It is not known if the cognitive effects of nAChRs are mediated by NA or by DA, or whether the modulation of both of these systems is important. There is evidence that NA is required for the attribution of salience to rewarding or aversive stimuli (Ventura *et al*, 2008). In the context of the work presented in this thesis this suggests that targeting β2* nAChRs may therefore be of benefit to enhance attentional processing.

Many cognitive deficits in humans are complex and it can be difficult to define a single aspect that is impaired. Both β2* and α7 nAChRs are useful potential targets. ABT-089 is a drug with an interesting selectivity profile. It is a partial agonist at α4β2* and α7, and weak partial agonist with low potency at α3β4* nAChRs, giving a substantially reduced side effect profile compared to nicotine (Rueter *et al*, 2004). In a trial in adults ABT-089 (40 mg once or twice daily) was effective in reducing ADHD deficits with minimal adverse effects (Apostol *et al*, 2011), however a trial in a paediatric population (6-12 years) showed no efficacy of ABT-089 at doses of up to 1.4 mg/kg (Wilens *et al*, 2011). No adverse effects of this dose were reported and so it may be that the metabolism of ABT-089 is faster in the paediatric population, leading to the lack of effect. ABT-418, another selective partial agonist for α4β2* nAChRs has also been trialled for ADHD (1.4).

Some cognitive enhancement has been found with drugs that selectively target the α7 nAChR. The development of selective agonists has been hampered by the high degree of homology between α7 and the 5-HT_3R, meaning that many α7 agonists have 5-HT_3 activity. Many α7 selective compounds have been tested in a wide range of cognitive tasks in several species with positive results on episodic and working memory and sensory gating, but mixed results for attention with improvement found in impaired (e.g. by ketamine or PCP) but not normal animals (reviewed in Wallace and Porter, 2011). The interaction between α7 ligands and $5HT_3$ receptors can cause side effects. Many α7 agonists act to inhibit $5HT_3$ receptors. $5HT_3$ antagonists act as antiemetics, but can also slow colonic transit, causing constipation (Stables *et al*, 1987, Talley *et al*, 1990). Interestingly varenicline has been shown to be an agonist at $5HT_3$, explaining some of the gastrointestinal side effects seen (Lummis *et al*, 2011). The effects at $5HT_3$, whether agonist or antagonist activity, make the development of effective and selective α7 ligands difficult. Positive allosteric modulators (PAMs) of α7 nAChRs have been developed, including PNU-120596. These molecules bind at a site distinct to the acetylcholine binding site and so could represent a novel line of investigation. PNU-120596 slows desensitisation of the α7 nAChR, thus increasing functional responses, and this effect is lost in a $5HT_3$/α7 nAChR chimera, suggesting that there would be no $5HT_3$ mediated side effects (Bertrand *et al*, 2008). As they do not cause direct receptor activation PAMs could be used to increase

signal to noise ratio upon endogenous cholinergic signalling, which would help to maintain efficacy of treatments which sometimes fail due to the development of tolerance. Overall these results suggest that the responses to α7 are in different domains than the β2* mediated effects and so combined treatments may be useful for some patient populations where cognitive deficits are across multiple domains. A risk factor for the use of PAMs is that by increasing the signal to noise ratio they may increase the response to nicotine. As the α7 nAChR is not involved in mediating the hedonic properties of nicotine (Grottick *et al*, 2000) a selective α7 PAM may not greatly raise the risk of increased nicotine use.

2.6.4. GABA

As previously determined (2.3.2.5) choline induces release of [^{3}H]-NA via α3β4 nAChRs, this release is partially sensitive to the $GABA_A$ antagonist bicuculline (2.3.2.6). Nicotine mediated release in FC and HC is also partially attenuated by bicuculline application. GABA release from mouse synaptosomes from a variety of brain regions can be elicited by α4β2 or α4β2α5 nAChRs (McClure-Begley *et al*, 2009). T-type Ca^{2+} channels can be activated by α3β4 nAChRs on synaptic regions of GABAergic interneurons, leading to the release of GABA (Tang *et al*, 2011). This suggests that a portion of the release of [^{3}H]-NA in the two brain regions is mediated indirectly by GABA. The administration of GABA elicited the release of [^{3}H]-NA from both FC and HC prisms and this was inhibited by bicuculline. The $GABA_B$ antagonist CGP54626 caused no reduction in GABA mediated [^{3}H]-NA release. The involvement of $GABA_A$ in neurotransmitter release has been observed before (Barik and Wonnacott, 2006; Leslie *et al*, 2002). As GABA is traditionally thought of as an inhibitory neurotransmitter, there arises the question of whether these responses are due to the inhibition of tonically active inhibitory interneurons, or due to a direct excitatory effect of GABA stimulation.

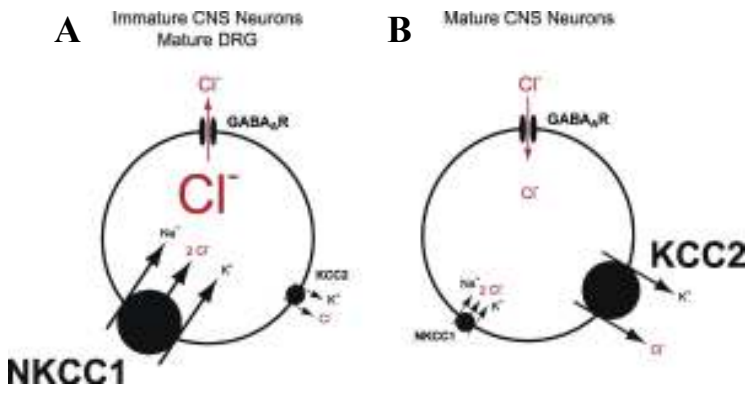


Figure 2-27 Developmental Regulation of Chloride Homeostasis in Neurons
The regulation of Cl^- gradients in **A)** immature and **B)** mature neurons. Dorsal root ganglion (DRG) cells have the gradient defined in **A)** The size of the lettering for the transporters depicts their relative importance, and the relative size for Cl^- depicts the gradient across the membrane. Reproduced from Stein and Nicoll, 2003 with permission.

During brain development there is a switch of chloride (Cl^-) cotransporter in neurons from the NKCC1, to the KCC2 (Plotkin *et al*, 1997; Stein and Nicoll, 2003). In development NKCC1 predominates to give high intracellular Cl^-, which means that upon $GABA_A$ activation Cl^- exits the cell, leading to depolarisation (Figure 2-27A). In mature neurons the KCC2 predominates to give low intracellular Cl^-, so receptor activation leads to influx of Cl^- and which supports hyperpolarisation upon receptor activation (Figure 2-27B).

NKCC1 has been observed in adult-born neurons suggesting that this difference in response is present until the individual neurons have matured. This means that adult-born neurons have an 'immature' excitatory phenotype. NKCC1 has also been shown to be important in the migration of neuroblasts during post-natal neurogenesis; however this may not be directly linked to its role in GABAergic signalling (Mejia-Gervacio *et al*, 2011). NKCC1 has also been shown in dendrites (but not somatic regions) in mature neurons, further suggesting a possible excitatory action of GABA (Romo-Parra *et al*, 2008, Khirug *et al*, 2008).

In order to test this theory, inhibitors of the NKCC1 and KCC2 Cl^- cotransporters were used (Figure 2-13). The NKCC1 inhibitor, bumetanide, reduced GABA mediated [^{3}H]-NA release showing a role for the developmental transporter form and supporting the excitatory GABA theory. Further work in other systems would be required to ascertain if this was due to dendritic NKCC1 or adult-born neurons (See 2.6.7).

The baseline effect of DIOA to elicit [^{3}H]-NA release in the absence of other stimulation (2.3.2.6) may suggest perturbation of the maintenance of the mature hyperpolarising gradient. This suggests that this is also an important component in the prism preparation. Together these results suggest that both excitatory and inhibitory GABAergic responses are occurring within the prisms from both FC and HC, consistent with a mixture of immature and mature cells, or dendritic vs. somatic actions of GABA. This fits with a review from Marty and Llano (2005) which discusses the complexity of the GABAergic system in terms balance between inhibition and excitation. The role of adult-born neurons in memory formation has been reviewed by Koehl and Abrous (2011). Selectively enhancing the function of these neurons may help enhance encoding of memories in neurodegenerative disorders. Inhibition of the reconsolidation of fear memories has been shown with a variety of neurotransmitter systems, including $GABA_A$ (Diergaarde *et al*, 2008) and may be a useful therapy in post traumatic stress disorder; however the exact mechanisms involved have yet to be elucidated. The $GABA_A$ mediated effects on [^{3}H]-NA release do not seem to differ between FC and HC, although responses and inhibition are slightly clearer in HC. It might be expected that the immature component of the GABA response would be larger in HC, as there is substantial neurogenesis in this region (Gross, 2000). The lack of distinction between these brain regions suggests that

dendritic GABA may be responsible for the excitatory component. The coexistence of excitatory and inhibitory GABA, with an overall inhibitory phenotype *in vivo* means that selective pharmacological targeting is difficult.

Another explanation for the effect of bumetanide is a direct inhibitory effect at $GABA_A$ receptors, as discussed by Russell (2000). As no effect was seen at concentrations below 100 μM this may be a possibility as only concentrations below 10 μM can be reliably determined to be selective for NKCC. This has led to the development of the NKCC cotransporter knockout mouse by Delpire *et al* (1999) however this mouse has a variety of developmental problems and so the relevance of information gained on neurotransmission by electrophysiology is limited. To the authors knowledge there is no conditional knockout for this gene. This lack of distinction between brain regions, when only the HC would be expected to contain developing neurons again suggests that bumetanide as used in these experiments may be acting more as a $GABA_A$ antagonist than NKCC inhibitor.

2.6.5. *In vivo* nicotine treatment

2.6.5.1. Single injection

The dose of nicotine administered within these assays (0.4 mg/kg) was high enough to induce locomotor depression, although this was not quantified. The time of tissue collection (30 min post injection) was such that nicotine in the brain should be near peak (peak is at 15 min, Turner, 1975). Singer *et al* (2004) show that brain nicotine levels are at 88 % of maximum at this time. There were no significant differences in the responses to any of the agonists tested between saline and nicotine treated animals. This suggests that acute receptor occupancy or desensitisation does not affect *in vitro* responses. The washing of the tissue may to some extent explain the lack of a significant difference in response to nicotine in the two groups as nicotine may have been washed out. Bullock *et al* (1994) show a development of tolerance to chronic nicotine treatment (7 days pretreatment *in vitro*), however the changes in nicotine response were quickly eliminated (~1 hour), suggesting that receptors can recover from desensitised states quickly, especially when undergoing washes. The lack of effect of a single nicotine treatment is useful information as it shows that acute desensitisation or receptor occupancy is washed out over the course of the assay, and unlikely to affect results, so any differences after chronic treatment are not due to these factors and so are due to changes in receptor expression or subunit stoichiometry.

2.6.5.2. Multiple injections

Nicotine (0.4 mg/kg) was administered to the animals once daily by s.c. injection for 14 days and tissue was collected 30 min after the final injection to produce maximal

brain concentrations as before. In this experiment there was no significant difference in the responses of tissue from nicotine and saline treated animals. The dose of nicotine administered was high enough to induce locomotor depression on initial days, although tolerance to this effect developed within 3-4 days suggesting chronic changes. Differences after repeated nicotine injection in response to a nicotine challenge on both locomotor activity and DA changes measured by microdialysis from PFC have been seen (Nisell *et al*, 1996). DA release in the mesoaccumbens, involved in locomotor stimulation and reinforcement of self-administration, is sensitised after repeated nicotine administration (Reviewed in Balfour *et al*, 1998). There are strain differences in sensitisation to repeated nicotine treatment, with evidence that Sprague-Dawley rats show a greater sensitisation than Lister hooded rats (Iyaniwura *et al*, 2001). After repeated nicotine administration as well as tolerance to the locomotor depression caused by acute nicotine a locomotor stimulant effect is seen (Stolerman *et al*, 1973) although Iyaniwura et al (2001) showed a disconnect between sensitisation of mesoaccumbens dopamine release and locomotor responses. An increase in operant responding for an audiovisual cue has been shown to be dependent on repeated nicotine exposure (Barrett and Odum, 2011), however it is unclear if this is a reward enhancement or due to the locomotor sensitisation caused by repeated nicotine administration. The sensitisation outlined above is centred on the dopaminergic system, with no evidence of sensitisation in NA release seen, although Barik and Wonnacott (2006) saw changes in NA release after nicotine administration, although this is during withdrawal from sustained rather than intermittent treatment.

There are no significant differences in [^{3}H]-NA release in response to nAChR stimulation between treated and untreated animals in either brain region. There are also no differences in responses to the range of nAChR ligands after 3 days of withdrawal in either brain region. It can be concluded that any changes caused by repeated nicotine injection are masked in this assay. The time period required for loading of [^{3}H]-NA, and the extensive washing required to ensure an accurate result may have meant that any differences caused by the repeated nicotine administration *in vivo* could not be observed in this *in vitro* assay.

The three day withdrawal time point was chosen on the basis of results from Barik and Wonnacott (2006) which show that changes in α7 nAChR function can be measured after 3 days of withdrawal, but without a concurrent change in receptor binding levels. The previous study used osmotic minipumps in order to deliver a constant dose of nicotine. The difference in results in the current study could therefore reflect differences between continuous and intermittent nicotine administration, as the whole of one dose is metabolised before the next administration, allowing desensitised receptors to revert to the resting state.

Constant infusion of nicotine (1 mg/kg/hour tartrate salt) has been shown to increase [^{3}H]-Epi binding to a greater extent than twice daily 2 mg/kg (tartrate) s.c. injection (Ulrich *et al*, 1997). Due to the differing total doses between injection and infusion studies it is often difficult to ascertain if differences in the responses are due to constant elevated plasma nicotine, or the total dose of nicotine being larger. Smokers maintain a steady state plasma level, as well as administering nicotine intermittently so both models of administrations have some experimental validity, but both also have shortcomings as they are simplified models. The minipump model has been used here to investigate if sustained elevated plasma nicotine concentrations *in vivo* are required to produce changes in response to nAChR agonists *in vitro*.

2.6.5.3. Minipumps

In this experiment nicotine (4 mg/kg/day) was administered via osmotic minipump. No differences were observed in agonist responses after chronic nicotine treatment or withdrawal (2 or 3 days). The profile of responses in the two brain regions was the same in control as treated animals, and these profiles did not differ from naïve animals. There was however an increase in basal [^{3}H]-NA efflux in HC after 2 days of withdrawal, basal responses did not differ at any other time or in the FC. Different methods of presenting results may have led to an apparent increase in NA release in response to agonist at this time across different treatments, hence percentage of buffer stimulated efflux is a good measure to use to normalise responses. An increase in NA in mice withdrawn from nicotine in drinking water was seen by Gaddnas *et al*, (2000) in post-mortem brain tissue homogenates. The difference in baseline efflux may be important as it could correlate to an increased HC NA level during nicotine withdrawal, something that may be linked to some of the signs of withdrawal such as anxiety. Somatic signs of withdrawal from nicotine infusion can be observed at 16 hours after withdrawal, but have largely subsided by 40 hours (Malin *et al*, 1992). This suggests that at the 48 hour timepoint used here, when a baseline change in NA efflux was seen, somatic signs will be minimal. Affective signs of withdrawal such as anxiety have been shown after spontaneous withdrawal. Intracranial self stimulation thresholds are elevated from 4 hours after spontaneous withdrawal, and this elevation remains significant for at least 4 days (Epping-Jordan *et al*, 1998).

There were significant changes in [^{3}H]-Epi binding after chronic treatment, which were maintained at 2 days of withdrawal, showing upregulation of nAChRs. An elevated nAChR binding was still seen at 3 days withdrawal; however this was no longer significant. A disparity between receptor binding and functional changes has been observed previously (Barik and Wonnacott, 2006; Jacobs *et al*, 2002). Recent work has suggested a role for nicotine as a pharmacological chaperone to increase the assembly and trafficking of

nAChRs to the cell surface (Lester *et al*, 2009; Srinivasan *et al*, 2011). It has already been shown that there is a change in stoichiometry after chronic nicotine treatment (Srinivasan *et al*, 2011). The high affinity $(\alpha4\beta2)_2\beta2$ stoichiometry is preferentially upregulated (Son *et al*, 2009), potentially leading to higher levels of binding but lower ion flux across the membrane (1.3.4), therefore explaining the ability of nAChRs to upregulate without increased functional responses.

[^{3}H]-Epi binding takes place on P2 brain membranes, and so represents both internal and external receptor pools. It is possible that receptor numbers are increased after chronic nicotine treatment, but that the number available at the cell surface does not change, hence the unchanged functional responses. Internalisation of receptors in this manner could be tested by labelling of the surface population and western blotting. Desensitisation of receptors whilst maintaining them at the cell surface could also occur; this would lead to a reduced pool of available receptors for functional actions but with increased surface binding.

2.6.6. Novel compounds and binding models

A series of cytisine analogs were synthesised in the lab of Dr Tim Gallagher. Previous cytisine analogs with halogenations at C3 and C5 have been reported with C3 halogenation tending to lead to greater binding affinity (Abin-Carriquiry *et al*, 2006; 2010a). C5 halogenations on the other hand tended to reduce the binding affinity.

The compounds reported here are a series of C4 substituted cytisine analogs, as well compounds formed by the fusing of the bridged bicyclic framework of cytisine (cyfusine) or the addition of an aryl group (aryl-cytisine). The binding data presented in 2.5 shows that many of the substitutions at C4 decrease the ability of cytisine compounds to displace [^{3}H]-Epi binding. [^{3}H]-Epi binding gives an indication of binding at heteromeric nAChRs; however the rat P2 membranes contain a mixed population so subtype selectivity cannot be ascertained.

Using the docking model outlined above (2.1.4.1) C4 of cytisine was not identified as a critical region for the interaction between ligand and receptor, and accordingly most of the changes in binding affinity are relatively small. N-methylation of cytisine has previously been shown to be detrimental to nAChR binding due to the disruption of a hydrogen bond between ligand and receptor (Tasso *et al*, 2009). The fusion of the bridged bicyclic structure of cytisine was developed as a possible way to retain the structural rigidity of cytisine and orient the key elements in the same manner, whilst allowing a simpler synthesis. As previously reported cyfusine has lower binding affinity to the nAChR (Yohannes *et al*, 2008) and this was shown also in the current study. A C4 substitution of cyfusine (4-F-cyfusine) was shown to have little or no ability to displace [^{3}H]-Epi binding

to the receptor. Arylation of cytisine reduced binding affinity approximately 10-fold, suggesting that there is some disruption to the interactions between C3 and the receptor molecule.

Several of these cytisine analogs bind well in the mixed heteromeric population present. Testing these compounds for the ability to displace radioligand binding in homogenates from specific brain regions or cell lines expressing known subunit composition would allow assessment of the selectivity. Many of the known cytisine derivatives are selective for α4β2* over α3β4* nAChRs, an important distinction thought to improve the side effect profile of nicotinic drugs. Improving this selectivity ratio could allow further use of these compounds as pharmaceuticals.

2.6.6.1. Functional assays

The [^{3}H]-NA release assay data presented (Figure 2-23) shows that only PM111 elicits a comparable amount of release to cytisine. Several of the other compounds exhibited similar binding profiles to PM111 and so the inability of these to elicit release may indicate an extremely weak partial agonist or an antagonist effect. [^{3}H]-DA release was elicited by FA-18, but minimally with FA-10 and FA-14 (Figure 2-24). Again this assay does not give a precise indication of subtype selectivity; however the differences between striatal DA and HC NA suggest that PM111 may activate α3β4* nAChRs and FA18 may be more selective for β2*.

Cytisine and related compounds have previously been shown to act as partial agonists and so it is likely that the compounds tested here will behave in a similar manner. The release elicited by these compounds is indeed smaller than for nicotine; however this may be due to lower potency as full concentration response curves have not been examined. For those compounds with no significant activity on [^{3}H]-catecholamine release which do however show [^{3}H]-Epi displacement it is likely that their action is as a weak partial agonist/competitive inhibitor. The coincubation of the compounds with a range of nicotine concentrations would allow assessment of the degree of antagonism. In addition to smoking cessation another area of possible use of partial nAChR agonists is for treatment of ADHD and depression (1.4). If any of the compounds here has additional selectivity for α4β2* nAChRs over α3β4*, or could separate effects at α7 nAChRs from those at 5-HT$_3$, then they could represent a better tolerated pharmacological agent. This system is not ideal for such comparisons and expression of nAChR subunit combinations in oocytes and testing of the drugs for function would be a more accurate and reliable way of assessing this.

2.6.7. Further work

2.6.7.1. nAChR pharmacology in FC and HC

Investigations into the pharmacology of the responses in the two regions could be expanded with the use of selective antagonists e.g. α-conotoxins to determine if more complex subtypes of nAChR are involved.

Confocal microscopy on slices colabelled with antibodies for specific nAChR subunits and dopamine-β-hydroxylase could show the subtypes of nAChR expressed in noradrenergic terminals. This may be difficult as some nAChR subunit antibodies, especially for α7, show cross-reactivity with other subunits; however this can be overcome thanks to the development of a gold-conjugated α-Bgt (Jones and Wonnacott, 2004). Colabelling of nAChR subunit antibodies or labelled α-Bgt with markers for dopaminergic, serotonergic and glutamatergic neurons has already been shown (1.3.2).

2.6.7.2. GABA

Electrophysiology in acute slices could be used to confirm the excitatory mechanism of action. Romo-Parra *et al* (2008) showed a shift from depolarising to hyperpolarising responses to GABA during neuronal development was slower in apical dendrites than in somatic regions and basal dendrites. Repeating these experiments in tissue from older animals would allow the assessment of the spatial restriction of depolarising GABA responses at later timepoints although responses to GABA stimulation were seen to reduce through early postnatal stages, suggesting that fewer neurons are responding in an excitatory manner.

2.6.7.3. Treated

After chronic nicotine treatment withdrawal could be precipitated by injection of mecamylamine. This gives a stronger withdrawal syndrome and a shorter withdrawal timecourse than simply removing the osmotic pumps and so changes in basal [^{3}H]-NA release could be assessed. We would expect this to cause a larger change than spontaneous withdrawal and there may be a correlation between this elevated NA baseline and withdrawal symptoms such as anxiety (as measured by reduced open arm time on the elevated plus maze for example) if these are modulated by the HC. It may also be the case that a shortened withdrawal timecourse allows changes to be seen in basal NA in the FC. This may reveal differences in nAChR modulation of [^{3}H]-NA within the preparations.

The observation that basal HC NA release is elevated *in vitro* during nicotine withdrawal would be interesting to test *in vivo* by the use of microdialysis. The changes are small, and so this would only be feasible to test if mecamylamine increases the size of this response and shortens the timecourse.

2.6.7.4. Novel compounds

The novel compounds reported here could be tested for the ability to displace the binding of α-Bgt to ascertain binding at α7 nAChRs. This has been done for the FA series of compounds, with all of the compounds showing binding in the μM range (53-84 μM) as opposed to the nM range for heteromeric receptors. This suggests similar selectivity for heteromeric over homomeric receptors as cytisine, and is likely to be the case for the other cytisine compounds presented here.

To further test the functional profile of these novel compounds their actions as partial agonists or competitive antagonists could be assessed by coincubation with a maximally effective concentration of nicotine. Effects on both HC NA and striatal DA would be tested as the responses may differ depending on the subtype selectivity of the compounds.

To further test the selectivity of the compounds that show activity the electrophysiological responses caused in oocytes expressing different subunit combinations could be examined. Changes in the selectivity of the compounds in comparison to cytisine may correlate with the changes made to the molecules and so may help inform future drug development in combination with docking models.

2.6.8. Conclusions

Overall the results presented in this chapter indicate that the subtypes of nAChR involved in the release of [^{3}H]-NA are different between FC and HC. An increase in [^{3}H]-Epi binding was seen after chronic nicotine treatment which was not accompanied by a change in functional responses either in magnitude nor sensitivity to a range of selective agonists. Acute nicotine treatment also had no effect on *in vitro* responses, suggesting a rapid recovery from any receptor desensitisation. This suggests that *in vivo* models may be better suited for testing the effects of chronic nicotine administration.

There is also the suggestion in the data presented that a proportion of the nAChR mediated [^{3}H]-NA release is via excitatory $GABA_A$ receptor stimulation, although this needs to be further elucidated in other systems.

Selective stimulation of certain regions of the brain with selective agonists is a real possibility, so docking models that allow more accurate prediction of the binding and functional potency may allow the design of better drugs. The docking model shown here is to a certain extent validated by the binding work presented, however the functional assays need to be extended to allow assessment of the predictive ability of the model, especially in terms of subtype selectivity.

The differences in nAChR subtypes involved in [^{3}H]-NA release between FC and HC are important as the function of these regions differs and so it may open a route for

pharmacologically selective treatments for cognitive conditions. *In vitro* work, however, does not always translate into the clinical setting and so ascertaining if the subtypes of nAChR involved also differ between brain regions *in vivo* is an important next step in the development of this work.

Chapter Three

3. NAChR modulation of monoamine release *in vivo*

3.1. Introduction - Microdialysis

The results outlined above (Chapter 2) show that there are differences *in vitro* in the nAChR subtypes involved in [^{3}H]-noradrenaline release between frontal cortex (FC) and hippocampus (HC). *In vitro* results however may not be translatable to *in vivo* situations due to the dissociation of tissues and isolation of brain regions from modulatory inputs. To measure changes in neurotransmitters in response to drug administration *in vivo,* microdialysis can be used.

3.1.1. Intracerebral microdialysis

In this section the limitations and advantages of *in vivo* microdialysis coupled to high performance liquid chromatography with electrochemical detection (HPLC-ECD) for sampling and detection of monoamines will be examined. *In vivo* microdialysis coupled to HPLC is a well established technique to measure extracellular neurotransmitter concentrations in the rodent brain, having been used to measure responses to nicotine as early as the 1980s (Damsma *et al*, 1988) as well as use in many studies over the years into a variety of systems. Simply, a concentric microdialysis probe (Figure 3-1) is implanted into the brain region of interest and continuously perfused with artificial cerebrospinal fluid (aCSF). Neurotransmitters diffuse across the semipermeable membrane portion of the probe and are collected in the aCSF flow and HPLC-ECD is used to analyse samples. This is a highly sensitive method that can detect small amounts of electro-active compounds by oxidisation on a glassy carbon electrode. The amount of compound in the sample is proportional to the area of peak on a chromatogram (see Appendix 3 for examples).

The advantages of this technique are that the sampling takes place continuously across the timecourse of a response from a conscious, freely moving animal with minimal manipulation during the sampling period. The effect of modulatory pathways from elsewhere in the brain can be examined and clearance and production of neurotransmitters (NTs) will be occurring, unlike in dissociated tissue *in vitro*. The main limitations are temporal resolution, sample recovery and detection sensitivity. These are interlinked, as a faster flow rate (better temporal resolution) will lead to poorer sample recovery and so the need for more sensitive detection techniques. These factors will be discussed in more detail below.

3.1.2. Development of microdialysis

Hernandez *et al* (1983) is the earliest report of the development of intracerebral dialysis in rats, showing increases in DA after amphetamine administration. This method was an improvement on earlier push-pull techniques as it minimised brain distension,

however the probe was somewhat larger than those used for push-pull extraction as the in and out flows were in parallel tubes in a U-shape formation. Horizontal probes were also developed, which consist of a length of dialysis tubing implanted horizontally through the region of interest (e.g. Benveniste and Diemer, 1987). The development of concentric probes (Hernandez *et al* 1986) meant that minimal tissue disruption was caused upon implantation, while also allowing more precise targeting than side-by-side or horizontal probes. A diagram of a concentric probe as used in the present study is shown (Figure 3-1). The microdialysis probe can be very accurately placed within selected structures of the brain by the use of stereotaxic coordinates; however there is still some limit on the spatial resolution due to the diameter of the probe (0.6 mm).

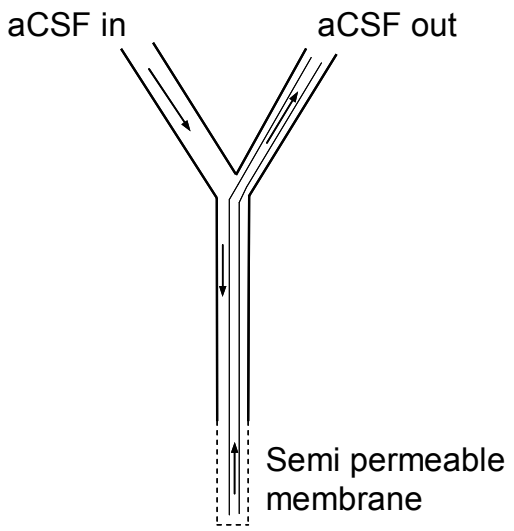


Figure 3-1 Cross section of concentric microdialysis probe
The microdialysis probes used in this study consist of a rigid Y shaped structure, with a concentric central arm with a diameter of 0.6 mm. The base of the probe is comprised of a semipermeable membrane with a molecular weight cut off of 14 KDa, across which neurotransmitters can diffuse. This allows sampling of neurotransmitter levels without alteration of the cerebrospinal fluid (CSF) volume. Artificial CSF flows into the probe, past the semipermeable membrane and is then pushed back up the probe to the outlet where it can be collected.

3.1.3. Timing of microdialysis sampling

Microdialysis can be undertaken in anaesthetised or awake animals. While the anaesthetised animals can be used immediately a study by Westphalen *et al* (2009) shows that isoflurane, even at subanaesthetic concentrations, has an effect on nicotine induced hippocampal NA release (from isolated synaptosomes). Anaesthetics bind to a site within the plasma membrane domain of many pentameric ligand-gated ion channels, including the nAChR where they are inhibitory (Nury *et al*, 2011). As well as direct effects of anaesthetics on nAChRs there are likely to be global changes in the anaesthetised vs. awake brain. Therefore the use of unanaethesised animal is important to give an accurate picture of the changes happening in response to drug administration. This also allows the technique to be combined with behavioural measurements, a successful correlation of locomotor activity

and DA increases after amphetamine administration has recently been demonstrated (Jackson *et al*, 2009). Attempts have also been made to correlate behavioural task performance with dialysate changes (e.g. Ostlund *et al*, 2011); however the temporal resolution limits the conclusions that can be drawn (3.1.5).

Another major consideration is that the probe is a foreign body which causes damage to the surrounding tissue upon insertion which may lead to neurochemical perturbations. Benveniste and Diemer (1987a) used histological techniques to assess reactions at varying timepoints to a microdialysis fibre implantation. At 1-2 days post implantation minimal changes were observed in the tissue with a heterogeneous distribution of small areas of oedema and some minor haemorrhage. On later days haemorrhage and oedema decreased, however phagocytic cells and increased glial fibrilary acidic protein were observed. Another paper from the same group (Benveniste *et al*, 1987b) showed that local cerebral glucose metabolism and blood flow are disturbed during the two hours following surgery, and these disturbances have normalised by around 24 hours after surgery. Combining these factors suggests that around 24 hours after surgery is the best time to perform microdialysis experiments and so in these studies microdialysis was performed the day following surgery (3.3.2). Animals were placed into the microdialysis chambers and connected to the perfusate flow immediately after surgery and allowed to recover and acclimatise overnight.

3.1.4. Sampling methodology

Due to the continuous flow of perfusate the level of neurotransmitter will never equalise to that in the surrounding tissue. The amount that can be assessed is known as the recovery of the probe. Factors affecting the recovery include the dimensions of the active membrane area, molecular weight cut-off of the membrane, the diffusion of NTs through the extracellular space, the flow rate of the perfusate and the charges associated with molecules (Stenken, 1999). Recovery can vary between probes of identical design and manufacture, this is more common with probes made ‘in house’ than with commercially available probes and so although there is a large cost difference the decision was made to purchase probes for these studies to minimise variability.

In vitro compared to *in vivo* recoveries are postulated to be different due to the influence of composition of tissue *in vivo* on the creation of diffusion gradients (Cano-Cebrian *et al*, 2005). The volume from which sampling takes place *in vivo* is smaller than *in vitro* as it is only from the extracellular volume. The presence of cells also means that the diffusion path is extended. The diffusion is difficult to quantify as the tissue composition and drug metabolism may be important factors. Keeping to the same probe type minimises the variation in recovery caused by membrane area and composition. The factors outlined

above also affect the delivery of drug across the probe. Although quantifying absolute recovery is difficult, the relative changes in neurotransmitter levels can be determined removing the need to quantify recovery *in vivo*. The issue of recovery is further discussed below (3.6.1.3).

3.1.5. Sample analysis

The collection of small, dilute samples means that sensitive detection techniques are needed. HPLC-ECD has been used for many years for the determination of monoamine NT levels in samples. The main issues are chromatogram separation, sensitivity of peak detection and sample throughput.

To increase sample throughput faster flow rates through the HPLC system can be used, however this impacts negatively on peak separation. Separation of peaks is also affected by the buffer composition and temperature. The usual factors altered to optimise peak separation are pH and the amount of ion-pairing agent (in these studies OSA). The solvent (methanol) content of the buffer can also be altered. Changing the methanol or OSA content tends affect all peak times, with those that are retained longer being changed by a larger amount than those early in the separation. The effect of changing pH is dependent on the molecule; there is a much bigger effect on acids or bases of pH manipulation than on neutral molecules which allows peaks to be moved in relation to one another not just spread further over time.

Good peak separation improves the ability to detect small amounts of the compound of interest however the limit of detection of ECD is still an issue in analysing the samples derived from microdialysis. The development of more sensitive detection methods has been useful in alleviating this limitation. The use of mass spectrometry in the analysis of microdialysates is becoming more common as it analyses the molecular weight of compounds and so removes the barrier of interfering peaks, although ECD is still the preferred method due to much wider availability. Mass spectrometry will not be further covered here, see Zhang and Beyer (2006) for a review.

3.1.5.1. Alternatives to microdialysis

The main *in vivo* alternative to microdialysis allowing better temporal resolution is cyclic voltammetry. In this technique carbon fibre electrodes are inserted into the selected brain sites and oxidisation occurs on the surface of the electrode. This technique has better spatial (fibres measure 5-30 μM diameter) and temporal (second scale) resolution than microdialysis, but is less sensitive and is limited to electroactive molecules. A review of this technique is found in Robinson *et al* (2003).

3.1.6. Nicotine in microdialysis studies

Nicotine has been used in a number of microdialysis studies, mostly looking at the effect on DA levels in dopaminergic areas such as striatum and nucleus accumbens (Mifsud *et al* 1989; Benwell and Balfour, 1992; Nisell *et al* 1994; Wonnacott *et al* 2000). There are fewer studies on the effect of nicotine on NA; many of these have focussed on the HC although other regions have also been examined.

Systemic nicotine

In HC acute nicotine has been shown to increase NA when administered systemically by i.p. or s.c. injection (Singer *et al* 2004; Brazell *et al*, 1991; Mitchell, 1993). Singer *et al* (2004) used fluoxetine or desipramine in the perfusion buffer to decrease neurotransmitter reuptake in some experiments in order to increase detection. This may in itself have caused increases in neurotransmitter levels, but uptake inhibitors were included in the perfusion buffer for 2 hours before sampling to allow baseline stabilisation and remained throughout the experiment. The inclusion of reuptake inhibitors in the perfusion fluid is now considered to be scientifically invalid, as it compromises neuronal function (discussed in Borland *et al*, 2005).

When administered systemically nicotine increased NA in FC (Liang *et al*, 2008; Rossi *et al* 2005; Singer *et al* 2004; Summers and Giacobini 1995) although the increase was in general smaller than that of DA. Summers and Giacobini (1995) found the greatest increases in NA at 1.2 mg/kg nicotine; this high dose induced seizures and so is untenable. However, significant changes in NA, DA, ACh and 5HT were found at 0.2 mg/kg in the same study. Studies using antagonist (typically mecamylamine) have confirmed that the responses after nicotine in both FC and HC are nAChR mediated.

Local administration

Drug can also be administered locally via the microdialysis probe (referred to as reverse dialysis). This method will be used in this study to mirror the *in vitro* experiments. When administered locally nicotine (1 μM via the probe) gave an increase in NA in both FC and HC, however the same administration gives a rise in DA in FC and a decrease in the HC (Shearman *et al* 2005). Marshall *et al* (1997) showed that nicotine administered locally to FC, striatum and nucleus accumbens (1-10 mM) elicited DA release with the FC being less sensitive. Possible reasons for the requirement of much higher concentration of nicotine in the probe than in Shearman *et al* (2005) are discussed below (3.6.1.3).

Other studies

A few studies have failed to find changes in NA levels in HC or FC e.g. Rossi *et al* (2005) with 0.3 mg/kg systemic nicotine. Kawahara *et al* (1999) infused nicotine into LC and found no change in PFC NA. Toth *et al* (1992) administered nicotine locally to striatum, substantia nigra, cerebellum, hippocampus, cortex (frontal, cingulate), and pontine

nucleus, and found no change in FC, HC, cerebellum or striatum but saw rises in NA in substantia nigra, cingulate cortex, and pontine nucleus.

Subtype selectivity

Relatively few studies have investigated the effect of selective nAChR agonists or antagonists on NA release. Those that have seem to suggest a role of both homomeric (α7) and heteromeric receptors in mediating the responses in HC (Fu *et al*, 1999a; 1999b).

3.1.7. Study design

The studies outlined above have confirmed a role for nAChRs in modulation of HC and FC NA levels *in vivo*, however nicotine is a non-selective agonist that is does not discriminate markedly between nAChR subtypes (Figure 2-5). In view of the large differences in sensitivity *in vitro* to 5IA between FC and HC it was decided that this was a good candidate with which to study the nicotinic responses operating locally *in vivo* and determine if differences are apparent. Previously 5IA has been used in one microdialysis study from this laboratory (Livingstone *et al,* 2009), showing robust increases in DA in the PFC at the concentrations tested (100 μM and 1 mM).

3.1.8. Aims and Hypotheses

The microdialysis experiments contained in this study aim to elucidate differences in the nAChR subtypes involved in NA release between FC and dorsal HC *in vivo* upon local stimulation. This will be done through local administration of the β2* nAChR selective agonist 5IA with the hypothesis that FC will be more sensitive to 5IA but that maximal responses will not differ between the regions, in line with the results from *in vitro* work.

These experiments also aim to confirm that the 96-well plate *in vitro* release assay gives translatable responses to aid in defining questions to study by microdialysis *in vivo*.

3.2. Introduction - Neurochemical fingerprinting

Increased neurotransmitter levels can be produced by several mechanisms in the brain such as increased release, reduced reuptake or reduced breakdown. The profile of metabolites (the 'fingerprint') differs for drugs that target these different mechanisms of action, although changes in NA or DA levels may appear the same when measured by microdialysis (Heal *et al*, 2009). For example, a monoamine oxidase inhibitor will reduce the breakdown of DA to DOPAC and 3MT to HVA thereby increasing the level of 3MT. A drug that prevents uptake of dopamine will increase synaptic DA levels, impede intraneuronal catabolism of DA to DOPAC, but have no effect on the extraneuronal catabolism of this monoamine, reflected by no change in HVA. The different mechanisms

of action may affect the abuse liability of a drug. The major metabolic pathways for DA, NA and 5HT are shown in Figure 3-2.

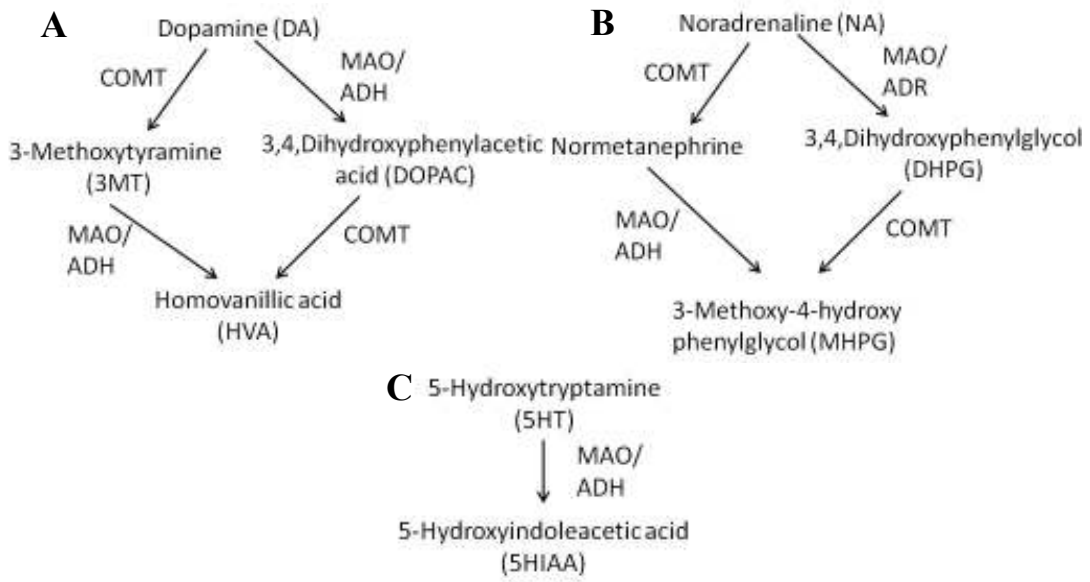


Figure 3-2 Major metabolites of Noradrenaline, Dopamine and Serotonin
The major metabolic pathways in the breakdown of **A)** Dopamine **B)** Noradrenaline and **C)** Serotonin within the CNS. MAO Monoamine oxidase; ADR, Aldehyde reductase; ADH, Aldehyde dehydrogenase; COMT, Catechol-O-methyl transferase

For this method animals are injected with drug and killed after a set period of time (Tani *et al*, 1997; Heal *et al*, 2009). The selected brain regions are rapidly dissected and frozen to prevent post-mortem changes and then homogenised and centrifuged. Samples of supernatant are analysed by HPLC-ECD with reference to an internal standard contained in the homogenising solution to correct for sample degradation.

3.2.1. Advantages and limitations

Neurochemical fingerprinting allows the assessment of a range of neurotransmitters and their metabolites at a single timepoint after drug administration. The analysis of a wider range of compounds than from microdialysates is made possible due to the higher levels found in homogenates. The analysis of multiple compounds is an advantage over microdialysis as it gives a picture of the metabolic changes occurring as well as the simple changes in e.g. NA. The limitation to a single timepoint means that the timecourse of a response to drug is more difficult to assess. A timecourse can be examined by conducting analyses at different timepoints in separate groups of animals. The measurement of the compounds is from homogenised tissue, which means that it is the entire content not just extracellular or intracellular, and a whole brain region is used so the spatial localisation is not as good as with microdialysis. Administering the drug *in vivo* means that metabolic pathways are intact, whereas *in vitro* with dissociated tissue this may not be the case.

3.2.2. Previous work

A previous study (Tani *et al*, 1997) used similar methodology to assess the effect of nicotine on a range of monoamines and metabolites. They found that administration of 1 mg/kg nicotine increased 5-HT, MHPG and DOPAC, but had little effect on NA or DA in homogenates of whole mouse brain. There was a small but non-significant decrease in 5HIAA levels. These changes were reversed with mecamylamine but not hexamethonium, a peripherally acting antagonist. The lack of response in total NA and DA levels with a rise in their metabolites suggests increased turnover, with production matching the level of breakdown. Chronic nicotine decreased NA and 5-HT in FC with no significant effect on 5HIAA, DA or HVA (Kirch *et al*, 1987). DOPAC was decreased in striatum but no other changes were seen in this region. This suggests that after chronic nicotine treatment there is desensitisation to the releasing effects, and so reduced monoamine turnover. Administration of a monoamine oxidase inhibitor (MOAI) has been used as a positive control. Previously MOAIs have been shown to increase dopamine and decrease DOPAC, HVA and 5HIAA in striatum (Iurlo *et al*, 2001). As well as decreases in DOPAC and HVA, Heal *et al* (2009) show that MAOI administration increases 3MT in striatum.

3.2.3. Study design

The effect of nicotine on DA and 5-HT metabolism and NA levels has been investigated here. The range of metabolites investigated differs from Tani *et al* (1997) who observed NA, MHPG, DA, DOPAC, 5-HT and 5HIAA. In the present study NA, DA, DOPAC, HVA, 3-MT, 5HIAA and 5-HT were assessed. This gives a more detailed picture of DA metabolism. A refinement to Tani *et al* (1997) is the dissection of striatum and frontal cortex selected. These regions were chosen as they are expected to differ in their monoamine content and release monoamine in response to nAChR stimulation. The half life of nicotine is known to be very short in mice (~6.9 min Petersen *et al*, 1984) however the timecourse of response seen by Tani *et al* (1997) showed significant responses up to an hour after nicotine treatment. Therefore, a pilot study to determine the optimal timepoint was conducted. Mice are less sensitive to the acute effects of nicotine than rats (see Matta *et al*, 2007) although whether this is purely a pharmokinetic effect is unknown. Doses of 0.3, 1 and 3 mg/kg were chosen based on the 0.04-5 mg/kg range in Tani *et al* (1997), and suggestions of dosing in Matta *et al* (2007).

3.2.4. Aims and hypothesis

These experiments aim to investigate nAChR mediated changes in catecholamine metabolism *in vivo* as measured from brain homogenates. The hypothesis is that nicotine will increase the metabolism of DA and NA by increasing release in both regions, with a larger effect detectable in striatum for DA due to the higher DA content of this region.

3.3. Materials and methods

3.3.1. Chemicals and Reagents

5-Iodo-A85380 (5IA) was purchased from Tocris Cookson (Avonmouth, UK). Tranylcypromine HCl, noradrenaline (NA), 3,4-dihyroxyphenylacetic acid (DOPAC), 3,4-dihydroxybenzylamine HBr (DHBA), 3-methoxytyramine HCl (3-MT), 5-hydroxyindoleacetic acid (5-HIAA), serotonin creatinine sulphate (5-HT), (-)-nicotine hydrogen tartrate, dopamine HCl (DA), homovanillic acid (HVA) and all other standard chemicals of analytical grade were obtained from Sigma-Aldrich Co.

3.3.2. Microdialysis

3.3.2.1. Animals

Male Sprague-Dawley rats were purchased from Charles River Laboratories (Margate, Kent, UK). They were group housed (4-6 per cage) with *ad libitum* access to food and water until the time of surgery. Animals weighed 280-320g at time of experiments. Lights were on a 12 hour cycle, with lights on at 8 am. All procedures were in accordance with the Animals (Scientific Procedures) Act of 1986.

3.3.2.2. Probe implantation

Animals were anaesthetised with isoflurane (5 % for induction; 2.5 % maintenance; O_2 1 L/min) and the head shaved and disinfected. They were then placed into a Kopf stereotaxic frame with the incisor bar set at -3.3 mm from the interaural line. An incision was made to reveal bregma from which coordinates were taken. Holes were then drilled for the placement of two anchor screws and a concentric microdialysis probe with 2mm membrane into FC or dorsal HC (MAB9.14.2, Microbiotech; coordinates of placement FC AP +3.2, ML +2.5, DV -4.0 mm.; dorsal HC AP -5.1, ML +4.8, DV -5.1 mm). Using dental cement the probe, anchor screws and a copper wire tether were secured in place and the wound was then sutured. Following surgery rats were housed in individual circular chambers with the microdialysis probe connected to a liquid swivel and a counterbalanced arm to allow free movement (see appendix 2 for diagram of setup). To aid recovery from anaesthesia animals were given an injection of saline (1 ml/kg) and a heated blanket was placed underneath the chamber to maintain body temperature. The probes were continuously perfused with artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3 mM KCl, 0.27 mM $Na_2HPO_4.H_2O$, 1 mM $MgCl_2$, 1.2 mM $CaCl_2.2H_2O$, 7.2 mM Glucose, pH7) at a rate of 1.6 µl/min and rats were allowed a recovery period of at least 16 hours with food and water available *ad libitum*.

3.3.2.3. Sample collection and drug administration

Samples of dialysis flow from freely-moving animals were collected at 15 minute intervals into 0.3 ml polypropylene sample vials containing 5 µl of a solution of 0.1 M perchloric acid (PCA) and 400 µM sodium metabisulphite to prevent oxidation of sample. Samples were collected for an hour for chromatogram optimisation, allowing baselines to settle after lights on. Samples were then collected for baseline and treatment conditions (see Figure 3-3 and Figure 3-5). Samples were placed onto dry ice immediately after collection, and stored at -80 °C for up to 14 days prior to analysis. Drugs were administered via the probe by inclusion in the infusion fluid. Syringes were primed and loaded onto syringe pump to ensure flow before switching between aCSF and drug. Syringes were switched in the same manner (between two separate aCSF syringes) for control animals.

At the end of the collection period animals were euthanized by the i.p. administration of 0.4 ml pentobarbitone (Euthatal), and brains removed and stored in 4 % formalin before probe placement verification. Coronal sections 50 µM thick were made using a vibratome, mounted on Superfrost Plus slides and stained with cresyl violet. The location of the probes was verified by reference to the atlas of Paxinos and Watson (1986).

3.3.2.4. Sample analysis

Detection and quantification of DA and NA in the samples used reverse-phase, ion pair HPLC-ECD. A reverse-phase column packed with 5 µM C_{18} silica based octadecyl silane material was used and mobile phase (16 % MeOH, 10.48 mM Na acetate, 8-30 mM OSA, 0.27 mM EDTA and 0.18 % glacial acetic acid, pH 4-4.5) circulated at a rate of 0.2 ml/min.

For the detection of NA alone the mobile phase contained 3.5 % MeOH, 0.15 M NaH_2PO_4, 20 mM octanesulphonic acid and 1 mM EDTA, adjusted to pH 4 with orthophosphoric acid. In each case the mobile phase was filtered through a 0.22 µm filter and degassed by sonication prior to use. Samples (20 µl) were injected onto the column by a Triathlon autosampler refrigerated to 4 °C. HPLC systems were calibrated to the average of two 40 fmol/20 µl (2 nM) standard samples daily. Standards of NA and DA (1 mM) were prepared weekly in a 1:1 mixture of deionised water and PCA (0.1 M) stored at 4 °C and diluted in aCSF daily to give 40 fmol/20 µl. Antec-Intro electrochemical detector was used in conjunction with an Antec wall-jet design cell (VT-03). The cell employs a high density glassy carbon working electrode combined with an Ag/AgCl reference electrode. The electrode signal was integrated using PowerChrom data acquisition system.

3.3.2.5. Data analysis

Data were plotted and analysed using SigmaPlot (version 11) for one way ANOVA and GraphPad Prism for 2 way ANOVA. Adjustments were made for multiple comparisons in all post tests.

3.3.3. Neurochemical fingerprinting

3.3.3.1. Animals

Experiments were carried out in conjunction with RenaSci, in male C57/BL6 JAX mice (20-25 g body weight; Charles River, UK). Animals were housed in groups of 4 on a 12 h/12 h light/dark cycle (lights on at 07.00 h), at an ambient temperature of 21 ± 2 °C and 55 ± 20 % humidity. Food and water were available *ad libitum*. Animals were allowed to acclimatise to these conditions for at least 5 days prior to the study. These studies took place at the Biomedical Services Unit of the University of Nottingham. All experiments were in accordance with the Animals (Scientific Procedures) Act (1986).

3.3.3.2. Pilot study to determine time course of response

Drug (or 0.9 % saline) was administered by intraperitoneal injection in a volume of 10 ml/kg body weight. The drugs were made up fresh and pH adjusted (pH 5-7) within 1-2 h of dosing. Saline-treated mice (n=3) were dosed and sacrificed by cervical dislocation at 15 minutes post-dose. Mice treated with nicotine (3 mg/kg) were dosed and sequentially sacrificed, (n=3 at 10, 15 and 30 minutes post-dose). All drug doses are expressed as the free base using the appropriate conversion factor of salt: free base.

3.3.3.3. Dose response study

Mice treated with saline, nicotine (0.3, 1 or 3 mg/kg) or tranylcypromine (5 mg/kg) i.p. were sacrificed by cervical dislocation, (n=8 at 15 min post-dose, determined by pilot study). Tranylcypromine is a MAOI which is used as a positive control for 3-MT analysis.

3.3.3.4. Sample collection

All brains were removed onto ice and the striata and prefrontal cortices rapidly dissected and snap frozen in liquid nitrogen. This procedure took less than 120 seconds to counter the effects of rapid post-mortem changes in monoamines and their metabolites. The frozen tissues were stored in individual plastic microcentrifuge tubes on dry ice and frozen at -80 °C until analysis.

The dissected tissues were weighed and homogenised in 5 (cortex) or 10 (striata) volumes of homogenising solution (0.1 M perchloric acid and 400 µM sodium metabisulphite containing 0.1 ng/µl DHBA as the internal standard). Homogenates were then microcentrifuged prior to determination of the following monoamine and metabolites from supernatant by HPLC-ECD: HPLC system 1: NA, DA, 5-HT, 5-HIAA, DOPAC and

HVA; HPLC system 2: 3-MT. Concentrations of monoamines and metabolites were calculated by reference to an internal standard, DHBA.

The monoamine system mobile phase contained 0.1M NaH_2PO_4, 1 mM EDTA, 14% MeOH, 1.2 mM OSA adjusted to pH 3.40. Flow rate 1.0 ml/min with a Spherisil C18 ODS2 5 µm 250 x 4.6 mm column (with Aquapore RP300 7 µm 30 x 4.6 mm guard column), ECD cell set at +0.70V, Range 0.5 nA/V (striatum) or 0.2 nA/V (PFC).

The 3-MT system mobile phase contained 0.01 M NaH_2PO_4, 8% MeOH, 0.6 mM OSA, 5.9 mM di-n-butylamine, adjusted to pH 3.80. The flow rate was 1 ml/min and the samples were separated on a Hypersil C18 ODS 5 µM 250 x 4.6 mm column (with Aquapore RP300 7 µM 30 x 4.6 mm guard column) electrode 1 set at +0.20V, range 100 µA and electrode 2 (monitoring) set at +0.45V, range 20 nA.

Each day HPLC systems were calibrated to two 5 µl replicates of a standard solution containing 0.04 ng/µl of 3-MT, 0.1 ng/µl of NA, DHBA, DOPAC, DA, 5-HIAA and 5-HT and 0.2 ng/µl of HVA. Sample supernatant (5 µl) was injected onto the HPLC and chromatograms obtained. DHBA, the internal standard was used to calibrate sample recovery. The limits of detection for the system are 3-5 ng/g brain tissue weight for each amine or metabolite.

3.3.3.5. Data analysis

In striatum DA levels are high, and so the chromatogram peaks go positively off scale. The area under the curve must be converted into ng/g tissue by the following method;

1. The peak area for the 0.5 ng (on column) DA standard is multiplied by 2, correcting to 1 ng.
2. The DA peak area for the sample is divided by the calculated area for 1 ng DA standard giving ng of DA in sample on column (in an injection volume of 5 µl).
3. The value from point 2 is multiplied by 200 to convert to ng DA/ml i.e. 1000/5 (µl).
4. The value from point 3 is multiplied by 10 to convert from ng DA/ml to ng DA/g tissue wet weight. This takes into account the 10-fold dilution of the sample with homogenising solution.
5. The overall effect of points 3 and 4 is to convert ng DA injected to ng DA/g tissue wet weight (multiply by 200 and then 10, therefore a factor of 2000 is applied).
6. The % recovery of the internal standard (X) for each sample is calculated by comparing the peak area of the internal standard in the sample to the mean area in the calibration standard.
7. The internal standard recovery is then applied (100/X) to each sample ng DA/g tissue to correct the value for losses during the homogenisation process.

In PFC NA was greater than DA and some NA responses were off scale. A similar calculation as to convert off scale striatal DA responses was performed however the dilution of PFC tissue is 5 fold, so the final conversion factor is 1000.

Analysis of pilot study data was by one way ANOVA on log transformed data to normalise variance. Comparison to vehicle was by multiple t-test. Analysis of main study data was by robust regression with treatment and study as factors, followed by Williams'

test to compare nicotine to vehicle, and multiple t-test to compare tranylcypromine to vehicle. Means are back-transformed and adjusted for differences between studies. SEMs are calculated from the residuals of the statistical model. These analyses were performed with the assistance of Richard Brammer at RenaSci.

3.4. Results - Microdialysis

3.4.1. Response of FC to nomifensine administration

In order to establish that local administration of drug via the perfusion fluid into the frontal cortex is a valid model, a known DA and NA uptake inhibitor, nomifensine (100 µM) was used. Rats were anaesthetised and a microdialysis probe was stereotaxically implanted into the FC (3.3.2). The probe was continuously perfused with aCSF and sampling took place the day after surgery. This study was performed at RenaSci Consultancy (Nottingham) and used a flow rate of 1.2 µl/min and a collection period of 20 min per sample. After 80 min baseline collection period nomifensine was included in the perfusion buffer for the remainder of the experiment to increase NA and DA levels by inhibiting reuptake. Levels of DA and NA measured from the dialysates. Both catecholamines were monitored in the same samples by HPLC with ECD.

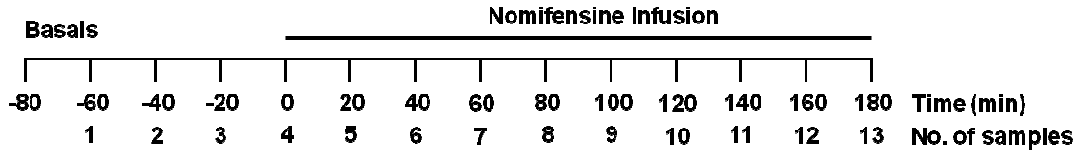


Figure 3-3 Nomifensine administration timeline
The probe was perfused with aCSF at a rate of 1.2 µl/min. After equilibration four basal samples were collected. Nomifensine (100 µM) was then administered via the probe for the remainder of the sampling period by switching syringes.

The mean ± S.E.M. basal levels of NA were 11.1 ± 1.1 fmol/20 µl (n=4), and for DA 2.3 ± 0.2 fmol/20 µl. Some basal samples (6 of 16) did not contain high enough levels of DA to detect, for statistical purposes these have been substituted with 2 fmol. All samples contained measurable NA. Figure 3-4 shows NA and DA as percentage of the baseline levels. DA increased from 2.3 ± 0.2 fmol/20 µl to a peak of 28.0 ± 5.9 fmol/20 µl ($F(13,42)=5.937$, $p<0.001$). This equates to 1190 ± 298 % of baseline levels (Figure 3-4A). Levels of NA increased from 11.1 ± 1.1 fmol/20 µl to 66.5 ± 11.2 fmol/20 µl by 40 min after the start of nomifensine administration ($F(13,42)=36.44$, $p<0.001$), this equates to 608 ± 62 % of basal levels (Figure 3-4B). *Post hoc* Holm-Sidak comparing each point to baseline (average of first four timepoints) showed that 100 µM nomifensine gives a significant increase in both DA (Figure 3-4A) and NA (Figure 3-4B) levels by 40 min after the start of drug infusion, which is stable across the remainder of the infusion period.

Overall it can be seen that the administration of a drug (in this case a reuptake inhibitor) via the microdialysis probe is an effective way to alter neurotransmitter levels

detected in a reliable manner. As a consequence 5IA was administered by the same route to examine effects on neurotransmitter levels in FC (Figure 3-7) and dorsal HC (Figure 3-9).

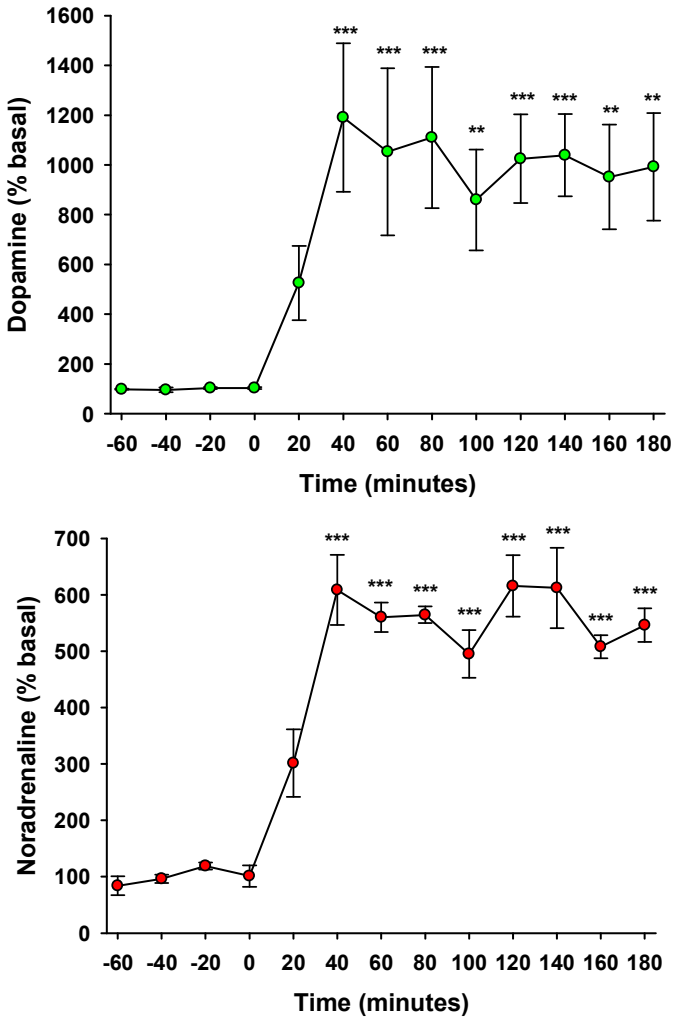


Figure 3-4 Catecholamine changes in the rat FC after nomifensine administration.
Rats were anaesthetised and a microdialysis probe with membrane length 2 mm was implanted stereotaxically into the FC (AP +3.2, ML +2.5, and DV -4.0 mm) and constantly perfused with aCSF at a rate of 1.2 µl/min. The following day sampling commenced; samples (24 µl) were collected into tubes containing 5 µl 0.1 M PCA and 400 µM Na metabisulphite. 4 basal samples were collected and nomifensine (100 µM) was then administered locally by inclusion in the perfusion fluid. **A)** DA levels as percentage baseline. $F(13, 42)=36.444$, $p<0.001$ **B)** NA levels as percentage baseline. Each point represents the mean ± SEM of 4 animals. Data analysed with a one way repeated measure ANOVA with Holm-Sidak multiple comparisons to control. Where samples contained levels of DA below the detection limit 2 fmol/20 µl sample was substituted. ** $p\leq0.01$ ***$p\leq0.001$ $F(13, 42)=5.937$, $p<0.001$

3.4.2. Administration of 5-I-A85380 to frontal cortex

A previous study reported from this lab (Livingstone *et al*, 2009) looked at the effect of 5-iodo-A85380 (5IA) in the FC, locally administered by reverse dialysis. Increases in DA levels were found after 5IA administration at two concentrations, 100 µM and 1 mM, and these responses were not different from one another, suggesting they were at the top of the dose-response curve. With this in mind lower concentrations of 5IA were assessed in FC. Two infusions at different concentrations were given to each animal,

separated by 90 min aCSF. This allows more data to be gathered from each animal whilst allowing transmitter levels to return to baseline between infusions and minimising desensitisation.

Probes were implanted as before (3.3.2) and perfused with aCSF. Local administration of 5IA took place via the microdialysis probe from time 0-30 and a second infusion at 120-150 min (Figure 3-5). The first administration was 1 or 10 µM; the second administration was 100 µM in each case. Control animals received aCSF throughout, with syringe changes at the same timepoints as treated animals to control for any effects of manipulation when switching between syringes. No behavioural responses were noted in the animals upon administration or washout of drug (although this was not formally scored).

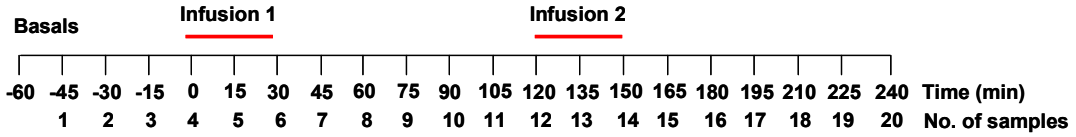


Figure 3-5 5-Iodo-A85380 administration timeline
The probe was infused with aCSF throughout the experiment for control animals. Local administration of 5IA took place via the microdialysis probe from time 0-30 and 120-150 min by switching syringes. Syringes were also switched for aCSF animals to control for effects of manipulation.

Probe placements for both control and treated animals were visually verified (3.3.2) and an example placement is shown below (Figure 3-6A) with the probe tract clearly visible in the left side, and the unimplanted right side as comparison. Figure 3-6B shows compiled probe placements in FC, all probes had the active membrane portion within frontal cortex.

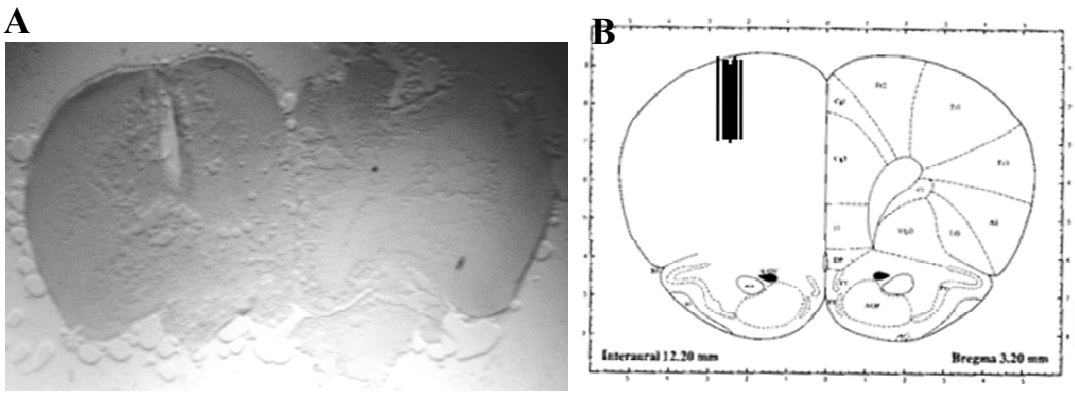


Figure 3-6 Probe placement in the frontal cortex.
Brains were removed, fixed in 4 % PFA to fix, and sectioned. Slices (50 µm) were mounted onto Superfrost slides and stained with cresyl violet. **A)** An example FC probe placement (left hemisphere) with unimplanted right hemisphere for comparison. **B)** Compiled probe placements for FC n=14. Adapted from Paxinos and Watson, 1986

Initially samples were analysed for levels of both NA and DA, however this posed many problems in achieving viable chromatography and baselines were often variable. For

DA in particular there was a very low baseline in many subjects, which fell below the limit of detection of the HPLC (as seen above 3.4.1). As attempting to measure both amines necessitated a compromise in the peak resolution it was decided to measure NA only, which gave more reliable results (see Appendix 3 for example chromatograms).

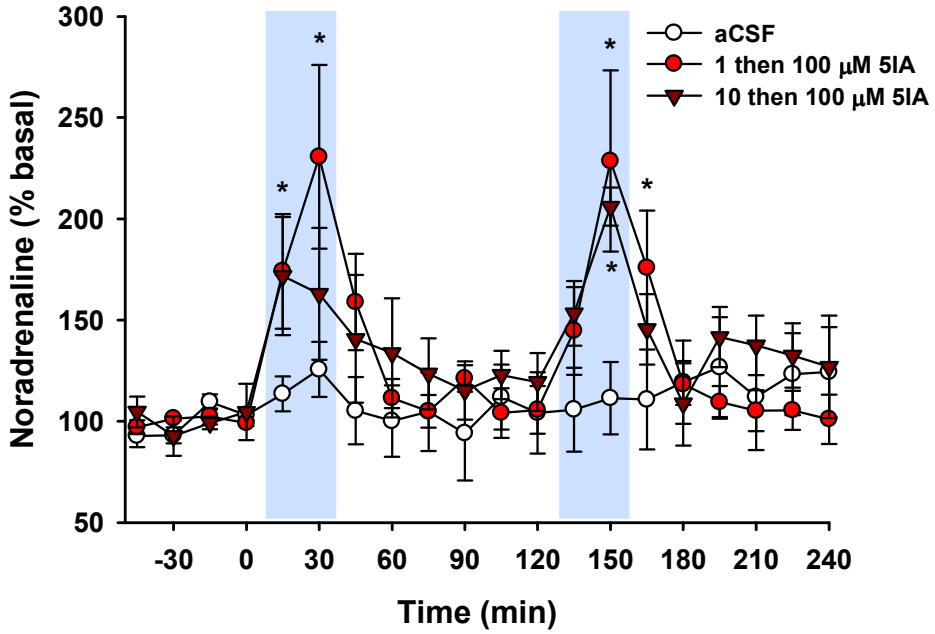


Figure 3-7 Effect of 5-I-A-85380 on noradrenaline levels in rat frontal cortex
Rats were anaesthetised and a microdialysis probe with membrane length 2 mm inserted stereotaxically into the frontal cortex (AP +3.2; ML +2.5; DV -4.0). The probe was continuously perfused with aCSF at 1.6 µl/min. The following day 15 min samples (24 µl) were collected into tubes containing 5 µl 0.1 M PCA and 400 µM Na metabisulphite. Four baseline samples were collected then syringes were changed so that 5-I-A-85380 (5IA; 1 or 10 µM) was infused from 0-30 min (shaded area). Animals were returned to aCSF for 90 min and a second infusion with 100 µM 5IA then took place (120-150 min; 2nd shaded area). Control animals received aCSF throughout the infusion period. Data are presented as % of the 4 baseline samples for each animal. Each point represents the mean ± SEM of 4-5 animals. * Significantly different from aCSF by 2 way ANOVA followed by pairwise Multiple Comparison Procedures (Holm-Sidak method). Infusion $F(2,211)=8.65$, $p=0.0002$, Time $F(19,211)=4.635$, $p<0.0001$, Interaction $F(38,211)=1.329$, $p=0.109$

In FC the basal quantity of NA was quite variable between animals (ranging from 12-60 fmol/20 µl); however baseline samples were very consistent within each animal (typical SEM ± 5 %). In the FC 5IA elicited significant increase in NA above baseline and aCSF at 1, 10 and 100 µM, with equal efficacy at all drug concentrations ($F(2,211)=8.65$, $p=0.0002$; Figure 3-7). The responses reached a maximum by the second sample after the start of drug infusion, and returned to baseline within 30 min of the removal of drug with a sharply defined peak shape. Treatment groups were larger than for nomifensine pilot due to the smaller responses expected to 5IA than to nomifensine.

At 1 µM 5IA NA levels reach a maximum of 230.6 ± 45.4 % of baseline in the second sample after the commencement of 5IA administration (Figure 3-7). At 10 µM 5IA, NA levels reached a maximum of 171.7 ± 29.2 % baseline in the first sample after the start of drug infusion which was not significantly different from control allowing for

adjustments for multiple comparisons (Holm-Sidak method). The response in the second sample after drug administration was slightly attenuated in comparison with 1 μM 5IA to 163 ± 32.6 % baseline.

At 100 μM both treatment groups achieved a peak response in the second sample after the start of drug administration of 228.6 ± 44.7 % and 206.0 ± 9.4 % (Figure 3-7) respectively for 1 μM and 10 μM initial dose groups. In the group that had previously received 1 μM this response remained significant at the following sample, the first after drug removal. There was no difference in the magnitude of the response to 100 μM 5IA between the two initial treatments.

3.4.3. Administration of 5-I-A-85380 into the hippocampus

In dorsal HC 5IA was administered at 1 and 100 μM following the same procedure as in FC (Figure 3-5). These concentrations were chosen on the basis that in the *in vitro* assay HC was less sensitive to 5IA than FC (Figure 2-8), and that FC had large responses to these concentrations *in vivo*. An example probe placement is shown below (Figure 3-8A) with the probe tract clearly visible in the left side, and the unimplanted right side as comparison. Figure 3-8B shows a compilation of the probe placements in the dorsal HC, although there is some variation in placement in the ML axis the active membrane portion of all probes was within the hippocampal formation.

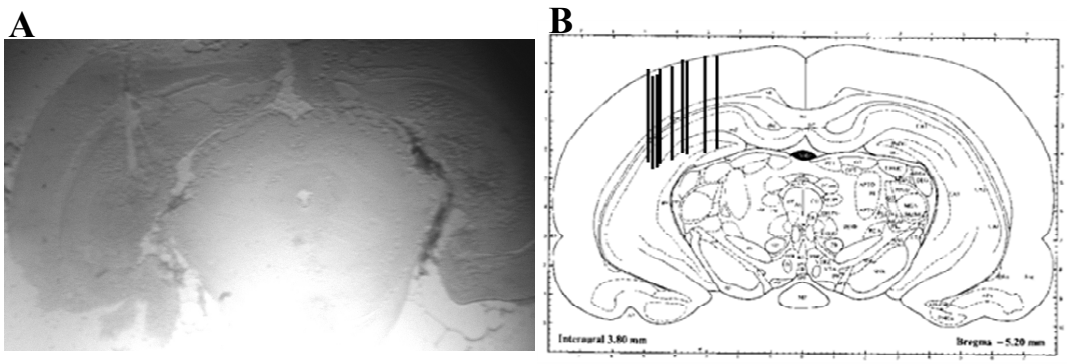


Figure 3-8 Probe placements in hippocampus
Brains were removed, fixed in 4 % PFA to fix, and sliced. Slices (50 μm) were mounted onto Superfrost slides and stained with cresyl violet. A) An example HC probe placement (left hemisphere) with unimplanted right hemisphere for comparison. B) Compiled probe placements for HC n=16 Adapted from Paxinos and Watson, 1986

In HC baseline was again variable between animals (ranging from 10-60 fmol/20 μl); however baseline samples were very consistent within each animal (the typical SEM was ± 5 %). Upon changing syringes an increase in NA in aCSF animals was observed (to 129 ± 18 % baseline). Although this effect was not significant above the basal time period, it may have masked changes in the drug treated group.

Levels of NA in the HC reached maxima of 160 ± 23.1 % and 173.6 ± 22.9 % of baseline in the second sample after administration of 1 μM and 100 μM 5IA respectively

(Figure 3-9). Using a two way ANOVA and *post hoc* comparisons (Holm-Sidak method) it is shown that although the curves as a whole differ there are no significant differences between treated and aCSF animals at any single timepoint (Treatment F(1,260)=9.726, p=0.002). As treatment had an effect overall it was decided to look at the area under the curve for each hour to see if the NA levels are different over longer time periods however no significant differences were observed (data not shown).

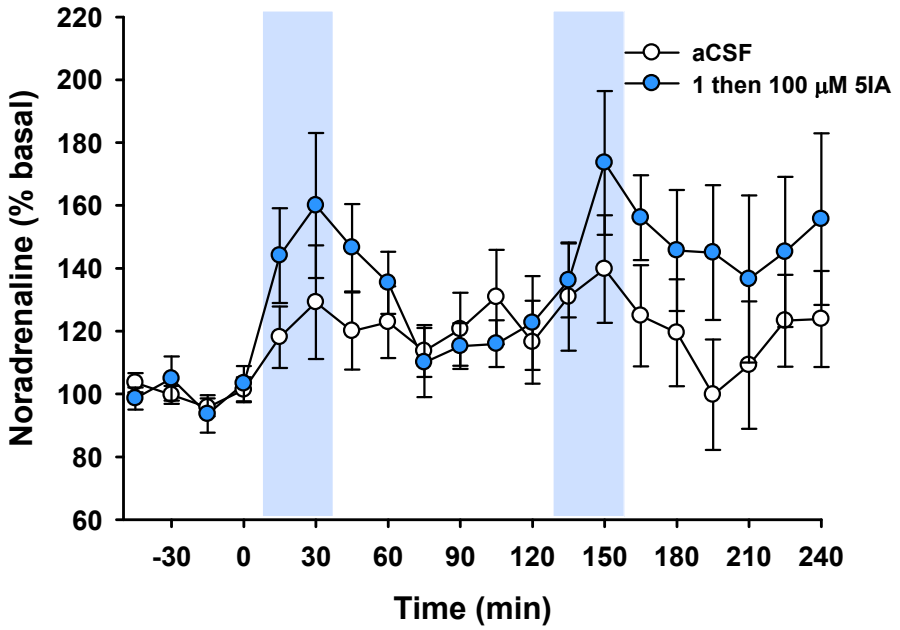


Figure 3-9 Effect of 5-I-A-85380 on noradrenaline levels in rat hippocampus
Rats were anaesthetised and a microdialysis probe with membrane length 2 mm inserted stereotaxically into the hippocampus (AP +5.2; ML +4.8; DV -5.1). The probe was continuously perfused with aCSF at 1.6 µl/min. The following day 15 min samples (24 µl) were collected into tubes containing 5 µl 0.1 M PCA and 400 µM Na Metabisulphite. Four baseline samples were collected then syringes were changed so that 5-I-A-85380 (5IA; 1 µM) was infused from 0-30 min (shaded area). Animals were returned to aCSF for 90 min and a second infusion with 100 µM 5IA then took place (120-150 min; 2nd shaded area). Control animals received aCSF throughout the infusion period. Data are presented as % of the 4 baseline samples for each animal. Each point represents the mean $\pm$ SEM of 8 animals. Analysed by 2 way ANOVA followed by pairwise Multiple Comparison Procedures (Holm-Sidak method) Treatment F(1, 260)=9.73, p<0.002, Time F(19,260)=2.249, p=0.0025, Interaction F(19,260)=0.6151, p=0.8939.

Although the responses are not significant there does appear to be an increase in NA levels after 5IA administration (Figure 3-9). The responses in the HC are smaller than those in the FC (173.6 $\pm$ 22.9 % vs. 228.6 $\pm$ 44.7 % after 100 µM 5IA respectively) and have a less sharp, defined peak shape. The significant NA levels in the FC and non-significant changes in HC even with a larger n (8) per condition to reduce variation indicate regional differences in the response to 5IA as seen *in vitro*.

Overall it can be seen that there is a significant response in NA levels to the administration of 5IA at 1, 10 and 100 µM in FC. In the HC this response is blunted and confounded by a more responsive baseline that leads to changes being statistically insignificant. The reasons for the different responses will be discussed below (3.6).

3.5. Results - Neurochemical fingerprinting

Local administration of drug, as in the microdialysis study presented above, allows the assessment of the involvement of terminal fields in neurotransmitter release; however this is not the full picture *in vivo*. Samples of tissue can be taken after drug administration to assess the action of systemic nicotine on a range of neurotransmitters and metabolites, giving the 'neurochemical fingerprint' of the response. It is not usually possible to assess this range of transmitters from microdialysates due to low levels of recovery and so tissue homogenates are used, limiting the technique to a single timepoint for each animal.

3.5.1. Standard curves

To ensure that the relationship between quantity of metabolite and area under curve (AUC) on chromatogram followed a linear relationship (and so could be calculated for unknowns) a standard curve was analysed. Calibration standard containing 0.08 ng/µl 3-MT, 0.2 ng/µl NA, DHBA, DOPAC, DA, 5-HIAA and 5-HT, and 0.4 ng/µl HVA in 0.1 M PCA and 400 µM sodium metabisulphite (200% daily standard) was made. Neat and ½, ¼ and 1/20 serial dilution (to give 200%, 100%, 50% and 10% standards) of this calibration standard were injected onto each system and chromatograms obtained.

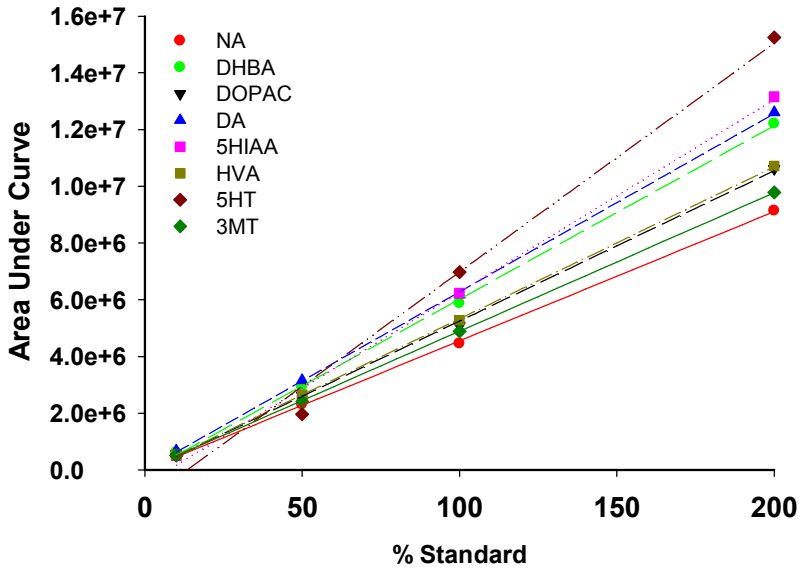


Figure 3-10 Standard curves for calibration of HPLC systems.
Two replicates of a solution (200% standard) and containing 0.08 ng/µl 3-MT, 0.2 ng/µl NA, DHBA, DOPAC, DA, 5-HIAA and 5-HT and 0.4 ng/µl HVA in 0.1 M PCA serial dilutions (100, 50 and 10%) was run through each system, and the AUC of peaks calculated from the resulting chromatograms.

Table 3-1 Coefficients of determination for standard curves
Rsq and p values were calculated from the data presented above (Figure 3-10)

	NA	DHBA	DOPAC	DA	5HIAA	HVA	5HT	3MT
Rsq	0.999	0.999	0.999	1.000	0.997	1.000	0.987	1.000
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

The relationship between concentration and AUC for each standard is shown (Figure 3-10). The relationship between the standard concentration and the AUC is linear. For each amine and metabolite a linear relationship was shown with all Rsq values above 0.987 (Table 3-1).

3.5.2. Pilot study

The time course of the response to nicotine was investigated in a pilot study (for detailed methods see 3.3.3). Briefly, 3 mg/kg nicotine was administered intraperitoneally to C57/BL6 mice and animals were sacrificed at 10, 15 and 30 min post-dose. Saline controls were sacrificed at 15 min post injection. Striata and PFC were rapidly dissected and tissues were prepared prior to determination of the monoamines and metabolites by HPLC-ECD (3.3.3). Locomotor depression was seen after injection in nicotine-treated animals, with recovery by 30 min post injection. This effect was not quantified.

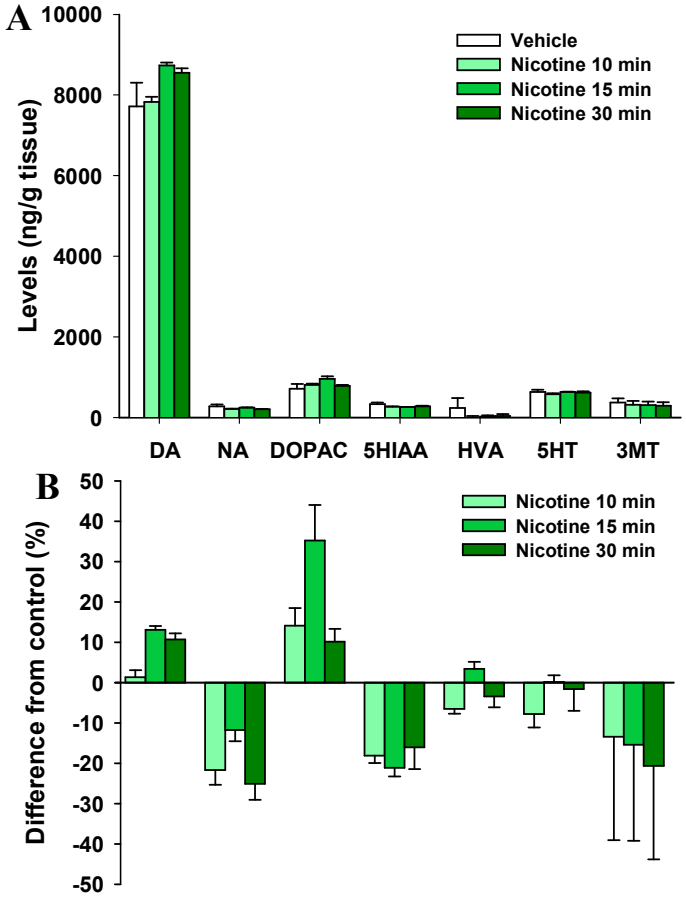


Figure 3-11 Neurochemical fingerprinting pilot - Striatum
Mice were treated with nicotine (3 mg/kg i.p.) and sequentially sacrificed by cervical dislocation, (n=3 at 10, 15 and 30 minutes post-dose). Saline-treated mice (n=3) were dosed and sacrificed at 15 minutes post-dose. Striata were removed and homogenised and levels of DA, NA, DOPAC, 5HIAA, HVA, 3MT and 5HT were determined by HPLC. Results are presented as ng/g wet weight **(A)** and as % change from saline control **(B)**. Bars represent mean ± SEM from 3 animals for each condition. Data analysed by one way ANOVA on log transformed data for each compound. See Appendix 4 for output.

In the striatum high levels of DA were found (~7800 ng/g tissue weight) reflecting the major dopaminergic innervations (Figure 3-11A). The levels of dopamine were calculated from the area under the curve on chromatograms using the procedure outlined above (3.3.3.5). NA, DOPAC, 5-HIAA, 5-HT and 3MT are each found at lower concentrations of 250-650 ng/g tissue and HVA at 1400 ng/g tissue. As the levels of different amines and metabolites varied in comparison to one another data have been presented as percentage change from saline treated control (Figure 3-11B).

No significant changes were observed in striatum at any timepoint (Figure 3-11) however some of the non-significant results showed a trend towards significance ($P<0.1$) at the 15 min time point, notably DA, DOPAC and 5-HIAA (highlighted in Table 3-2).

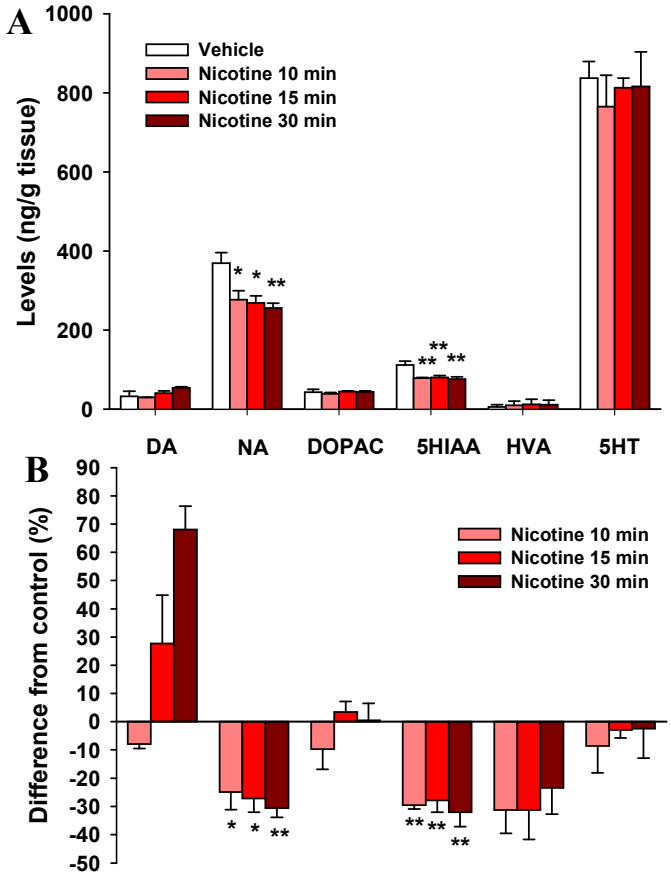


Figure 3-12 Neurochemical fingerprinting pilot - PFC
Mice were treated with nicotine (3 mg/kg i.p.) and sacrificed by cervical dislocation, (n=3 at 10, 15 and 30 minutes post-dose). Saline-treated mice (n=3) were dosed and sacrificed at 15 minutes post-dose. PFC was removed and homogenised and levels of DA, NA, DOPAC, 5HIAA, HVA and 5HT were determined by HPLC. Results are shown as ng/g **(A)** and as % change from saline control **(B)**. Bars represent mean ± SEM from 3 animals for each condition *p<0.05, ** p<0.01. Data analysed by one way ANOVA on log transformed data for each compound. See Appendix 4 for output.

In the PFC the more diffuse dopaminergic innervation is reflected in the lower level of DA and its metabolites, with less than 100 ng/g tissue DA, HVA and DOPAC (Figure 3-14A). 5-HT is the most abundant component in the PFC with levels of ~550 ng/g. NA levels are slightly higher in PFC than striatum (340 vs. 291 ng/g).

Significant decreases were seen in NA and 5HIAA at all timepoints in the PFC (Figure 3-12B). No other significant changes were observed again due to the small number of animals studied for this pilot but there was a trend towards significance in HVA at 10 and 15 min (Table 3-2). 3-MT is not reliably detectable in PFC (only above limit of detection in 5 of 12 samples; data not shown), and so was not assayed in this region for the main study. On the basis of these data and those for the striatum the 15 min time point was chosen for the main study.

Table 3-2 Mean (ng/g), SEM and p values for each treatment
Mice were injected with 3 mg/kg nicotine and Striata and frontal cortices dissected and frozen at the appropriate timepoints before homogenisation and analysis by HPLC-ECD.

Treatment	Mean ng/g)	SEM	p		Treatment	Mean ng/g	SEM	p
Striatal DA					**Frontal cortex DA**			
Vehicle	7722	585			Vehicle	32.2	13.5	
Nicotine 10 min	7824	135	0.82		Nicotine 10 min	29.7	0.5	0.799
Nicotine 15 min	8731	73	0.06		Nicotine 15 min	41.1	5.5	0.46
Nicotine 30 min	8549	114	0.11		Nicotine 30 min	54.1	2.7	0.137
Striatal NA					**Frontal cortex NA**			
Vehicle	278	52			Vehicle	369	27	
Nicotine 10 min	217	10	0.13		Nicotine 10 min	277	23	0.019*
Nicotine 15 min	245	8	0.41		Nicotine 15 min	269	18	0.012*
Nicotine 30 min	208	11	0.08		Nicotine 30 min	256	12	0.006**
Striatal DOPAC					**Frontal cortex DOPAC**			
Vehicle	713	125			Vehicle	43.4	7.3	
Nicotine 10 min	813	31	0.36		Nicotine 10 min	39.2	3.1	0.489
Nicotine 15 min	964	63	0.06		Nicotine 15 min	44.9	1.6	0.812
Nicotine 30 min	785	23	0.5		Nicotine 30 min	43.6	2.6	0.971
Striatal 5HIAA					**Frontal cortex 5HIAA**			
Vehicle	331	42			Vehicle	112	9.7	
Nicotine 10 min	271	6	0.09		Nicotine 10 min	78.9	1.5	0.005**
Nicotine 15 min	261	7	0.05		Nicotine 15 min	80.7	4.7	0.008**
Nicotine 30 min	278	18	0.14		Nicotine 30 min	76.2	5.8	0.003**
Striatal HVA					**Frontal cortex HVA**			
Vehicle	1626	242			Vehicle	121.6	5.8	
Nicotine 10 min	1521	20	0.55		Nicotine 10 min	83.6	10.1	0.053
Nicotine 15 min	1682	28	0.76		Nicotine 15 min	83.7	12.7	0.053
Nicotine 30 min	1570	44	0.76		Nicotine 30 min	93.1	11.3	0.145
Striatal 5HT					**Frontal cortex 5HT**			
Vehicle	633	64			Vehicle	837	42	
Nicotine 10 min	583	21	0.37		Nicotine 10 min	765	79	0.449
Nicotine 15 min	634	11	0.99		Nicotine 15 min	813	24	0.8
Nicotine 30 min	623	34	0.86		Nicotine 30 min	816	87	0.825
Striatal 3MT								
Vehicle	370	111						
Nicotine 10 min	321	95	0.74					
Nicotine 15 min	313	88	0.7					
Nicotine 30 min	294	86	0.59					

3.5.3. Main study

Forty C57/BL6 mice were used for this portion of the work. They were allocated into 5 groups, saline, tranylcypromine (5 mg/kg; positive control), and 0.3 mg/kg, 1 mg/kg and 3 mg/kg nicotine. Doses were based on Tani *et al* (1997) and Matta *et al* (2007). All nicotine doses are expressed as free base. Animals were sacrificed 15 min after i.p. injection and striata and PFC rapidly dissected and frozen before preparation of homogenates as before (3.3.3).

Two animals (both 3 mg/kg nicotine group) died during the course of treatment, probably due to respiratory depression. No abnormalities were found at postmortem. Tissue was not collected for analysis from these animals. Samples from the corresponding time point in the pilot study (and saline controls) were included in the analysis to increase the n in the 3 mg/kg group with adjustments made to the statistical analyses to ensure there were no group differences between 'pilot' and 'main study' data. A dose of 3 mg/kg nicotine is quite high, and is likely to have caused toxicity. The effect of this on the results will be further discussed in section 3.7.

3.5.3.1. Striatum

The monoamine oxidase inhibitor tranylcypromine was used in this study as a positive control as it has a well characterized profile of effects (Heal *et al* 2009). Through inhibition of monoamine oxidase, tranylcypromine prevents the metabolism of DA to DOPAC and so increases the extracellular metabolism of DA to 3-MT. The increase in 3-MT is further emphasized because it is not then broken down into HVA, therefore giving a reduction in HVA levels.

It can be seen that the levels of DA are far in excess of the level of any other compound measured within the striatum (Figure 3-13A). For this reason data is also presented as a percentage change from control, so changes in the other compounds can be more clearly seen (Figure 3-13B).

In this study tranylcypromine increased DA by 7.4 % ($p=0.014$), reduced DOPAC to 47.8% of control ($p<0.001$), decreased HVA to 84.2 % control ($p=0.023$) and increased 3-MT to 189.3 % control. There was also a reduction in 5-HIAA to 83.7 % control ($p=0.029$). There was no significant change in NA or 5-HT, although there was a trend toward an increase in 5HT (112 % control; $p=0.074$). The only significant response in the striatum with nicotine was an increase in DOPAC at the 3 mg/kg dose to 110.9 % control ($p=0.048$). Increases in DOPAC were also seen at 0.3 and 1 mg/kg, although these were not significant. A decrease in 5HIAA (mirroring that produced by tranylcypromine) was seen at 1 mg/kg nicotine, again this was non-significant.

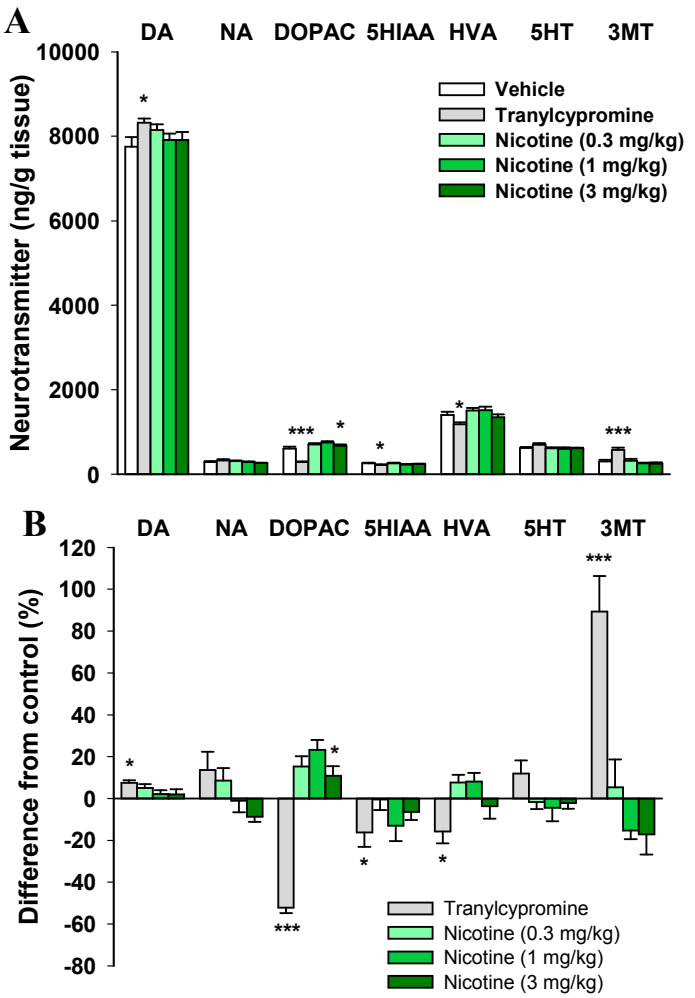


Figure 3-13 Neurochemical fingerprint of striatum after nicotine administration
Nicotine (0.3, 1 or 3 mg/kg), tranylcypromine (5 mg/kg) or saline was administered to mice intraperitoneally. 15 min later mice were sacrificed by cervical dislocation and striata rapidly dissected on ice. Tissue was homogenised and levels of amines and metabolites quantified by HPLC with reference to an internal standard (DHBA) A) Amine and metabolite levels in the striatum. DA levels are high in this region. B) Amines and metabolites in striatum as % change from saline control. Bars represent mean $\pm$ SEM from 8-11 animals for each condition *p<0.05, ** p<0.01, *** p<0.001. Data was analysed by robust regression with treatment and study as factors, followed by William's test to compare nicotine to control and multiple t-tests to compare tranylcypromine to control. See Appendix 4 for output.

3.5.3.2. Frontal cortex

In FC levels of DA are much lower than in striatum, however levels of 5-HT and NA are higher than those of the other compounds (Figure 3-14A). Figure 3-14B shows the responses in the frontal cortex as a percentage change from saline control for each amine and metabolite in order that the changes can be more clearly observed. Tranylcypromine induced a significant increase in DA levels (210 % of control; p<0.001) and decreased NA (76.1 %; p<0.001), DOPAC (59.2 %; p=0.006) and 5HIAA (65.9 %; p<0.001).

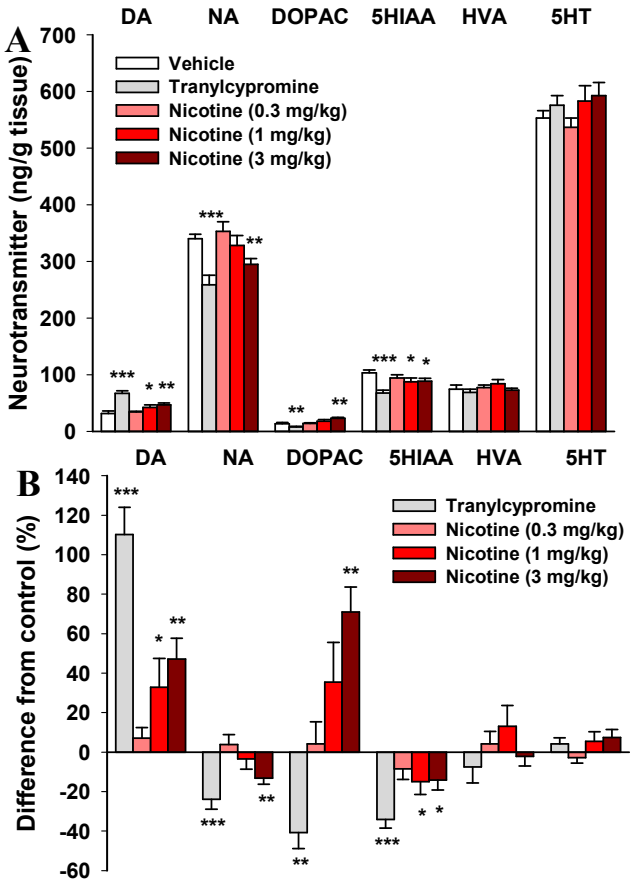


Figure 3-14 Neurochemical fingerprint of the PFC after nicotine administration
Nicotine (0.3, 1 or 3 mg/kg), tranylcypromine (5 mg/kg) or saline was administered to mice intraperitoneally. 15 min later mice were sacrificed by cervical dislocation and PFC rapidly dissected on ice. Tissue was homogenised and levels of amines and metabolites quantified by HPLC with reference to an internal standard (DHBA) A) Amine and metabolite levels in the PFC. B) Amines and metabolites as % change from saline control. Bars represent mean ± SEM from 8-11 animals for each condition *p<0.05, ** p<0.01, *** p<0.001. Data was analysed by robust regression with treatment and study as factors, followed by William's test to compare nicotine to control and multiple t-tests to compare tranylcypromine to control. See Appendix 4 for output.

Nicotine induced an increase in DA at both 1 mg/kg (132.8 % control; p=0.039) and 3 mg/kg (147.1 %; p=0.003; Figure 3-14). DOPAC is also increased at 3 mg/kg nicotine (171 % control, p=0.003). There are significant decreases in NA (3 mg/kg 86.7 %; p=0.006) and 5HIAA (1 mg/kg, 85.1 %, p=0.043; 3 mg/kg, 85.8 %, p=0.031). Nicotine induced no significant changes in HVA or 5-HT levels.

All of the responses to nicotine are smaller than those to tranylcypromine in both regions, although not all responses are in the same direction. In particular DOPAC is increased after nicotine treatment and reduced after tranylcypromine. Overall it can be seen that the responses to nicotine are larger in the PFC than the striatum, in particular the increases in DA and DOPAC. There are also larger reductions in NA and 5HIAA in PFC than striatum. There are no significant differences in HVA or 5-HT in either region. These differences between the drugs and the brain regions will be discussed below.

3.5.3.3. Ratios

The ratio of amines to their metabolites can also give insights into turnover so the ratios of several combinations of the amines were calculated. This may allow changes that are not significant for a particular amine or metabolite to be more clearly seen, indicative of changes in monoamine turnover.

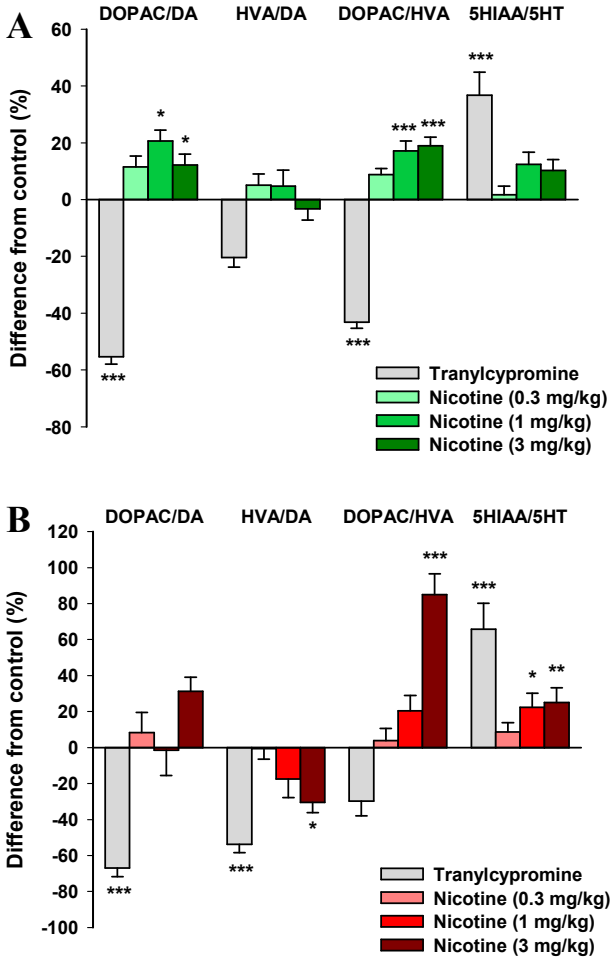


Figure 3-15 Striatal and PFC neurochemical ratios
Nicotine (0.3, 1 or 3 mg/kg), tranylcypromine (5 mg/kg) or saline (0.9 %) was administered to mice i.p. 15 min later mice were sacrificed by cervical dislocation and PFC and striata rapidly dissected on ice. Tissue was homogenised and levels of amines and metabolites quantified by HPLC with reference to an internal standard (DHBA). The ratios of amines and metabolites in **A)** striatum and **B)** PFC were calculated and are expressed as % change from control. Bars represent mean $\pm$ SEM from 8-11 animals for each condition *p<0.05, ** p<0.01, *** p<0.001. Data was analysed by robust regression with treatment and study as factors, followed by William's test to compare nicotine to control and multiple t-tests to compare tranylcypromine to control. See Appendix 4 for output.

Here the ratios are shown as % changes from control ratio. In striatum tranylcypromine decreases the ratio of DOPAC to DA ($p \leq 0.001$; Figure 3-15A). The 5HIAA/5HT ratio was increased after tranylcypromine treatment (p=<0.001; Figure 3-15A) with a trend towards an increase in the 3 mg/kg nicotine dose (p=0.078). These changes are indicative of decreased DA turnover. Nicotine increases the ratio of DOPAC to DA (1 mg/kg p=0.024; 3 mg/kg p=0.016), as well as the DOPAC/HVA (1 and 3 mg/kg; p< 0.001)

ratio with no significant effect on HVA/DA ratio. An increased DOPAC/DA ratio is indicative of increased turnover, supporting nAChR mediated DA release as an important mechanism in this region.

The ratios of the data from FC were also calculated and are presented as percentage changes from control (Figure 3-15B). Tranylcypromine decreased the DOPAC/DA, HVA/DA, and DOPAC/HVA ratios in the PFC in comparison with saline (to 33 %, $p<0.001$, 46.2 %, $p<0.001$ and 70.2 %, $p=0.013$ respectively; Figure 3-15B) and increased the 5HIAA/5HT ratio to 165.8 % control ($p<0.001$; Figure 3-15B). This suggests again that tranylcypromine reduces dopaminergic neuronal firing. Nicotine decreased the HVA/DA ratio to 69.9 % control ($p<0.016$) and increased the DOPAC/HVA ratio to 185 % control ($p<0.001$). The 5HIAA/5HT ratio is also increased by nicotine (122.3 %, $p=0.024$ and 125 %, $p=0.008$ at 1 and 3 mg/kg respectively). Unlike striatum there is no significant effect on the DOPAC/DA ratio, suggesting that this metabolic route is less affected by nicotine in FC than striatum. In contrast the changes in HVA/DA are larger in FC than striatum.

In summary, there are differences in the profile of changes in ratios of amine and metabolites between tranylcypromine and nicotine. In particular the change in ratio of DOPAC to DA is reversed in striatum. In FC there is no difference in DOPAC/DA ratio in nicotine treated animals, whereas there are significant differences in striatum. Possible reasons behind these differences are discussed below (3.7.7).

3.6. Discussion - Microdialysis

To verify that local administration of drug via the microdialysis probe can induce increases in NA levels in our hands, nomifensine administration was used as a positive control. Nomifensine (100 μM) elicited significant increases in both NA and DA in the FC as expected. Due to the large *in vitro* differences in the potency of 5IA in eliciting [^{3}H]-NA release the responses *in vivo* elicited by local administration were measured by microdialysis.

In the FC the β2* nAChR selective agonist 5IA elicited significant release above aCSF of NA at 1 and 100 μM, with equal efficacy of both drug concentrations. The responses reached a maximum by the second sample after the start of drug infusion, and returned to baseline within 30 min to an hour of the removal of drug. The response to 10 μM 5IA was not significant, although it was not significantly different to the responses elicited by the other concentrations of drug.

In HC there appeared to be small responses to 5IA administration (1 and 100 μM), although these were not significantly different to aCSF control. The responses at the two drug concentrations were not different from one another, although a sustained increase in NA was seen after the second (100 μM) administration.

3.6.1. Critique of the microdialysis technique

3.6.1.1. Spatial resolution

For most brain regions in the rat (FC, HC etc) the probe is easily placed within the boundaries of the structure, indeed all of the probe placements in the current study were within the areas of interest (Figure 3-6 and Figure 3-8). Some fine detail (e.g. the NAcc shell) may be more difficult to target, especially taking into account the diffusion gradients established within the area surrounding the probe. Finer probes are being developed (primarily for use in mice) to help target these smaller structures more precisely. These finer probes also help reduce tissue damage around the probe tip. One technical drawback of the use of finer probes is the increase in back pressure produced if the flow rate is not reduced (Chen, 2008).

Slow flow rates can affect recovery and delivery across the probe. A slow flow rate will allow more complete recovery, but generate smaller sample volumes and samples take longer to reach the collection tubes. This leads to greater degradation of neurotransmitters and also effects temporal resolution for collected samples. This can be overcome by the addition of a carrier flow after sample collection (See Figure 3-16 Cremers *et al*, 2009). Although this lessens the lag-time between brain and collection tube and increases the sample volume, there is also significant dilution of the sample. This leads to the next major consideration in microdialysis studies, the sensitivity of sample analysis.

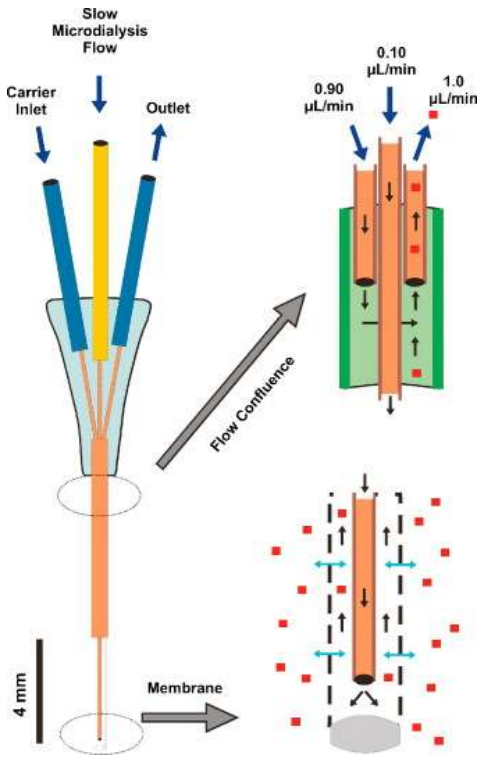


Figure 3-16 A MetaQuant probe
For ultraslow flow microdialysis a different probe type can be used in which the slow dialysis flow is supplemented by a carrier flow to aid faster sample collection. An artificial cerebrospinal fluid is typically delivered at 0.1 µl/min through the middle (yellow) inlet. The carrier flow (typically ultra-purified water) is delivered through the left (blue) inlet at 0.9 µl/min. The right outlet thus yields a combined outflow of 1 µl/min, consisting of 0.10 µl/min dialysate and 0.9 µl/min water. Reproduced from Cremers *et al* 2009 with permission.

3.6.1.2. Sample analysis

One of the main technical limitations of microdialysis is in the analysis of the samples by HPLC-ECD. Although this technique is sensitive (measuring fmol amounts per sample) the resolution of chromatograms and the presence of interfering peaks from other compounds often means that in practice the limit of detection is higher in samples than from standards. Alterations of the buffer can go some way to moving interfering peaks away from the peak of interest, however when wishing to analyse multiple components (e.g. both NA and DA) compromises must often be made to retain peak resolution. Within this study it was decided to assess NA alone as attempts to analyse both NA and DA gave variable baselines and poor detection of DA. The assessment of NA alone was much more successful as it allows the optimal separation of early running peaks (such as NA) on the chromatogram. An alternative to HPLC-ECD is the use of capillary electrophoresis and laser-induced fluorescence detection (Parrot *et al*, 2004). This method uses microdialysis to collect samples but require much smaller sample volumes for analysis, allows better temporal resolution (around 20 sec) than other detection methods. As with mass

spectrometry the equipment for this technique is expensive and was unavailable for the present study.

3.6.1.3. Drug delivery

Nomifensine (100 μM) was effective in increasing NA and DA levels in FC (3.4.1). *In vitro* it was seen that nomifensine (0.5 μM) was effective in inhibiting NA uptake into FC prisms (2.3.1). This large difference in drug concentration is in part due to efficiency of delivery of drug across the probe membrane. Several studies have sought to address the question of drug concentration in tissue after local delivery via the probe, with differing results. Marshall *et al* (1997) showed that over a 15-min period 0.5% of the nicotine delivered was found in the brain, leading to a calculation that the perfusion of 3 mM nicotine for 15 min at 4 μl/min gave approximately μM concentrations in the tissue. Shearman *et al* (2005) used longer infusion periods and a slower flow rate (30 min at 1 μl/min) and found that with 1 μM nicotine, levels in the brain were 10- 20% of that in the probe, with this increasing with longer perfusion periods. These estimates are quite different, as are the *in vitro* recoveries of neurotransmitter (6.9% in Marshall *et al* vs. around 20 % in Shearman *et al*) meaning that the estimation of drug concentrations delivered via a microdialysis probe is very difficult. These large differences between labs further emphasise the importance of consistency in probe types used. Drug diffusion over the probe membrane will depend on the molecular weight and the charges associated with the molecule as well as the active membrane area (Stenken, 1999) and so cannot be assumed to be the same from one drug to another, or the same as recovery of neurotransmitter. Diffusion away from the probe also gives a concentration gradient within the tissue. Although radioiodinated forms of 5IA exist due to its development as a PET ligand, they are not generally available so a drug diffusion study was not possible to quantify the exact concentrations within the brain tissue.

3.6.1.4. Synaptic overflow

Microdialysis does not measure levels of neurotransmitter at the synapse directly due to the size of the probe. Overflow of neurotransmitter around the synapse can be detected, and is influenced by release, diffusion and reuptake of the transmitter. For dopamine and noradrenaline sufficient diffusion away from the synapse occurs to give consistent recovery. This overflow has been shown to be action potential mediated by reduction by TTX and Ca^{2+} sensitivity (Westerink, 2000, di Chiara, 1990). For glutamate, synaptic transmission is much more tightly regulated; with a smaller amount of synaptic overflow occurring. The synaptic density of a region will affect the extrasynaptic monoamine content and so establishment of a stable baseline for comparison is important (Plock and Kloft, 2005). The increase in NA and DA seen in the current study (3.4.1) in

response to an infusion of nomifensine (100 μM) confirms the role of transporter mediated reuptake in modulating the levels of neurotransmitter. This is as expected as nomifensine is a monoamine transporter inhibitor, which inhibits both NET and DAT (Tuomisto, 1977).

The differential regulation of glutamate and the monoamines has led to the theory of 'wired transmission', mainly the GABA/glutamate system, and 'volume transmission' with the monoamines as a modulatory system (Fuxe *et al*, 2010). The 'volume' referred to is the extracellular space, the very place accessed by microdialysis techniques. This hypothesis was formed based on observations of extraneuronal DA fluorescence after amphetamine treatment (Fuxe and Ungerstedt, 1970). These observations were supported by the high incidence of non-synapse forming varicosities demonstrated by Descarries *et al* (1975). It has been seen that a single noradrenergic neuron arising from the LC has as many as 170,000 varicosities within the HC of which only around 15 % form synaptic connections (Umbriaco *et al* 1995). This evidence suggests that the measurement of extrasynaptic overflow of NA is therefore valid as a marker of physiological changes. The TTX sensitivity of NA release from varicosities is thought to be due to the propagation of action potentials after the opening of preterminal nAChRs (Vizi *et al* 1995; Sershen *et al*, 1997).

As neurotransmitters and other substances are removed by microdialysis there is the possibility that this will affect the extrasynaptic levels of DA or NA. Sam and Justice (1996) investigated this possibility by using a range of flow rates for sample collection. They did not find any significant effect of flow rate on DA estimation using a zero net flux method. This suggests that microdialysis will cause minimal neurotransmitter depletion, and have little effect on transmission once a concentration gradient has been established.

3.6.2. Study design

Local administration of drug via the microdialysis probe was chosen for this study to reflect the terminal field stimulation that takes place in the *in vitro* assay. Livingstone *et al* (2009) administered 5IA locally via the probe into FC and found increases in DA. In the current study two infusions of drug at different concentrations were given to animals, separated by 90 min aCSF. This allowed more data to be gathered from each animal whilst allowing transmitter levels to return to baseline between infusions and minimising desensitisation. The timing of the second infusion was based on data from Livingstone *et al* (2009), showing that the DA level has returned to baseline by 90 min after 100 μM or 1 mM 5IA infusion. In the present study the response to the second (higher concentration) drug infusion did not differ between the two groups with differing initial drug administration in FC, suggesting that desensitisation is minimal by the time of the second administration.

3.6.3. Response to 5IA in frontal cortex

The level of DA in several of the basal samples was below the limit of detection of the system. This meant that the calculation of percentage basal for the DA responses was less accurate than for NA, leading to greater variability. As DA was not consistently detected it was decided to assess NA alone for this portion of the study. This also allowed better separation of the NA peak as discussed above (3.1.5), so responses were clearer.

NA levels increased comparably in FC with the infusion of 1, 10 or 100 μM 5IA although the responses were much smaller than with nomifensine. Finding increases in NA levels that are comparable with a range of concentrations suggests that maximal responses at the top of the dose-response curve are being assessed. Taking into account the large differences between the findings of Marshall *et al* (1997) and Shearman *et al* (2005) a reasonable estimate of drug delivery across the probe may be around 5 %. This would give an estimate of drug concentrations in the nM range in the region of interest, depending on diffusion away from the probe. This shows that the potency of 5IA is extremely high as seen *in vitro*. Further experiments with a lower concentration range may be warranted and would be required to find a local EC_{50}. Other than Livingstone *et al* (2009) there is no published work on the effects of 5IA on microdialysates to the author's knowledge.

The responses to 5IA were smaller than those to nomifensine, which may be a good thing as increases in catecholamines that are too high cause dysregulation of FC and possibly HC function (1.2). The increases are similar to responses seen with nicotine previously and so any cognitive benefits may be of a similar size, although the relative magnitude of the responses after systemic injections would be a better measure in terms of a clinically relevant administration protocol.

The responses have fast kinetics, with rises in the first sample after start of drug infusion, which reach a maximum at the second sample after the start of administration. With 10 μM 5IA, the response does not reach the same peak and is slightly lower in the second than the first sample after the start of drug administration. Although this could be due to receptor desensitisation the same attenuated peak NA response is not seen with 100 μM 5IA treatments and so desensitisation may not be the cause of this effect.

The NA levels return to baseline swiftly upon the removal of drug with little or no residual effect. This shows a fast clearance of 5IA from the FC either by washout or metabolism or a desensitisation of the nAChRs to 5IA. Saji *et al* (2002) examined the metabolism of [^{125}I]-5IA after systemic injection. Around 90 % of radioactivity in brain homogenates could be extracted, and formed a single peak on HPLC indicating that no metabolised 5IA was present within the brain in samples taken at various times up to one hour after injection. In blood, the contribution of unchanged 5IA to the overall radioactivity fell quickly over time, with two additional metabolite peaks on HPLC. By 15 min after

injection only 18 % of the radioactivity could be attributed to unchanged 5IA. These results indicate that either brain metabolism is minimal or that any 5IA metabolised within the brain is quickly cleared. The much faster decline in 5IA levels in blood than brain suggests that the former of these explanations is the case, and so after administration via a microdialysis probe directly to the brain, as in the present study, minimal metabolism of 5IA will take place. This suggests that the fast return to baseline in NA responses after the removal of 5IA from the perfusion flow is due to washout or to receptor desensitisation.

3.6.4. Response to 5IA in hippocampus

As the HC was shown to have lower sensitivity of 5IA *in vitro* (Figure 2-8) it was decided on the basis of the results from FC to test at 1 and 100 μM 5IA. In HC responses to 5IA were markedly less than in FC and NA release did not reach significance above control at 1 μM or 100 μM. No significance was found for single timepoints, although when analysed by two-way ANOVA the aCSF and drug treated curves did differ. This is suggestive of subtle effects that were not significant at any timepoint in *post hoc* analyses.

The baseline response to manipulation seen in this study may have masked any significant drug effects. There was an increase in NA that correlated with the syringe changes in aCSF treated animals. This did not lead to significant differences from baseline; however this may have masked drug responses. The larger response to syringe manipulation in HC than FC is possibly due to the different function of the two brain regions as described in the introduction and further discussed below. Another explanation is that small pressure changes upon tubing switches may perturb the local environment, although as the perfusate flow is separated from the brain by the membrane this should be minimal. Changes to the aCSF in the 'old' vs. 'new' syringes (containing aCSF from the same batch) may occur whilst the syringe is flowing (and so not refrigerated) overnight, but this is unlikely to affect HC whilst leaving FC unaffected so should not be a significant factor. The HC is more involved in stress responses and spatial tasks, so it is probable that this larger response in aCSF animals is due to reaching over the animals to switch syringes although no overt stress responses were observed. This seems the most likely reason as the other explanations should affect FC and HC equally and such changes in aCSF NA levels were not seen in FC.

3.6.5. Regional differences

In HC a larger n (8) was used than in FC (4-5) to attempt to reduce variation, and overall it is no different to that from the FC data. The lack of significant response in HC is therefore likely to be due to differences in the magnitude of the response or the more responsive baseline than to a lack of numbers. Comparing the curve to those obtained from FC suggests that any response is of smaller magnitude with a less well defined peak shape.

Differences in the peak response to locally administered nicotine were found by Shearman *et al* (2005), with the FC showing a slightly larger peak response than the HC but with more variability over the administration period.

Although the responses were not significant in HC there seemed to be a slower return to baseline in HC than FC after drug administration. Local feedback via NA α_2-adrenoceptor autoreceptors may inhibit further NA release in the FC, leading to a faster termination of response to drug although α_2-adrenoceptors have been shown to be active in HC both *in vitro* (Allgaier *et al*, 1993; Milusheva *et al*, 1994) and *in vivo* (Abercrombie *et al*, 1988; Thomas and Holman, 1991). This suggests that autoreceptor modulation is similar between the two regions and so not responsible for the differences in NA peak shape.

Extrapolating from *in vitro* results, that are suggestive of α3β4* in HC as opposed to β2* nAChRs in FC, it may be possible that the slower return to baseline is due to nAChR subtype differences between these two regions. Differences in desensitisation of α3β4 and β2* have been demonstrated using electrophysiology in oocytes expressing known subunit combinations, with α3β4 nAChRs having a slower desensitisation and recovery in comparison with β2 containing nAChRs (Fenster *et al* 1997; Wu *et al* 2006). The subtypes involved could also be more complex, as discussed in section 2.6.3.2.

Overall the data presented here support differences in the subtype of nAChR involved in modulating NA release, with higher sensitivity to β2* selective agonist in FC than HC.

3.6.6. Further experiments

Further work on the actions of 5IA in inducing NA release is required. There is a need to test lower concentrations of 5IA administered through the probe in order to find a dose response relationship due to the extremely high potency observed so far. This would be important in order to compare EC_{50} values between FC and HC.

Antagonism of the response of FC DA to 5IA has already been shown with DHβE (Livingstone *et al*, 2009), and is likely to be the same for FC NA as inhibition has been seen *in vitro* (Figure 2-8). Antagonism with systemic mecamylamine of the HC responses may allow confirmation of a specific nicotinic effect causing the non-significant increase above aCSF. The use of a selective antagonist such as DHβE given locally would allow further confirmation of the subtypes of nAChR involved in FC and HC for comparison with *in vitro* data. As DHβE is a competitive antagonist, achieving an appropriate concentration to inhibit responses is critical as the inhibition may be overcome by high concentrations of agonist which may lead to the assumption that there is no β2* mediated component when in actuality it is just responding maximally.

To test the theory of differential sensitivity to desensitisation between FC and HC two possible protocols could be tested; either a longer infusion period of drug, with assessment to see whether responses decline over time, to separate washout from desensitisation, or repeated administration of the same concentration of 5IA to see if the magnitude of the responses stays the same. In order to optimise conditions and for comparison with Fu *et al* (1999), the interval between infusions could also be altered to investigate the timecourse of desensitisation and recovery of nAChRs.

Testing the effects on NA and DA of systemic administration of 5IA in various brain regions may give an indication of the functional profile of these responses. Systemic administration would be interesting to show if there are regional differences in responses. This may differ from local administration due to cell body vs. terminal field effects. 5IA readily penetrates the blood brain barrier (as it was designed to do so) meaning that it is a good candidate for systemic administration. Most studies with 5IA have so far focussed on its role as a PET ligand, and so use tracer doses to show β2* receptor availability (Saji *et al*, 2002). Systemic administration of 5IA has been recently shown to be effective in a number of behavioural tasks, in both cognitive enhancement and drug discrimination to nicotine (Smith *et al* 2007). Some studies have now been done with 5IA at behaviourally active doses, studying the effects on cognitive and learning tasks. In particular the responses in 5CSRTT and the odour span task have been assessed and 5IA has been show to be active at doses of 6 μg/kg compared with nicotine at 0.025-0.1 mg/kg (Shaoib *et al*, personal communication).

The tasks used are frontal cortex based, no reports of HC tasks are available to the author's knowledge, and so it may be that these doses are selectively targeting β2* nAChRs. NA may not be the main mediators of the behavioural responses as β2* nAChRs are the most common nAChR subtype, and so likely to have effects on many transmitter systems including DA. Systemically mediated behavioural effects may involve the interaction of multiple brain regions and neurotransmitters. Now that some studies have been done with systemic 5IA, behaviourally active doses are known and so work would focus on observing the changes in NA seen with these doses, and whether the responses in HC as well as FC. We would expect DA changes in the striatum as this region is known to contain β2* nAChRs and DA levels are higher than in FC and so more easily detectable. There may be responses in HC NA levels if β2* nAChRs are on cell bodies rather than terminal fields, or if there are network effects. The fact that all the noradrenergic neurons assessed from the LC contained β2* nAChR mRNA (Lena *et al*, 1999) suggests that β2* nAChR mediated effects may be important in modulating NA responses more widely than is found by terminal field stimulation.

3.7. Discussion - Fingerprinting

3.7.1. Neurochemical fingerprinting

The technique of neurochemical fingerprinting involves the assessment of a range of neurotransmitters and metabolites in tissue homogenates after drug administration. The restriction of this technique to a single timepoint for each animal limits the ability to create a timecourse. As with microdialysis the ability to create dose-response curves is limited in comparison with *in vitro* assays. The main advantage over microdialysis is the analysis of multiple compounds in a manner not possible for dialysates due to the larger quantities contained in homogenates. The use of homogenates means that assessment of compounds is in terms of total tissue content, not extracellular release. Whilst this allows the examination of changes in metabolism, it does not necessarily equate to changes in transmission. As entire brain regions are used for this technique it is less spatially defined than microdialysis and variation in dissection my affect results. Neurochemical fingerprinting is a much higher throughput technique than microdialysis, with a single injection of drug and collection of brain tissue at the appropriate time as opposed to surgery, a recovery period and lengthy sample collection periods. This means that although only a single timepoint or drug dose can be assessed for each animal it is possible to create dose response or time profiles relatively quickly although consideration must be given to minimising animal usage.

3.7.2. Study design

In this study it was chosen to assess the response to nicotine in the frontal cortex and in the striatum. These regions were chosen as they are expected to differ in their monoamine content, have accepted monoamine release in response to nAChR stimulation and are easy to dissect quickly, a prime concern to prevent post-mortem metabolism occurring. The range of metabolites observed differed from Tani *et al* (1997) who observed NA, MHPG, DA, DOPAC, 5HT and 5HIAA. In the present study NA, DA, DOPAC, HVA, 3MT, 5HIAA and 5HT were assessed. This gives a more detailed picture of DA metabolism, likely to be the main aspect to vary between these regions.

In order to determine the optimal timepoint at which to assess the effect of nicotine injection on monoamine metabolism a pilot study was undertaken. The times investigated were based on knowledge of the fast metabolism of nicotine in the mouse ($t_{½}$ ~6.9 min) and a similar previous study (Tani *et al*, 1997) that found maximal effects in most parameters at 15-30 min after nicotine administration. Tani *et al* (1997) also influenced the dose selection, as did Matta *et al* (2007). Two mice in the highest dose group (3 mg/kg) died of respiratory failure during the main experiment. Other papers had previously used this and higher doses and all the animals in the pilot study received this dose, so this problem was unexpected. The ED_{50} for induction of clonic seizures (a known side effect of high dose

nicotine) is >5 mg/kg in most strains (Matta *et al*, 2007). Data from the appropriate timepoint in the pilot study were included in the analysis of the main study data.

As 3 mg/kg nicotine is also associated with significant toxicity the results from 0.3 and 1 mg/kg nicotine groups are more reliable. In these groups the only significant response was an increase in DA in the PFC. Another factor that must be taken into consideration in the analysis of these results is that acute nicotine is anxiogenic, and the brain regions investigated respond to stress. This may change the interpretation of the results as it changes the assumption that any increase in neurotransmitter turnover is due to a nAChR mediated release in striatum or PFC, and raises the possibility of more global effects on the catecholaminergic pathways innervating these brain regions.

3.7.3. DA release and metabolism

Figure 3-17 illustrates DA release, reuptake and metabolism pathways. From the knowledge of these pathways the different profiles given by drugs with different mechanisms of action can be explained. The administration of *d*-Amphetamine increases 3-MT and reduces DOPAC and DA due to a combination of DA release, and reuptake inhibition, forcing metabolism down the extracellular route. A DA reuptake inhibitor such as GBR-12909 will decrease DA turnover, leading to reduced DOPAC levels, and a monoamine oxidase inhibitor (MAOI) such as tranylcypromine leads to an increase in 3-MT and a decrease in DOPAC and homovanillic acid (HVA).

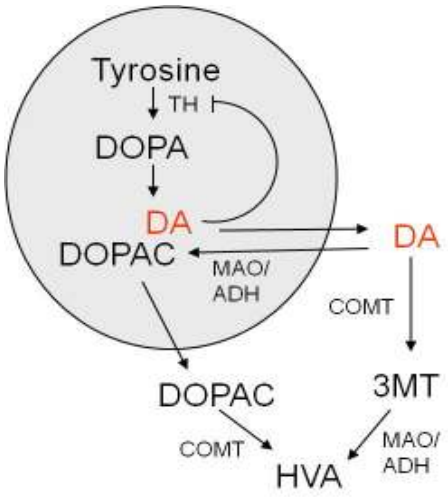


Figure 3-17 A schematic diagram of the release of DA and its metabolism
Metabolism of DA by the intra- and extracellular routes. MAO - Monoamine oxidase, ADH - Aldehyde dehydrogenase, COMT – Catechol O-methyl transferase, TH – Tyrosine hydroxylase, DA – Dopamine, DOPA – Dihydroxyphenylalanine, DOPAC - 3,4-Dihydroxyphenylacetic acid, HVA – Homovanillic acid, 3MT - 3-Methoxytyramine

3.7.4. Control of DA metabolism

Tyrosine hydroxylase (TH) is the rate limiting enzyme is the formation of catecholamines. As the catecholamines can be toxic their formation is tightly regulated.

Tyrosine hydroxylase is regulated by many different mechanisms including feedback inhibition by the catecholamines (Kumer and Vrana, 1996). There are two DA binding sites on the tyrosine hydroxylase molecule, a high and a low affinity site (Gordon *et al*, 2008). The high affinity site has very slow dissociation kinetics and is generally fully occupied at physiological cytosolic dopamine concentrations. The low affinity binding site is thought to be the main regulator of catecholamine synthesis DA binding at this site reduces TH activity by around 12-fold (Gordon *et al*, 2008).

Upon release of DA from the cell, vesicles are loaded from the cytosolic pool lowering the concentration of DA within the cell. This reduces the binding at the low affinity site, relieving the inhibition of TH and therefore increasing the production of DA. When DA levels increase binding occurs and TH activity is reduced. This dynamic regulation of TH activity gives close control of catecholamine levels within the cell, ensuring that a releasable pool is readily available, without the risk of toxicity from oxidized catecholamines.

3.7.5. Tranylcypromine

Tranylcypromine gives the expected results for its mechanism of action as a MAOI. This blocks the intracellular route of DA metabolism leading to a rise in DA, a fall in DOPAC and HVA which both depend on MAO for their formation, and a rise in 3MT. The MAO metabolism of 5HT to 5HIAA is also inhibited.

In striatum tranylcypromine increased DA, reduced DOPAC, decreased HVA and increased 3MT (Figure 3.13). There was also a reduction in 5-HIAA. There was no significant change in NA or 5-HT, although there was a trend toward an increase in 5HT. There was a decrease in the DOPAC/DA ratio, indicative of a reduction in firing. There is also a reduction in the DOPAC/HVA ratio, which is due to the decrease in intracellular metabolism, as expected from the known mechanism of action of tranylcypromine. The increase in 5HIAA/5HT ratio is suggestive of an increased turnover of 5HT.

In the PFC tranylcypromine again shows the effects that would be expected from its known MAO inhibition; however there is a decrease in NA, which is likely to reflect a marked reduction in synthesis with normal levels of release by exocytosis. Another explanation is that the decrease in NA is due to increased metabolism by COMT extracellularly, with insufficient synthesis to replenish cellular content.

3.7.6. Nicotine

The profiles of changes in metabolites differ between tranylcypromine and nicotine. The only significant response in the striatum with nicotine was an increase in DOPAC at the 3 mg/kg dose which as discussed above may be irrelevant due to the toxicity of nicotine at this dose (3.7.2). The ratio of DOPAC/DA is increased after 1 mg/kg nicotine

treatment, suggesting that there is increased DA turnover (a surrogate for increased firing), which is not seen as clearly by observing the individual compounds. The ratio of DOPAC/HVA is also increased after 1 mg/kg nicotine treatment as opposed to a decrease after tranylcypromine treatment and suggests that the increased DOPAC seen in this region is due to the increase in DA to DOPAC metabolism being faster than that of DOPAC to HVA, leaving a relative excess of DOPAC.

In FC nicotine induced an increase in DA at both 1 mg/kg and 3 mg/kg. DOPAC only increased at 3 mg/kg. There are decreases in NA (at 3 mg/kg) and 5HIAA at 1 and 3 mg/kg. Nicotine induced no significant changes in HVA or 5-HT levels. The ratios of transmitters and metabolites mostly show a similar profile of responses as seen in striatum; however DOPAC/DA is not significant, whereas HVA/DA is in FC. The ratio of DOPAC/HVA is strongly changed in favour of a relative increase in DOPAC. These changes to the ratio indicate that once again the increase in production of DOPAC due to higher turnover outstrips the increase in rate of HVA production, suggesting COMT as a rate limiting step in comparison with MAO/ADH action. The changes in 5HIAA/5HT are also significant in FC at 1 mg/kg nicotine where they were not in striatum; however the magnitude of the changes is similar.

The changes in striatum in response to nicotine suggest an increased turnover of DA, which is to be expected upon nicotine administration as it elicits release. It may be expected to see increased HVA also as the end metabolite of DA; this is not seen possibly due to a rate limiting effects of COMT, or rapid clearance of HVA, preventing a buildup.

In FC there is an increase in DA as well as DOPAC. Keeping in mind that the levels measured are total tissue content, and not extracellular content as for microdialysis, this is counter intuitive within the model of DA metabolism presented above where DOPAC is formed from DA that has been released and then taken back up into cells. A possible explanation for this may be a differing reuptake mechanism in FC from striatum, as has been previously postulated (see 2.6.2 and 3.7.7).

3.7.7. Differences between PFC and striatum

The changes induced differed between the two brain regions. There is evidence that the nAChR subtypes involved in DA release in FC and striatum differs with α4β2* nAChRs mediating release in FC and the striatum also containing around 30 % α-conotoxin MII sensitive α3/α6β2* nAChRs (Kulak *et al*, 1997; Grady *et al*, 2007; Livingstone *et al,* 2009). Differentiating between these in this assay may be difficult due to a lack of selective ligands. α-conotoxin MII is a selective antagonist of α3/α6β2* containing nAChRs, however it does not cross the blood brain barrier and intracranial administration would not be feasible as it is invasive and the distribution of the drug may not be wide enough. As

both regions are mainly regulated by β2* containing nAChRs (Livingstone et al, 2009), it is unlikely that there are large enough differences in the subtypes involved to have caused the differences observed in these experiments.

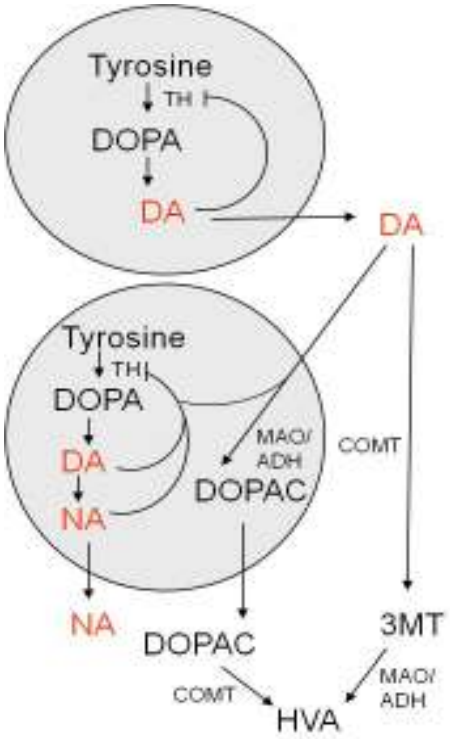


Figure 3-18 A schematic diagram of the release, and metabolism of DA and NA in FC
MAO - Monoamine oxidase, ADH - Aldehyde dehydrogenase, COMT – Catechol O-methyl transferase, TH – Tyrosine hydroxylase, NA – Noradrenaline, DA – Dopamine, DOPA – Dihydroxyphenylalanine, DOPAC - 3,4-Dihydroxyphenylacetic acid, HVA – Homovanillic acid, 3MT - 3-Methoxytyramine

The striatum is a region of the brain with dense dopaminergic innervation, whereas the dopaminergic innervation of the frontal cortex is sparser. It has been suggested that the reuptake mechanism of DA differs between FC and striatum, with the NET playing an important role in the FC (Williams and Steketee, 2004). Inhibition with nisoxetine which was shown to be selective for the NET over DAT by Wong and Bymaster, (1976), significantly reduces DA uptake at concentrations known to be selective for the NET. A possible model of the reuptake and metabolic pathways in the frontal cortex with a contribution of NET to DA uptake is shown (Figure 3-18). In this model DA is released as normal then taken up by NAdrenergic cells in the frontal cortex, within noradrenergic cells DA is metabolised to form DOPAC. This explanation leads to the assumption that the different route of uptake removes the inhibition on tyrosine hydroxylase DA production (3.7.4) in the initial dopaminergic cells; however as tyrosine hydroxylase is also the rate limiting enzyme in the production of NA, the DA taken up into noradrenergic cells may inhibit NA production. This model explains the concomitant rise in both DA and DOPAC after nicotine administration in this region as well as the reduction in NA.

The different reuptake mechanism between cortical regions and striatum may be due to the different levels of DAergic innervation. DAT levels vary between these regions both in terms of number of terminals and transporters per terminal (Sesack *et al*, 1998a; 1998b) meaning that once DA has diffused away from its releasing cell it is more likely to be taken up by the NET as they are more prevalent. This mechanism has also been recently suggested to play a role in DA regulation in HC (Borgkvist *et al*, 2011). Another transporter mechanism that is widespread within these regions is the plasma membrane monoamine transporter (PMAT) which is a high volume, low affinity transporter with selectivity for DA and 5-HT over NA, epinephrine and histamine (Engel *et al*, 2004; Dahlin *et al*, 2007). This may provide an additional mechanism for monoamine clearance in those regions where spill over from the synapses is expected.

The use of a selective NA uptake inhibitor (such as atomoxetine) in the current paradigm would allow the contribution of NET in DA clearance in FC compared to striatum to be assessed. Atomoxetine is known to be an effective treatment for ADHD, which is primarily treated with stimulant medications which increase DA levels. Stimulant medications also increase NA in PFC, as well as increasing DA in PFC and striatum. It has also been shown that the most efficacious drugs clinically increase both NA and DA transmission (Heal *et al,* 2008). Atomoxetine has been shown to increase both NA and DA in the PFC (Bymaster *et al* 2002). The rise in DA levels only happens in the FC (Swanson *et al*, 2006) whereas NA levels rise in all regions tested, suggesting that a different method of DA regulation (with significant uptake by NET) exists in PFC as suggested in Figure 3-18. This paper however did not find detectable baseline levels of DA in HC, and so comparison with the suggestion of Blomkvist *et al* (2011) that HC DA is also taken up by the NET is not possible. To the author's knowledge there are no selective inhibitors of PMAT available yet, and so assessing the contribution of this transporter would not be possible at the present time.

3.7.8. Further experiments

Use of selective agonists to assess regional differences in nAChR subtype involvement may not reveal many differences as both known to mainly be regulated by β2* nAChRs as far as DA release is concerned. The use of antagonists is limited due to lack of selectivity, and poor blood brain barrier penetration of conotoxins. The use of α7 nAChR selective drugs may be possible in this paradigm, especially with the ability of a selective positive allosteric modulator such as PNU-120596 to increase the α7 nAChR mediated portion of the effect.

The assay could also be extended to the HC to attempt to measure differences in the subtypes involved to link with *in vitro* work. This may pose problems as the HC is more

difficult to dissect within the time frame needed to prevent post-mortem changes in the metabolite profile. To see if there are differences between FC and HC (as for *in vitro* preparations from rat see 2.3.2) a selective drug such as 5IA would be used, this drug has been shown to be systemically active only recently and so the doses that would activate one population of nAChR selectively are difficult to estimate. As a smaller subset of nAChRs would be activated by 5IA there may be a smaller risk of toxicity than for nicotine.

The administration of atomoxetine would allow the contribution of different reuptake mechanisms between striatum and FC to be assessed. Based on Bymaster *et al* (2002) differences in the profile of changes in the two regions would be expected, with little change to DA metabolites in striatum and larger changes in FC. There is also evidence that the HC, again relatively sparsely innervated by DAergic neurons also has a proportion of DA reuptake mediated via NET (Borgkvist *et al*, 2011).

Another modification to the assay would be to observe the changes in NA metabolites. The system used was optimised for the DA metabolites as well as NA and 5HT, to analyse the NA metabolites in more detail would mean losing the detail for the DA metabolites. As NA is generally at lower concentrations within the brain due to sparser innervation the metabolites are also likely to be at lower concentrations and so there may be issues with the sensitivity of detection for this type of analysis.

3.8. Summary and implications

The data presented in this chapter suggest that the regulation of monoamines varies across the brain. NA release in response to local β2* nAChR stimulation varies between FC and HC, with greater responses that are cleared more quickly in FC. The regulation of DA in FC is different to that in striatum, with evidence to suggest that a model including clearance by NET may be appropriate, although this has not been tested in this study.

The use of nicotinic ligands as therapeutics has been discussed in sections 1.4 and 2.6.3. The data presented in this chapter show that there are many factors to consider when attempting to optimise neurotransmitter function. Both release and reuptake affect transmitter levels, with the regulation of metabolism of drug and transmitters also a factor. This emphasises the need for drugs to be tested in a variety of paradigms. The precise mechanisms of action of some drugs that have been shown to be behaviourally and clinically efficacious are not well known. Elucidating these mechanisms by the use of microdialysis and examination of the neurotransmitter metabolite profile may help define pharmacological targets for the future.

Chapter Four

4. Summary and Future Directions

4.1. Summary of results

4.1.1. In vitro

The use of a high throughput 96-well plate based filtration assay in Chapter 2 allowed the assessment of [^{3}H]-NA release from rat brain prisms. This thesis presents evidence that the regulation of [^{3}H]-NA release from FC prisms is regulated by β2* nAChRs. This is contrasted with the HC, where results are in agreement with α3β4* nAChRs as the main subtype involved in modulating NA release. This difference is in spite of the fact that both regions contain primarily α4 and β2 subunits although HC does indeed contain a higher proportion of β4 nAChR than does the cortex (Gotti and Clementi, 2004). A proportion of the response in both regions was mediated indirectly via the actions of GABA, possibly acting in an excitatory manner through $GABA_A$ receptors. Interaction with the GABA system has previously been postulated by Barik and Wonnacott (2006) and Leslie *et al* (2002).

Neither acute nor chronic *in vivo* nicotine treatment altered the profile of *in vitro* nicotinic responses in FC or HC. This is in agreement with Barik and Wonnacott (2006), although that paper found α7 nAChR mediated NA release from HC prisms which was increased during withdrawal. In the current study basal [^{3}H]-NA efflux was increased in HC during withdrawal from chronic nicotine treatment but there were no differences in response to choline. [^{3}H]-epibatidine binding was increased in tissue from chronically treated animals, and this upregulation persisted during early nicotine withdrawal.

A number of analogs of cytisine were tested for their ability to displace [^{3}H]-epibatidine binding from P2 brain membranes. Most of the compounds tested displaced the binding with similar or lower potency to cytisine itself. A number of the compounds were selected for trial in neurotransmitter release assays. Most of the selected compounds showed little or no ability to elicit neurotransmitter release, suggesting they act as weak partial agonists or competitive antagonists. None of the compounds were more efficacious in eliciting neurotransmitter release than cytisine showing that they remain partial agonists.

4.1.2. In vivo

In vivo microdialysis was used to monitor NA levels in the brain. Local administration of nomifensine increased the level of NA, showing that administration of drug via the perfusate flow is efficacious. In FC significantly increased NA in response to local administration of selective β2* nAChR agonist 5IA was observed. Increased DA levels in this region during 5IA administration have previously been reported (Livingstone *et al*, 2009). In HC with administration of the same concentrations of 5IA the increase in

NA level was not significant. Full differentiation of FC and HC responses was difficult, although the HC responses seemed to be blunted in comparison with FC. Overall the differences in responses may support *in vitro* work suggesting differing nAChR subtypes in the terminal field in these two brain regions, although further work is needed to confirm this.

The profile of neurotransmitters and their metabolites 15 min after systemic administration of nicotine was observed in homogenates of mouse brain. In striatum, a richly dopaminergic region, nicotine produced a profile of responses consistent with increased turnover of DA. In FC the profile was broadly similar, although there is an increase in DA as well as its metabolites, indicating increased production. It is proposed that differences in the reuptake of DA within the FC may account for this as some DA is taken up by the NET in FC (Williams and Steketee, 2004).

4.2. Comparison of techniques

As discussed the main drawback of the 96-well *in vitro* assay is the very fact that it is *in vitro*, therefore lacking the connectivity and tonic innervation present *in vivo*. Its main advantage is to greatly increase throughput and allow the rapid construction of concentration response curves. *In vivo* techniques such as microdialysis are not good for the construction of dose-response curves as only 1-2 concentrations per animal can be reliably assayed for most drugs. The technique is costly in terms of time and animals used and so one of the aims of these assays was to confirm the translatability of the *in vitro* release assay for guidance to design microdialysis studies and define the questions to be asked. Validation of the translatability of *in vitro* to *in vivo* assays is difficult due to the small number of conditions examined in the microdialysis study, however finding differences in the terminal field responses of FC and HC as seen *in vitro* is encouraging. The difficulty in accurately determining the concentration of drug in the tissue due to diffusion gradients makes direct comparison of the concentrations administered by the two techniques complicated. A true test would be to examine full dose-response curves, but of course the point of the *in vitro* work is to reduce the number of animals used *in vivo*.

Overall the results from both techniques indicate that the nAChR subtypes involved in NA release differ between the HC and FC. This shows that the *in vitro* assays are a good indicator of terminal field responses *in vivo*. Extension of the microdialysis work to include systemic injection of agonist may show that the extensive interactions between brain regions have modulatory influences and so that targeting particular regions is less straightforward than simply targeting selected nAChR populations. Dopaminergic inputs into the HC from the VTA may increase their firing after stimulation by β2* agonists, so although the terminal field is not stimulated directly HC responses may be affected.

4.3. Subunit differences

The majority of noradrenergic projections in the brain arise from the LC. Although all cells examined in the LC contain β2 nAChR mRNA (Lena *et al*, 1999) the present work, in agreement with previous studies, has found a lack of involvement of β2 selective ligands on NA release in HC. The finding that FC NA release is influenced by β2* nAChRs shows that the mRNA can be translated and form functional receptors in the terminal regions of these cells. Hill *et al* (1993) showed that expression of the β2 subunit is widespread, and greatly in excess of that which arrives the plasma membrane as functional receptors. This may explain the relative abundance of β2* mRNA. Alternatively the β2 subunits may not be expressed in the terminal field of noradrenergic neurons in HC. Gotti and Clementi (2004) immunoprecipitated nAChRs from various brain regions and found high levels of β2 in both cortex and HC, indicating that this subtype are indeed expressed in this region although the neuronal type was not identified. As discussed in Chapter 2 it is possible that the nAChRs on noradrenergic terminals in the HC contain β2 subunits, but in combination with β4 subunits. As occupation of two binding sites on the receptor is required for optimal function the lower affinity binding site may determine the perceived affinity of the receptor as a whole. This would give a much less sensitive receptor in HC if β2 is found in combination with β4 subunits.

Overall, no matter the reason, β2* nAChRs are more important in the modulation of NA release in FC than HC, and this difference could open therapeutic avenues.

4.4. Importance of differences

As discussed earlier the differences in subtypes of nAChR involved in NA release between FC and HC may be important for targeting certain functions, or enhancing function whilst avoiding side effects. Many of the side effects of nicotinic drugs (nausea etc) are mediated by α3β4 nAChRs, therefore HC responses would be difficult to enhance as this is a major subtype involved in the release of NA in this region. FC NA could be more effectively targeted by use of β2* nAChR agonists, however striatal DA is also strongly influenced by β2* and so full agonists may have some abuse liability. Indeed the β2 subunit is important for drug discrimination and self-administration (Besson *et al*, 2006; Smith *et al*, 2007). 5IA is self administered by rats previously trained to administer cocaine (Liu *et al* 2003) and so may have abuse potential. Partial agonist therapy has been used with some success as a smoking cessation therapy however selectivity is often an issue still. The novel compounds presented here seem to show similar or reduced efficacy and potency at heteromeric nAChRs; however the selectivity profile has not been ascertained.

Systemic administration of drugs (as would be required for a therapeutic application) may give differing profiles of responses to local administration whether *in*

vitro or *in vivo*. The many interconnections between cortical areas and the midbrain in particular suggest powerful feedback loops that may influence responses to drugs. The HC also has extensive connections with other brain regions and is innervated by midbrain dopaminergic neurons. These are likely to be sensitive to β2* selective ligands, and so there may be extensive modulation of HC activity not seen in the current experiments.

Another way to target neurotransmitter levels is with selective reuptake inhibitors. Atomoxetine is in use for ADHD to increase FC NA levels. It also increases FC DA due to the uptake by NET in this region (Williams and Steketee, 2004). It has recently been demonstrated that the NET regulates DA uptake in the HC also (Blomkvist *et al*, 2011). Atomoxetine does not target the FC selectively and has a range of side effects. Nicotinics may be a useful adjunct as they could increase the release of NA and DA in FC, which would then remain elevated for longer due to the reuptake inhibition. This may allow usage of lower doses of atomoxetine and of the nicotinic agents than would be effective alone, assuming that the side effect profile is due to effects in other brain regions.

The usefulness of animal models must also be considered. Whilst giving a good surrogate for some aspects of behaviour, many animal models require much inference to extrapolate to clinical models. Models of many disorders only characterize some of the deficits seen in the clinic. Modelling complex neurodegenerative disorders such as Parkinson's disease for example requires the acute administration of neurotoxin, which whilst potentially providing an excellent model for neurotoxic lesions may not accurately reflect the progression of a disorder. Another drawback is the existence of species differences. Whilst for many cellular processes these are of minimal importance there is evidence that the predominance of different nAChR subtypes, for example, varies greatly between species with very little expression of α2 in mice, some expression in rats and widespread expression in primates. This means that some ligand interactions or prospective targets may not be discovered in rodent based animal studies, potentially risking side effects or missing an important therapeutic target.

4.5. Future directions

The development of lines of nAChR knockout mice has allowed the separation of some aspects of the function of nicotine, but it is difficult to know to what extent the remaining subunits compensate for those removed. This may mean that conclusions drawn from the use of knockout mice about the effect of the loss of a specific subunit may be due, in part, to increased expression of another (Champtiaux *et al*, 2003). The absence of some subunits may affect brain development; extensive signalling with α7 nAChRs is known to be important for the maturation of GABAergic system (Liu *et al*, 2007; Campbell *et al*, 2010).

The development of more selective ligands for each possible subunit combination may help address these issues, however they would not be able to separate the more complex subtypes with two (or three) distinct binding sites. The generation of concatamers of nAChR subunits can allow the investigation of how more complex subtypes respond to ligands, however this expression will be in heterologous systems, and so may not represent subunit compositions that exist *in vivo*.

Although targeting subtypes more effectively can help overcome side effects and so improve drug tolerability ligands that are more selective may not be the answer as therapeutics, for example the actions of antipsychotic drugs are across a broad range of receptor types, and the balance of these actions appears important for their efficacy. Investigation of the effect of targeting two or more known nAChR subtypes in combination may show synergistic effects and give new strategies for improving unmet treatment needs in many conditions.

Whilst the work in this thesis goes some way to improving the definition of the nAChR subtypes involved in the regulation of NA release in the rodent brain, there is still work to be done.

Appendices

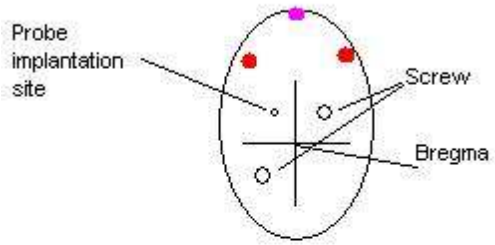
Appendix 1

TABLE 1. Numbering of ligand-binding residues in AChBP compared to *Torpedo* and muscle α_1, γ, and δ and human α_7 subunits

Loop	AChBP	α_1	γ	δ	α_7
A	Tyr89	Tyr93			Tyr93
B	Trp143	Trp149			Trp149
C	Tyr185	Tyr190			Tyr188
	Cys187	Cys192			Cys190
	Cys188	Cys193			Cys191
	Tyr192	Tyr198			Tyr195
D	Trp53		Trp55	Trp57	Trp55
	Gln55		Glu57	Thr59	Gln57
E	Arg104		Leu109	Leu111	Leu109
	Val106		Tyr111	Arg113	Asn111
	Leu112		Tyr117	Thr119	Gln117
	Met114		Leu119	Leu121	Leu119
F	Tyr164				
			Asp174	Asp180	Asp164

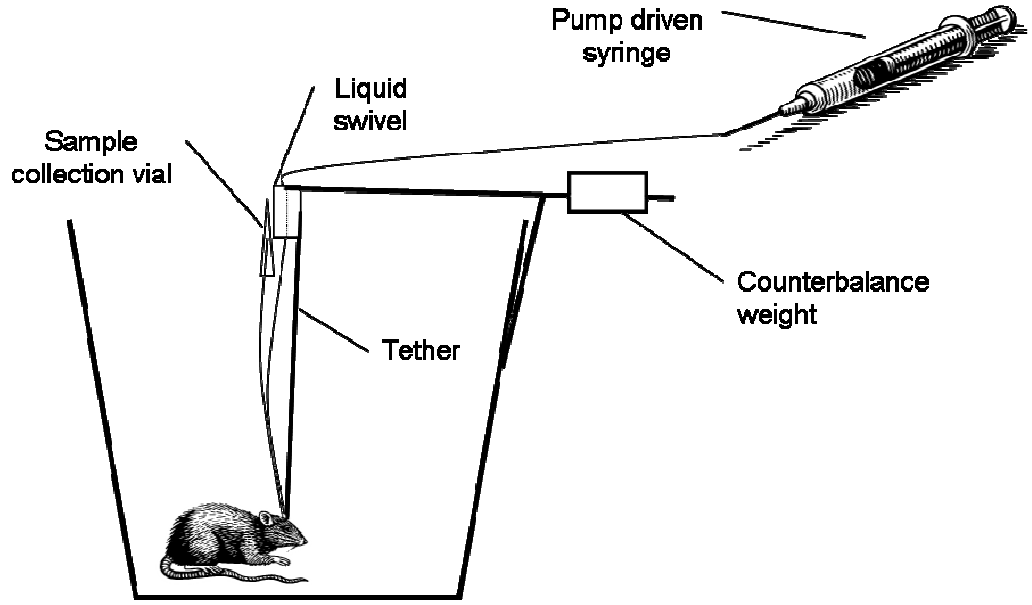
Reproduced from Smit et al, 2003 with permission

Appendix 2



Probe and screw implantation

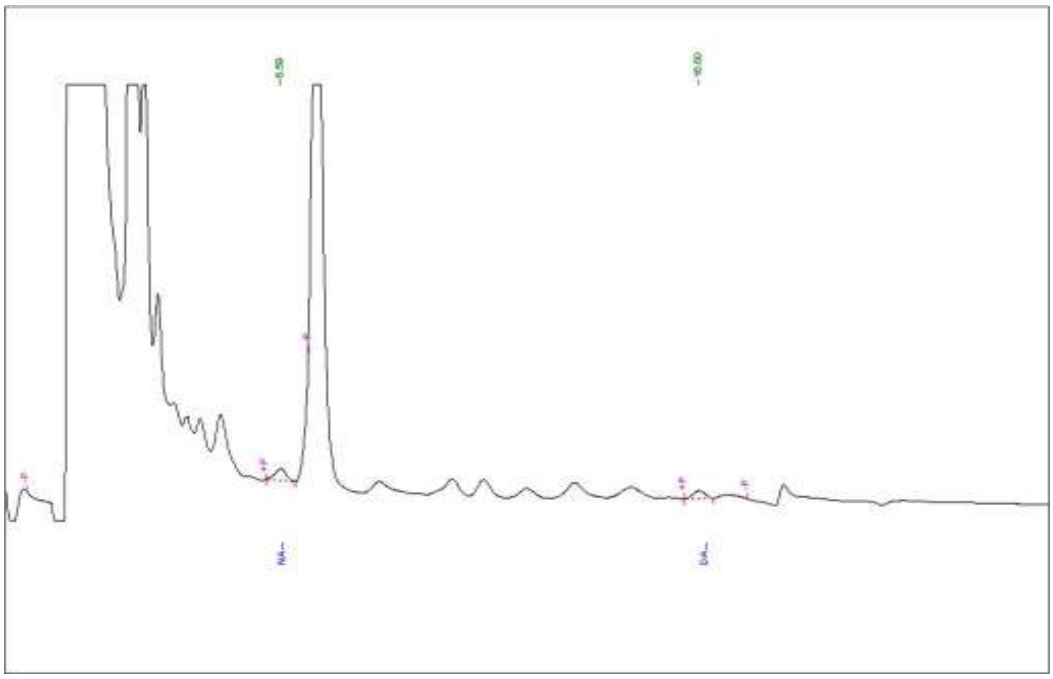
Holes are drilled for the probe and two anchor screws. For the frontal cortex anchor screws are placed in the contralateral anterior and ipsilateral posterior skull plates to ensure firm fixing, for the hippocampus the two posterior skull plates have screws implanted. The microdialysis probe is lowered into position and a copper wire tether is attached to one screw and aligned with the probe. Probe, screws and tether are enclosed within a layer of dental cement.



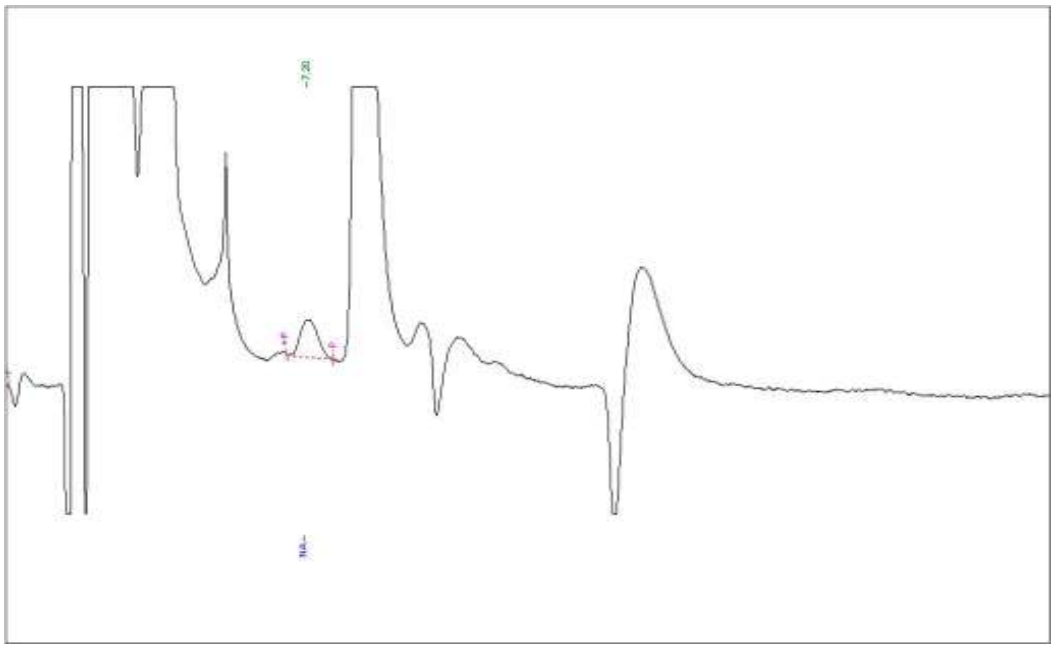
Microdialysis setup

After surgery the rat is attached to inlet and outlet tubing with aCSF flow driven by a syringe pump. Inlet tubing passes through a liquid swivel to allow free movement of the animal without twisting of the tubing. Samples are collected from the outlet tubing just below the level of the liquid swivel into polypropelene vials. A counterbalanced arm is used to take the strain of the animal's movement via a tether spring, which is attached to a copper wire tether implanted within the dental cement on the animal's skull. Bedding is provided and food and water are available *ad libitum* to the animal.

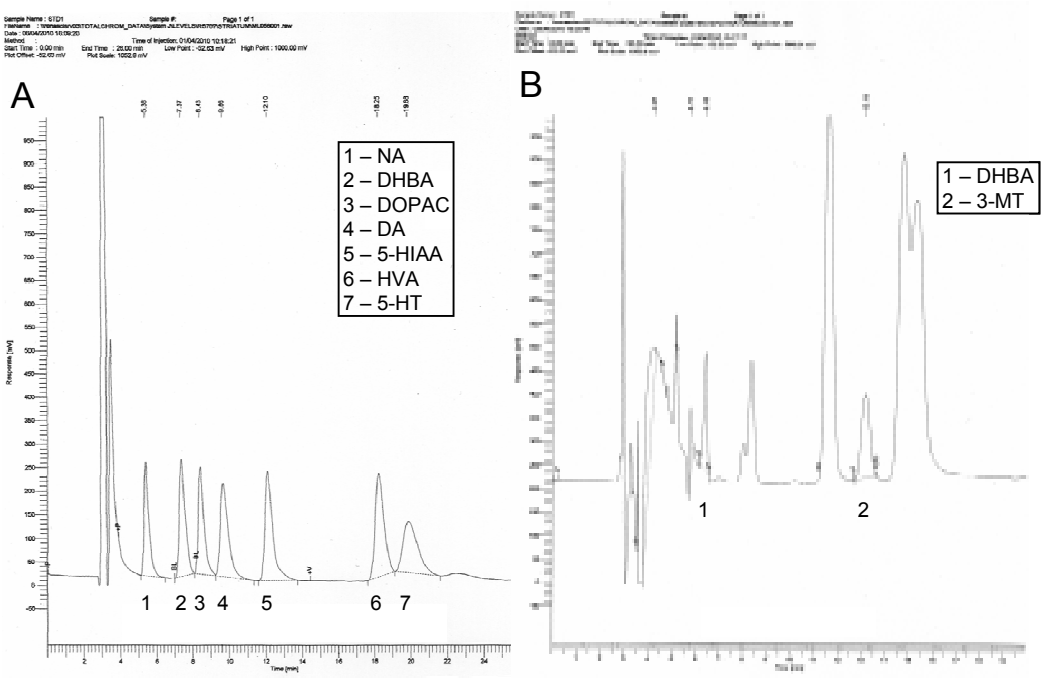
Appendix 3



Example chromatogram for NA and DA together
Trace is the first nomifensine sample from one animal containing 17.5 fmol NA and 5.5 fmol DA. Basal sample DA levels were below 2 fmol, with some below the limit of detection. This was the case for each set of samples analysed with the mix method, and detection of NA was sometimes compromised by the shifting of peaks between sample sets.



Example chromatogram for NA alone
Trace is a representative example of analysis for NA alone. Peak is 64 fmol NA and is well resolved from neighbouring peaks.



Example standard chromatograms

A) monoamine and B) 3MT system. Five µl of calibration standard was injected onto each HPLC system. This equates to 0.2 ng of 3-MT, 0.5 ng of NA, DHBA, DA, DOPAC, 5-HIAA and 5-HT and 1.0 ng of HVA on column. Peak area is calculated from integration of the curve above baselines.

Appendix 4

FC pilot data

Dependent variables		FcDa	FcNa	FcDopac	Fc5hiaa	FcHva	Fc5ht	
Treatments:	Group	A Saline	B 10 min	C 15 min	D 30 min			
Transformation:	Log							
ANOVA table for			FcDa					
Source	df	ss	ms	F	p			
Group	3	0.12454	0.04151	1.49	0.29			
Residual	8	0.22363	0.02795					
Shapiro-Wilk test for normality				p=	0.087			
ANOVA table for			FcNa					
Source	df	ss	ms	F	p			
Group	3	0.04598	0.01533	5.7	0.022*			
Residual	8	0.02151	0.00269					
Shapiro-Wilk test for normality				p=	0.614			
ANOVA table for			FcDopac					
Source	df	ss	ms	F	p			
Group	3	0.00602	0.00201	0.36	0.784			
Residual	8	0.04468	0.00558					
Shapiro-Wilk test for normality				p=	0.71			
ANOVA table for			Fc5hiaa					
Source	df	ss	ms	F	p			
Group	3	0.0542	0.01807	7.49	0.010*			
Residual	8	0.01931	0.00241					
Shapiro-Wilk test for normality				p=	0.871			
ANOVA table for			FcHva					
Source	df	ss	ms	F	p			
Group	3	0.05295	0.01765	2.29	0.155			
Residual	8	0.06169	0.00771					
Shapiro-Wilk test for normality				p=	0.286			
ANOVA table for			Fc5ht					
Source	df	ss	ms	F	p			
Group	3	0.00247	0.00082	0.23	0.875			
Residual	8	0.02903	0.00363					
Shapiro-Wilk test for normality				p=	0.5			

Means and comparisons (The LSD test) for							FcDa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	32.215	13.55					
B	3	29.657	0.549	A	92.1	44.6,190.0	-0.26	0.799
C	3	41.126	5.511	A	127.7	61.8,263.5	0.78	0.46
D	3	54.134	2.678	A	168	81.4,346.9	1.65	0.137
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							FcNa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	368.79	26.96					
B	3	276.81	23.13	A	75.1	59.9,94	-2.94	0.019*
C	3	268.64	17.93	A	72.8	58.2,91.2	-3.25	0.012*
D	3	256.06	12.07	A	69.4	55.5,86.9	-3.74	0.006**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							FcDopac	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	43.355	7.308					
B	3	39.155	3.135	A	90.3	65.3,124.9	-0.73	0.489
C	3	44.88	1.554	A	103.5	74.9,143.1	0.25	0.812
D	3	43.582	2.563	A	100.5	72.7,139.0	0.04	0.971
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Fc5hiaa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	111.97	9.661					
B	3	78.87	1.529	A	70.4	56.9,87.2	-3.79	0.005**
C	3	80.721	4.727	A	72.1	58.3,89.2	-3.54	0.008**
D	3	76.197	5.808	A	68	55.0,84.2	-4.17	0.003**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							FcHva	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	121.55	5.821					
B	3	83.557	10.06	A	68.7	47.0,100.6	-2.27	0.053
C	3	83.651	12.72	A	68.8	47.0,100.7	-2.26	0.053
D	3	93.124	11.26	A	76.6	52.4,112.1	-1.61	0.145
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Fc5ht	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	837.17	42.27					
B	3	765.04	79.48	A	91.4	70.4,118.7	-0.8	0.449
C	3	812.71	24.2	A	97.1	74.8,126.1	-0.26	0.8
D	3	815.82	87.2	A	97.5	75.1,126.5	-0.23	0.825
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Striatum Pilot data

Dependent variables		StrDa	StrNa	StrDopac	Str5hiaa	StrHva	Str5ht	Str3mt
Treatments:	Group	A Saline	B 10 min	C 15 min	D 30 min			
Transformation:	Log							
ANOVA table for			StrDa					
Source	df	ss	ms	F	p			
Group	3	0.0065	0.00217	2.43	0.14			
Residual	8	0.00712	0.00089					
Shapiro-Wilk test for normality				p=	0.053			
ANOVA table for			StrNa					
Source	df	ss	ms	F	p			
Group	3	0.02868	0.00956	1.64	0.255			
Residual	8	0.04651	0.00581					
Shapiro-Wilk test for normality				p=	0.234			
ANOVA table for			StrDopac					
Source	df	ss	ms	F	p			
Group	3	0.02691	0.00897	1.7	0.243			
Residual	8	0.04212	0.00527					
Shapiro-Wilk test for normality				p=	0.249			
ANOVA table for			Str5hiaa					
Source	df	ss	ms	F	p			
Group	3	0.0188	0.00627	2.02	0.189			
Residual	8	0.02479	0.0031					
Shapiro-Wilk test for normality				p=	0.151			
ANOVA table for			StrHva					
Source	df	ss	ms	F	p			
Group	3	0.00321	0.00107	0.32	0.808			
Residual	8	0.02642	0.0033					
Shapiro-Wilk test for normality				p=	0.017*			
ANOVA table for			Str5ht					
Source	df	ss	ms	F	p			
Group	3	0.00258	0.00086	0.41	0.751			
Residual	8	0.01683	0.0021					
Shapiro-Wilk test for normality				p=	0.577			
ANOVA table for			Str3mt					
Source	df	ss	ms	F	p			
Group	3	0.01613	0.00538	0.11	0.951			
Residual	8	0.38808	0.04851					
Shapiro-Wilk test for normality				p=	0.154			

Means and comparisons (The LSD test) for							StrDa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	7722.1	585.2					
B	3	7824.3	135.3	A	101.3	89,115.30	0.23	0.821
C	3	8731.2	72.85	A	113.1	99.4,128.7	2.19	0.06
D	3	8549.1	114.4	A	110.7	97.3,126	1.81	0.107
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							StrNa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	277.65	51.99					
B	3	217.29	10.12	A	78.3	56.2,108.9	-1.71	0.126
C	3	245.22	8.306	A	88.3	63.5,122.9	-0.87	0.411
D	3	207.97	10.85	A	74.9	53.8,104.2	-2.02	0.079
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							StrDopac	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	713.07	124.9					
B	3	813.38	31.08	A	114.1	83.3,156.2	0.96	0.363
C	3	964.35	62.79	A	135.2	98.7,185.2	2.21	0.058
D	3	785.2	22.96	A	110.1	80.4,150.8	0.71	0.5
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Str5hiaa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	331.14	42.45					
B	3	271.06	6.055	A	81.9	64.3,104.2	-1.91	0.092
C	3	261.13	6.566	A	78.9	61.9,100.4	-2.27	0.053
D	3	278.21	18.34	A	84	66,107	-1.66	0.135
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							StrHva	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	1625.9	241.9					
B	3	1520.7	20.11	A	93.5	72.9,120	-0.62	0.553
C	3	1681.8	27.52	A	103.4	80.6,132.7	0.31	0.763
D	3	1570.3	43.73	A	96.6	75.3,123.9	-0.32	0.756
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Str5ht	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	632.8	64.21					
B	3	583.38	21.38	A	92.2	75.6,112.5	-0.94	0.373
C	3	633.51	11.13	A	100.1	82.1,122.1	0.01	0.99
D	3	622.67	33.68	A	98.4	80.7,120	-0.19	0.856
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Str3mt	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	3	370.44	110.8					
B	3	320.66	95.2	A	86.6	33.3,224.9	-0.35	0.736
C	3	313.46	88.03	A	84.6	32.6,219.9	-0.4	0.697
D	3	294.09	86.44	A	79.4	30.6,206.3	-0.56	0.593
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

FC main study

Dependent variables					FcDa	FcNa	FcDopac	
Treatments:	Group	A Saline	B Tranycypro-mine	C 0.3 mg/kg	D 1 mg/kg	E 3 mg/kg		
Blocks:	Study							
Transform:	Log							
Robust regression tests for				FcDa				
Source	df	Chi squared	p					
Study	1	0	0.968					
Group	4	41.46	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
p3A	A	75.8	4.136926					
4	A	61.2	3.1316453					
Shapiro-Wilk test for normality				p=	0.096			
Robust regression tests for				FcNa				
Source	df	Chi squared	p					
Study	1	1.02	0.313					
Group	4	46.72	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
28	B	175.4	-3.807218					
14	D	219.9	-3.92188					
Shapiro-Wilk test for normality				p=<0.001***				
Robust regression tests for				FcDopac				
Source	df	Chi squared	p					
Study	1	0.97	0.326					
Group	4	36.68	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
24	A	3.2	-4.784402					
Shapiro-Wilk test for normality				p=	0.032*			
Robust regression tests for				Fc5hiaa				
Source	df	Chi squared	p					
Study	1	1.64	0.201					
Group	4	35.12	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
20	A	169.9	4.7731595					
28	B	107	4.3241555					
7	C	137	3.5308075					
19	D	157.1	5.5924255					
Shapiro-Wilk test for normality				p=<0.001***				

Robust regression tests for				FcHva				
Source	df	Chi squared	p					
Study	1	0.54	0.461					
Group	4	3.18	0.528					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
20	A	158.9	4.699276					
Shapiro-Wilk test for normality				p=	0.139			
Robust regression tests for				Fc5ht				
Source	df	Chi squared	p					
Study	1	7.16	0.007**					
Group	4	6.45	0.168					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
14	D	449	-3.078997					
Shapiro-Wilk test for normality				p=	0.295			
Means and comparisons (The LSD test) for							FcDa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	31.97	3.958					
B	8	67.195	4.411	A	210.2	161.6,273.3	5.73	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							FcNa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	340.01	7.818					
B	8	258.78	17.35	A	76.1	68.6,84.5	-5.3	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							FcDopac	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	13.532	2.37					
B	8	8.0104	1.144	A	59.2	41.2,85	-2.93	0.006**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Fc5hiaa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	103.26	5.505					
B	8	68.064	4.45	A	65.9	56.8,76.5	-5.69	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Means and comparisons (The LSD test) for							FcHva	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	74.293	7.404					
B	8	68.613	5.992	A	92.4	73.1,116.6	-0.69	0.494
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Fc5ht	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	553.01	12.6					
B	8	575.55	16.88	A	104.1	95,114	0.89	0.379
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						FcDa		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	31.97	3.958					
C	8	34.24	1.737	A	107.1	81.6,140.6	0.53	0.6
D	8	42.458	4.7	A	132.8	100.0,183.6	2.19	0.039*
E	9	47.023	3.397	A	147.1	108.8,198.9	3.19	0.003**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						FcNa		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	340.01	7.818					
C	8	352.91	16.74	A	103.8	93.2,115.6	0.72	1
D	8	328.33	18.48	A	96.6	86.7,107.6	-0.68	0.599
E	9	294.75	9.719	A	86.7	76.9,97.7	-2.98	0.006**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						FcDopac		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	13.532	2.37					
C	8	14.099	1.452	A	104.2	71.6,151.7	0.23	0.82
D	8	18.331	2.746	A	135.5	93.1,197.2	1.7	0.113
E	9	23.143	1.717	A	171	112.8,259.4	3.22	0.003**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						Fc5hiaa		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	103.26	5.505					
C	8	94.465	5.508	A	91.5	78.4,106.7	-1.21	0.232
D	8	87.849	6.81	A	85.1	72,100	-2.15	0.043*
E	9	88.552	5.191	A	85.8	72,100	-2.3	0.031*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Means and comparisons (The Williams test) for						FcHva		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	74.293	7.404					
C	8	77.432	4.663	A	104.2	81.8,131.2	0.36	0.853
D	8	84	7.847	A	113.1	82.2,131.2	0.4	0.853
E	9	72.643	3.641	A	97.8	83.6,131.2	0.43	0.853
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						Fc5ht		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	553.01	12.6					
C	8	537.43	15.56	A	97.2	88.4,106.8	-0.64	1
D	8	583.13	26.74	A	105.4	96,115.90	1.18	0.292
E	9	593.19	23.27	A	107.3	98.2,117.1	1.68	0.124
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Striatum main study

Dependent variables	StrDa	StrNa	StrDopac	Str5hiaa	StrHva	Str5ht	Str3mt	
Treatments:	Group							
Blocks:	Study							
Transformation:	Log							
Robust regression tests for				StrDa				
Source	df	Chi squared	p					
Study	1	18.22	<0.001***					
Group	4	7.71	0.103					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
p3A	A	7221.4747	-3.876681					
34	A	6403.4791	-3.883954					
27	E	6747.3646	-3.166531					
Shapiro-Wilk test for normality				p=	0.023*			
Robust regression tests for				StrNa				
Source	df	Chi squared	p					
Study	1	0.2	0.653					
Group	4	8.36	0.079					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
p10A	A	461.2	3.0678656					
20	A	494.1	3.8165179					
Shapiro-Wilk test for normality				p=	0.023*			
Robust regression tests for				StrDopac				
Source	df	Chi squared	p					
Study	1	23.17	<0.001***					
Group	4	185.31	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
p3A	A	537.9	-3.547477					
Shapiro-Wilk test for normality				p=	0.228			
Robust regression tests for				Str5hiaa				
Source	df	Chi squared	p					
Study	1	0.22	0.636					
Group	4	7.92	0.095					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
19	D	420.9	4.3998556					
Shapiro-Wilk test for normality				p=<0.001***				

Robust regression tests for				StrHva				
Source	df	Chi squared	p					
Study	1	14.26	<0.001***					
Group	4	14.88	0.005**					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
p3A	A	1216.6	-3.329819					
21	D	1030.1	-3.045726					
Shapiro-Wilk test for normality				p=	0.171			
Robust regression tests for				Str5ht				
Source	df	Chi squared	p					
Study	1	0.18	0.668					
Group	4	7.52	0.111					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
19	D	979.6	5.1294179					
Shapiro-Wilk test for normality				p=	0.006**			
Robust regression tests for				Str3mt				
Source	df	Chi squared	p					
Study	1	1.17	0.279					
Group	4	29.71	<0.001***					
Shapiro-Wilk test for normality				p=	0.064			
Means and comparisons (The LSD test) for							StrDa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	7753.5	222.7					
B	8	8326.4	96.24	A	107.4	101.5,113.6	2.57	0.014*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							StrNa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	291.21	20.77					
B	8	331.14	24.7	A	113.7	97,133.40	1.63	0.111
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							StrDopac	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	611.83	40.59					
B	8	292.65	15.9	A	47.8	40.9,56	-9.5	<0.001***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Str5hiaa	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	260.72	14.52					
B	8	218.16	17.57	A	83.7	71.4,98.1	-2.27	0.029*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Means and comparisons (The LSD test) for							StrHva	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	1401.4	78.43					
B	8	1180.6	50.8	A	84.2	72.8,97.6	-2.37	0.023*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Str5ht	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	626.4	23.3					
B	8	701.27	39.19	A	112	98.8,126.8	1.84	0.074
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Str3mt	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	306.55	34.47					
B	8	580.4	52.41	A	189.3	135.2,265.2	3.84	<0.001***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						StrDa		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	7753.5	222.7					
C	8	8145.7	136.7	A	105.1	97.2,108.7	1.06	0.351
D	8	7913.3	147.5	A	102.1	97.2,108.7	1.06	0.351
E	9	7910.1	191.4	A	102	97.6,108.7	1.14	0.332
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						StrNa		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	291.21	20.77					
C	8	316.35	17.09	A	108.6	92.1,128.2	1.05	1
D	8	288.09	15.93	A	98.9	83.9,116.7	-0.14	1
E	9	265.67	7.133	A	91.2	78.2,106.4	-1.25	0.276
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						StrDopac		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	611.83	40.59					
C	8	705.62	29.86	A	115.3	98,135.70	1.84	0.074
D	8	753.78	28.61	A	123.2	99,137.10	1.97	0.064
E	9	678.48	27.89	A	110.9	100.0,139.6	2.11	0.048*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						Str5hiaa		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	260.72	14.52					
C	8	259.45	13.2	A	99.5	84.4,117.4	-0.06	0.951
D	8	226.71	18.96	A	87	77.5,106.6	-1.28	0.246
E	9	244	9.809	A	93.6	77.5,105.4	-1.38	0.222
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Means and comparisons (The Williams test) for						StrHva		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	1401.4	78.43					
C	8	1508.9	57.59	A	107.7	92.7,125.6	1.05	1
D	8	1515	83.13	A	108.1	92.7,125.6	1.05	1
E	9	1349	66.19	A	96.3	83.5,110.9	-0.57	0.734
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						Str5ht		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	626.4	23.3					
C	8	616.16	21.99	A	98.4	86.5,111.9	-0.27	0.79
D	8	598.68	41.23	A	95.6	85.8,110.2	-0.53	0.734
E	9	613.46	16.91	A	97.9	85.8,109.2	-0.56	0.734
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						Str3mt		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	306.55	34.47					
C	8	322.95	41.19	A	105.3	74.3,149.4	0.31	1
D	8	259.51	12.95	A	84.7	59.9,120	-1	0.386
E	9	254.13	29.86	A	82.9	59.9,114.8	-1.21	0.296
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Ratio statistics main study

Dependent variables	StrDaDopacRat	StrDaHvaRat	StrDopacHvaRat	Str5hiaa5htRat				
Treatments:	Group	A Saline	B Tranycypro-mine	C 0.3 mg/kg	D 1 mg/kg	E 3 mg/kg		
Blocks:	Study							
Transform:	Log							
Robust regression tests for				StrDaDopacRat				
Source	df	Chi squared	p					
Study	1	11.08	<0.001***					
Group	4	321.34	<0.001***					
Shapiro-Wilk test for normality				p=	0.574			
Robust regression tests for				StrDaHvaRat				
Source	df	Chi squared	p					
Study	1	3.01	0.083					
Group	4	23.04	<0.001***					
Shapiro-Wilk test for normality				p=	0.927			
Robust regression tests for				StrDopacHvaRat				
Source	df	Chi squared	p					
Study	1	4.29	0.038*					
Group	4	371.87	<0.001***					
Shapiro-Wilk test for normality				p=	0.087			
Robust regression tests for				Str5hiaa5htRat				
Source	df	Chi squared	p					
Study	1	0.15	0.702					
Group	4	33.1	<0.001***					
Shapiro-Wilk test for normality				p=	0.771			
Robust regression tests for				FcDaDopacRat				
Source	df	Chi squared	p					
Study	1	0.34	0.558					
Group	4	87.68	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
24	A	0.1230769	-4.106303					
19	D	0.959375	3.5884041					
Shapiro-Wilk test for normality				p=	0.179			
Robust regression tests for				FcDaHvaRat				
Source	df	Chi squared	p					
Study	1	0	0.985					
Group	4	35.36	<0.001***					
Shapiro-Wilk test for normality				p=	0.219			

Robust regression tests for				FcDopacHvaRat				
Source	df	Chi squared	p					
Study	1	0.54	0.462					
Group	4	50.21	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
24	A	0.0626223	-3.889758					
Shapiro-Wilk	test	for	normality	p=	0.021*			
Robust regression tests for				Fc5hiaa5htRat				
Source	df	Chi squared	p					
Study	1	0.06	0.804					
Group	4	41.13	<0.001***					
Extreme observations with | Studentised residual|>3								
Subject	Group	Value	Z					
28	B	0.2113789	4.1809265					
Shapiro-Wilk test for normality				p=	0.033*			
Means and comparisons (The LSD test) for							StrDaDopacRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.0776	0.003					
B	8	0.0346	0.002	A	44.6	39.3,50.6	-12.9	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							StrDaHvaRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.1773	0.007					
B	8	0.1412	0.006	A	79.6	69.8,90.9	-3.49	0.001**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							StrDopacHvaRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.4298	0.012					
B	8	0.2441	0.009	A	56.8	52,62	-13.1	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Str5hiaa5htRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.4312	0.017					
B	8	0.3154	0.019	A	73.2	64.8,82.6	-5.21	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Means and comparisons (The LSD test) for							FcDaDopacRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.3757	0.06					
B	8	0.1238	0.018	A	33	24,45.3	-7.06	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							FcDaHvaRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	2.2079	0.28					
B	8	1.0198	0.1	A	46.2	34,62.7	-5.11	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							FcDopacHvaRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.1738	0.022					
B	8	0.1221	0.014	A	70.2	53.4,92.4	-2.61	0.013*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The LSD test) for							Fc5hiaa5htRat	
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.1917	0.01					
B	8	0.1156	0.01	A	60.3	50.8,71.6	-5.99	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						StrDaDopacRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.0776	0.003					
C	8	0.0865	0.003	A	111.5	100,100	1.74	0.09
D	8	0.0936	0.003	A	120.6	100.134.3	2.39	0.024*
E	9	0.0871	0.003	A	112.2	100.4,134.3	2.56	0.016*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						StrDaHvaRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.1773	0.007					
C	8	0.1864	0.007	A	105.1	91.7,120.5	0.77	1
D	8	0.1856	0.01	A	104.7	91.3,120	0.71	1
E	9	0.1715	0.007	A	96.7	85.2,109.9	-0.54	0.751
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						StrDopacHvaRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.4298	0.012					
C	8	0.4677	0.009	A	108.8	99.4,119.2	1.95	0.058
D	8	0.5033	0.015	A	117.1	105.1,130.5	3.65	<0.001 ***
E	9	0.5109	0.013	A	118.9	107.5,131.5	4.29	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

Means and comparisons (The Williams test) for						Str5hiaa5htRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.4312	0.017					
C	8	0.424	0.013	A	98.3	86.7,111.5	-0.28	0.781
D	8	0.3837	0.015	A	89	80,102	-1.77	0.097
E	9	0.3913	0.014	A	90.8	80,101.10	-1.9	0.078
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						FcDaDopacRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.3757	0.06					
C	8	0.4074	0.042	A	108.4	74.3,143.9	0.21	0.982
D	8	0.3705	0.053	A	98.6	74.3,143.9	0.21	0.982
E	9	0.4935	0.029	A	131.3	96.6,178.7	1.86	0.085
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						FcDaHvaRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	2.2079	0.28					
C	8	2.1944	0.13	A	99.4	72.4,136.5	-0.04	0.968
D	8	1.8218	0.228	A	82.5	60.1,113.3	-1.27	0.25
E	9	1.5359	0.125	A	69.6	48.9,98.9	-2.58	0.016*
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						FcDopacHvaRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.1738	0.022					
C	8	0.1805	0.012	A	103.8	78.2,138	0.28	0.782
D	8	0.2091	0.015	A	120.3	90.5,159.8	1.36	0.214
E	9	0.3216	0.02	A	185	135,253.50	4.88	<0.001 ***
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								
Means and comparisons (The Williams test) for						Fc5hiaa5htRat		
Treatment	n	Adj mean	SEM	compared to	%	95 % CI	t	p
A	11	0.1917	0.01					
C	8	0.1763	0.008	A	92	77,109.80	-0.99	0.327
D	8	0.1567	0.01	A	81.7	66.2,100	-2.39	0.024*
E	9	0.1533	0.01	A	80	65.7,97.3	-2.84	0.008**
Adj mean are back-transformed means adjusted for any unbalance in covariates/blocks								

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