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Serotonergic modulation of attentional processes in the rat prefrontal cortex.

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29/03/2006

SEROTONERGIC MODULATION OF ATTENTIONAL

PROCESSES IN THE RAT PREFRONTAL CORTEX

A dissertation submitted for the degree of

Doctor of the Philosophy

In the Life Science

Open University, London, U.K.

By

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September 2005

ProQuest Number: 13917274

ProQuest 13917274

In dedication to my parents, Dragojla and Leonard, my son Oliver,

and my lifelong companion Roberto Turri.

Declaration

This thesis has not been submitted in whole or in part for a degree or diploma or other qualification at any other University. The experimental work described in this thesis was performed by myself and includes work done in collaboration with Dr. Roberto W. Invernizzi.

Publications

Some of the experimental findings have been published:

Mirjana C, Baviera M, Invernizzi RW, Balducci C
The serotonin 5-HT$_{2A}$ receptors antagonist M100907 prevents impairment in attentional performance by NMDA receptor blockade in the rat prefrontal cortex.
Neuropsychopharmacology (2004) 29: 1637-47.

Ceglia I, Carli M, Baviera M, Renoldi G, Calcagno E, Invernizzi RW
The 5-HT$_{2A}$ receptor antagonist M100,907 prevents extracellular glutamate rising in response to NMDA blockade in the mPFC.
J Neurochem (2004) 91: 189-199.

Carli M, Baviera M, Invernizzi RW, Balducci C
Dissociaciable contribution of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in the medial prefrontal cortex to different aspects of executive control such as "impulsivity" and "compulsive" perseveration in rats.
Neuropsychopharmacology (2005) In press.

Calcagno E, Carli M, Invernizzi RW
The 5-HT(1A) receptor agonist 8-OH-DPAT prevents prefrontocortical glutamate and serotonin release in response to blockade of cortical NMDA receptors.
J Neurochem (2006) 96: 853-860.

Acknowledgements

ABSTRACT

The experimental evidence for the 5-HT system involvement in cognitive processes sensitive to dysfunction of the prefrontal cortex is sparse. The aim of this project was to examine serotonergic modulation of attentional performance deficits in the 5-choice serial reaction time (5-CSRT) task induced by blockade of NMDA receptors in the medial prefrontal cortex (mPFC) of rats. Using *in-vivo* microdialysis techniques attempts have been made to relate the behavioural effects of 5-HT receptors agonists and antagonists to changes in glutamate and 5-HT efflux induced by blockade of NMDA receptors in the mPFC.

The data clearly demonstrate that glutamate transmission in the mPFC is involved in the control of attentional performance on the 5-CSRT task. The data also show for the first time that enhanced glutamate and 5-HT release induced by blockade of NMDA receptors in the mPFC are not causally related to aspects of inhibitory response control as indexed by impulsive anticipatory and compulsive perseverative responses.

The important suggestion emerging from this study is that of distinct neuromodulation in the control of impulsive responding by 5-HT $_{2A}$/5-HT$_{2C}$ receptors and in compulsive behaviours by 5-HT$_{1A}$ and DA D $_2$ receptors in the prefrontal cortex. Intriguingly deficits in attentional accuracy might be associated with increased glutamate release in the mPFC.

Thus behavioural evidence obtained in animals with dysfunctional glutamate transmission performing the 5-CSRT task, as well as a series of pharmacological manipulations of 5-HT, DA and mGlu$_{2/3}$ receptor mechanisms have been presented that are consistent with the hypothesis that serotonergic mechanisms in the prefrontal

cortex play specific roles in attention and response selection particularly when these have a possible function in the strategic control of behaviour.

These behavioural and neurochemical studies provide new information on the physiological mechanisms involved in the control of various aspects of attentional performance as well as in cognitive deficits associated with some neuropsychiatric disorders.

CONTENT

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CHAPTER 1: GENERAL INTRODUCTION

Section1.   Human psychopathology-psychiatry of schizophrenia related to glutamatergic and 5-HT mechanisms

- Neuropsychology of schizophrenia

Whatever causes schizophrenic symptoms it is evident that the pathological process is played out in one or more domains of higher cognitive function.

Notwithstanding, impaired attentional processing has been suggested as one of the core deficits of schizophrenia. Patients with schizophrenia are unable to select, correctly interpret and appropriately respond to sensations. At certain times they find difficult to sustain and shift attention in response to environmental demands while on other times they may orient and fixate on trivial aspects of a situation or engage compulsively in some activity. Withdrawn schizophrenic patients may be essentially unaware of the outside world and attend to environmental stimuli only sporadically. In laboratory settings schizophrenic patients show deficits in tests of attention such as the continuous performance test (CPT), poor performance in tests such as Wisconsin Card Sorting test (WCST) but they also show deficits in tests of memory and language. The WCST is the best-known test of *"executive functions"*. This function embodies the concept of a supervisory system whose main purpose is to orchestrate the function of other systems in the performance of complex task such as comprehension, learning and reasoning. But it is still unclear to what extent they represent an emergent properties of interactions between specialized cognitive subsystems or the operation of a single central executive (Baddeley, 1986), possibly with dissociable components (Shallice and Burgess, 1993). Components may include the enhancement of information held temporarily or "on-line" (concept of "working-memory") (Goldman-Rakic, 1987), the marshalling of attentional resources (Shallice,

1982), the inhibition of inappropriate responses in certain circumstances (Shallice and Burgess, 1993) and the monitoring of behaviour with respect to affective or motivational state (Damasio, 1994; Petrides, 1996). However, the associations between positive and negative dimensions of symptoms and neurocognitive functioning of schizophrenic patients in tasks such as CPT and WCST were found to be weak, suggesting a relative independence of these disease processes (Nieuwenstein et al., 2001).

The non-unitary nature of attention and its different manifestations has been recognized and a number of different sub-forms have been described including focused, divided, effortful and controlled. Three largely independent components of attention have been described; selection, by which some information are given priority over others; vigilance, which ensures attentional persistence over time; and control, which serves to optimize performance by inhibition of concurrent activities (Parasuraman, 1998; Robbins, 2002). These attentional processes allow for the selection and integration of sensory inputs, for learning and remembering, and for organization and preparation of appropriate responses. Thus, impaired attentional processing may be associated with inattention, distraction, memory impairment, confusion, perseveration, or disinhibition, behaviours that are typically observed at one time or other in schizophrenic patients.

- Hyperglutamatergic hypothesis of schizophrenia

Although there is little doubt that patients with schizophrenia have attentional impairment and deficits in executive function it is quite difficult to specify their underlying neural and neurochemical basis. Schizophrenia has been associated with abnormal or reduced cortical connectivity and disruptions in the coherent activity of

cortical networks (Krystal et al., 2003). These disturbances in cortical network function have been suggested to underlie cognitive impairments. Abnormal glutamatergic transmission in the prefrontal cortex (PFC) has been associated with schizophrenia (Krystal et al., 2003). The reduced density of spines and synaptic proteins, reduced glutamatergic markers and hypofrontality suggest a decreased glutamatergic activity (Konradi and Heckers, 2003). However, hypofrontality appears associated with negative symptoms (Potkin et al., 1999). When patients had normal or reduced metabolism, the cortical activity increased in schizophrenic patients experiencing acute hallucinatory phase of disease (Catafau et al., 1994; Dierks et al., 1999; Shergill et al., 2000). Likewise, proton magnetic resonance studies reported higher than normal glutamate/glutamine levels in PFC of neuroleptic-naïve schizophrenic patients (Bartha et al., 1997; Theberge et al., 2002). The reduced GABA markers found in the PFC of schizophrenic patients (Lewis et al., 2005) may suggest a decreased local inhibitory GABA inputs and increased glutamate transmission. Additionally, the glutamate NMDA receptor antagonists such as phencyclidine and ketamine used to model clinical symptoms and cognitive deficits of schizophrenia in healthy human subjects (Javitt and Zukin, 1991; Krystal et al., 1994) increased frontal activation (Holcomb et al., 2005).

- 5-HT function in schizophrenia

Findings from post-mortem studies on 5-HT receptors in several brain areas as well as more recent studies using in vivo neuroimaging in schizophrenic patients in addition to studies of indices of 5-HT metabolism in CSF, platelet, and whole brain and blood have not provided a clear picture regarding 5-HT function in schizophrenia (Iqbal and van Praag, 1995; Joyce et al., 1997; Harrison, 1999; Lewis

et al., 1999; Yasuno et al., 2003). Nevertheless, there are several indications that 5-HT system might participate in higher cognitive functions; psychopharmacological studies show that LSD and psilocybin induce hallucinations (mostly visual), cognitive deficits and other perceptual changes by acting as agonists at 5-HT_{2A} receptors (Vollenweider et al., 1998). The atypical antipsychotics with high $5\text{-HT}_{2A}/D_2$ affinity ratio demonstrate superior efficacy compared to typical antipsychotics on test of verbal fluency, digit-symbol substitution, fine motor function, and executive function in schizophrenic patients (Keefe et al., 1999; Meltzer and McGurk, 1999; Muller et al., 2005). Interestingly, adding a 5-HT_{1A} receptor agonist, tandospirone, to typical antipsychotics treatment in patients improved their performance in WCST and in a test of verbal memory compared to those that did not receive tandospirone (Sumiyoshi et al., 2001b). Moreover, drugs without any DA receptor affinity but acting as antagonist at 5-HT_{2A} receptors such as M100907 behave as antipsychotic in some animal models although it does not have antipsychotic activity in schizophrenic patients (see (Krystal et al., 2003)). However, there is continuous debate as to whether atypical antipsychotics have pro-cognitive effects or have reduced cognitive liability (Carpenter and Gold, 2002).

Section 2.   Functions and neurochemical modulation of prefrontal cortex

- Cognitive and behavioural functions of primate prefrontal cortex

Several lines of evidence suggest that the prefrontal cortex (PFC) exerts a crucial role in visual attention in humans (see (Fuster, 1989)). Patients with PFC lesions show increased distractibility (Rylander, 1939; Chao and Knight, 1995), low alertness (Luria, 1966/1980) impaired sustained attention (Rylander, 1939; Wilkins et al., 1987) and diminished attention to novel stimuli (Daffner et al., 2000). In positron emission tomography (PET) studies a prominent prefrontal activation has been shown during the execution of tasks requiring selective attention (Corbetta et al., 1991) and sustained attention (Pardo et al., 1991). The PFC functions have been implicated in other cognitive processes such as working memory (Goldman-Rakic, 1987), decision-making (Damasio, 1994; Rogers et al., 1999b), planning (Shallice, 1982; Owen et al., 1990), initiation and execution of complex sequences of behaviour and cognitive flexibility (inhibitory control), which operate to monitor and revise them when they are unsuccessful (Milner, 1964).

Monkeys with PFC lesions show deficits in go/no-go situations, successive discrimination, discrimination reversal of stimuli and place, hyper-reactivity to novel but not familiar stimuli and deficits in delayed response task (Fuster, 1989). The deficits have been attributed to a supra-modal characteristic of the tasks. It has been argued that the tendency to perseverate, which is viewed as a form of proactive interference, make the performance vulnerable to internal interference thus biasing the response of animals in spite of continued errors (Mishkin, 1964). These deficits have been variously interpreted as deficits in working memory (Goldman-Rakic, 1987), temporal scheduling of behavioural sequences (Fuster, 1989), inhibition of

central sets (Mishkin, 1964), and drive and response inhibition (Brutkowski, 1965). A degree of functional heterogeneity has also been reported; dorsolateral aspects of the prefrontal cortex including the sulcus principalis has been suggested to be involved in "short-term" memory as measured by performance on the delayed response paradigm and ventral aspects, including the orbital cortex and the inferior convexity primarily involved in "behavioural disinhibition" responsible for deficits in discrimination reversal and "go/no-go" tasks (Fuster, 1989). The delay dependent deficits have also been reported in primates following damage to the orbitofrontal cortex (Mishkin, 1964; Kowalska et al., 1991).

The various PFC functions have been interpreted in a framework of *executive function* that embody the concept of an "central executive" (Baddeley, 1986) and "supervisory attentional system", identified with consciousness and the control of attention, which integrates information from different sources, schedules its processing according to the needs of the moment, and selects overall strategies of action (Robbins, 1998). Other views hold that the PFC is critical for the "on-line" maintenance of memory representations, which is necessary for the mediation of contingencies of action over time, especially under conditions of interference (Goldman-Rakic, 1998).

However, distinct forms of "executive" dysfunction occur after frontal lobe damage in humans and monkeys suggesting that executive function may not be unitary in nature and may involve dysfunctions along distinct neural circuitry (Robbins, 2000b). The prefrontal cortex itself has several distinct cytoachitectonic regions (Pandya and Yeterian, 1996). Thus discrete prefrontal regions and their differential connections with other brain areas might contribute distinct features to the nature and organization of these "executive/supervisory" functions.

It has been suggested that inhibitory control processes may be an intrinsic propriety of the prefrontal cortex (Robbins, 2000b) and functional differences between prefronto- cortical sub-regions might result from the kinds of information that are processed and the operations that are performed within these sub-regions. In fact, a loss of inhibitory response control may result in a deficit in shifting attention between supra-ordinate features (e.g. colour versus shapes) of visual stimuli (extradimentional set shifting) when the lesion is made to dorsolateral or in a deficit in the capacity to reverse affective association (rewarded rather than non-rewarded) to specific stimuli (reversal learning) when the lesion is centred on orbitofrontal portion of prefrontal cortex (Dias et al., 1996). However, the factors that are responsible for the functional heterogeneity are far from clear and may depend on how these sub-regions are organized, whether in a hierarchical or independent functional units, and whether their differences are primarily in the type of information they carry or the type of operational process they perform. The neuroimaging data suggest that dorsal and ventral aspects of PFC are not functionally organized according to stimulus modality but may be coding different operational processes (Owen, 1997).

- Cognitive and behavioural functions of rat prefrontal cortex

The rat prefrontal cortex (PFC) does not possess the morphological and functional differentiation of the primate brain and an unequivocal definition based upon single morphological or functional criteria has proved difficult. However, the rat PFC has been classically defined and delineated by anatomical criteria such as the area that possesses strong reciprocal connections with the mediodorsal nucleus (MD) of the thalamus (Rose and Woolsey, 1948; Leonard, 1969). This criterion has proved

useful to define what constitute the equivalent area across mammals. Based on this and other anatomical criteria several distinct regions of the PFC have been identified in the rat (Uylings and van Eden, 1990). The three major sub-divisions are: the *medial*, sub-divided into a dorsal region including precentral (PrC) and anterior cingulated (ACg) and a ventral component that comprises prelimbic (PrL), infralimbic (IL) and medial orbital (MO); the *lateral* region includes dorsal and ventral agranular insular (AID, AIV) and lateral orbital (LO); the *ventral* region that encompasses the ventral orbital (VO) and ventral lateral orbital (VLO).

These regions are schematically depicted in Fig. 1.
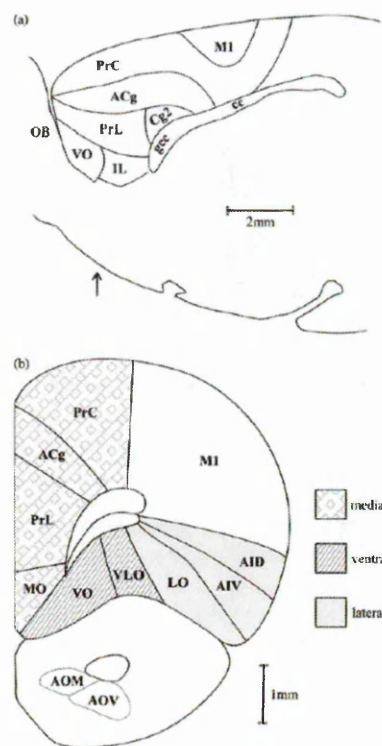
Figure 1. Illustrative diagrams of the rat prefrontal cortex (PFC). (a) Lateral view, 0.9 mm from the midline. (b) Unilateral coronal section, approximately 3.5 mm forward of the bregma (depicted by arrow above). The different shadings represent the three major subdivisions of the PFC (*medial, ventral and lateral*). Abbreviations: ACg, anterior cingulated cortex; AID, dorsal agranular insular cortex; AIV, ventral agranular insular cortex; AOM, medial anterior olfactory nucleus; AOV, ventral anterior olfactory nucleus; cc corpus callosum; IL, infralimbic cortex; LO, lateral orbital cortex; MI, primary motor area; MO, medial orbital cortex; OB, olfactory bulb; PrL, prelimbic cortex; PrC, precentral cortex; VLO, ventrolateral orbital cortex; VO, ventral orbital cortex. (from (Dalley et al., 2004).

The anatomical connections of PFC are characterised by its contribution of inputs to several levels of the neuraxis, thus enabling this region to participate in many aspects of control. The PFC efferents include backprojections to the striatal feedback loop circuitry, hypothalamus, amygdala, hippocampus, lateral septum, mesencephalon and the brain stem nuclei. It possesses thalamocortical connections and receives backprojections from the posterior parietal cortex and sensory cortical areas. The PFC targets in a reciprocal and topographical manner the main source of monoaminergic and cholinergic cells, the dopamine (DA) cells in the ventral tegmental area (VTA) and substantia nigra compacta (SNC), noradrenergic (NA) cells in the locus coeruleus (LC), serotonergic (5-HT) cells in the raphé nuclei, and cholinergic cells in the basal forebrain.

The medial PFC (mPFC) of the rats posses a degree of functional equivalence with that of non-human primates as shown by deficits in homologous functions such as working memory, behavioural flexibility, temporal sequencing of behaviour and attention (Heidbreder and Groenewegen, 2003; Dalley et al., 2004). Interestingly, rat mPFC appears to be functionally homologous to the dorsolateral PFC of monkeys. As previously discussed, dorsolateral (Brodman area 9) and orbital PFC in non-human primates show dissociable functions in attentional and affective set shifting. Similar dissociation in functions of medial and orbitofrontal cortices had been found in rats (Birrell and Brown, 2000; Brown and Bowman, 2002). In fact, rats with lesions to the mPFC were selectively impaired in extra-dimensional set shifting stage but not intra-dimentional or reversal stage of a rat version of the attentional set-shifting task (Birrell and Brown, 2000). In contrast, in the same task lesions to the orbital PFC (VLO/VO) impaired reversal but not intra-dimensional or extra-dimensional set-shifts (Brown and Bowman, 2002; McAlonan and Brown, 2003).

The perseverative nature of the deficits on this and other visual discrimination tasks suggest that rats with PFC lesions are unable to efficiently shift between new and old rules and strategies. It has been suggested that orbital PFC might be processing low order rules, based on affective value of objects and necessary for reversal learning whereas high order perceptual processing which is accomplished in medial PFC is necessary for extra-dimensional set-shifting. This may be compatible with the notion that activity in mPFC is necessary to preserve attentional selectivity.

Relevant for this project is a study by Muir et al. (Muir et al., 1996) showing that the mPFC but not post-genual cingulate, anterior dorsolateral and parietal cortex is crucial for attentional functioning in a five-choice serial reaction time (5-CSRT) task in rats. Additionally, bilateral excitotoxic lesions of mPFC caused increased perseverative responding whereas post-genual cingulated cortex lesions specifically increased anticipatory responses (Muir et al., 1996). The findings of this study have been on the whole replicated but with mPFC lesions causing some additional effects such as increased anticipatory responding (Passetti et al., 2002).

The dorsal and ventral regions of the medial PFC have been shown to be involved in different behavioural functions or in different aspects of the same function. Briefly, the dorsal part of the mPFC (dorsal ACg and dorsal PrL) appears to be primarily involved in memory for the temporal order (Kesner et al., 2000) and temporal patterning of behavioural sequences (Delatour and Gisquet-Verrier, 2001), timing of extrinsic stimuli (Dietrich and Allen, 1998), egocentric memory (Ragozzino and Kesner, 2001) and spatial win-shift behaviour (Seamans et al., 1995). Lesions to ACg do not affect locomotor activity, acquisition of spatial learning or switching from spatial to visual-cued learning and *vice-versa*, and "go/no-go" conditional discrimination (reviewed in (Heidbreder and Groenewegen, 2003)). In contrast, the

ventral part of mPFC comprising ventral PrL and IL as well as MO appears critical

for the flexible shifting to new strategies or rules in spatial and visual discrimination

(Ragozzino et al., 1998; Ragozzino et al., 1999b), cross-modal shifts in place-

response learning in a cross-maze (Ragozzino et al., 1999a), reversal learning in a

visual discrimination task (Li and Shao, 1998). The excitotoxic lesions of the PrL/IL

but not ACg altered the motor readiness (delay dependent speeding of reaction time)

and increased impulsivity (Risterucci et al., 2003). Recently, PrL and IL regions of

PFC have been shown to co-operate in the coordination of goal-directed actions and

habits; PrL appears crucial for voluntary control of goals, IL for automatic

responding (habit) (Killcross and Coutureau, 2003). This area of the mPFC has also

been involved in behaviours associated with anxiety and fear (reviewed in

(Heidbreder and Groenewegen, 2003)).

Recent studies by Chudasama et al. (Chudasama et al., 2003) with lesions restricted

to sub-regions of the mPFC such as ACg and IL and to orbitofrontal sectors of PFC

show that in a 5-CSRT task attentional selectivity is related to the ACg, impulsive

responding to IL and perseveration to the orbitofrontal cortex. Lesions of the PrL-IL

sector of mPFC have small effects on attentional selectivity but increased

perseverative responses (Passetti et al., 2002). Interestingly, presentation of visual

stimuli at unpredictable times from the start of the trial to rats performing a 5-CSRT

task impaired the accuracy of controls but did not affect accuracy of ACg lesioned

rats, suggesting that controls but not ACg rats were using temporal cues to perform

the task (Passetti et al., 2002). Similarly, excitotoxic lesions of the dorsal PFC (ACg

and PCr) but not ventral PFC produced deficits in a visuospatial task indicative of

impaired attention (Bailey and Mair, 2004). Therefore it could be suggested that

visual selectivity and the ability to use temporal cues to guide performance might

reside in the dorsomedial areas of mPFC (ACg) whereas ventral regions of the mPFC

and OFC appear critical for different aspects of inhibitory response control such as

impulsivity (area IL) and compulsive perseveration (PrL and OFC).

- Neurochemical modulation of PFC function

The pyramidal glutamatergic neurons of the PFC integrate various excitatory inputs

from cortical and subcortical (thalamic) areas and modulatory inputs arising from

brain stem monoaminergic and basal forebrain cholinergic nuclei (Azmitia and

Segal, 1978; Mesulam et al., 1983). Various GABA cells forming a complex

microcircuitry provide local inhibitory/excitatory inputs to pyramidal cells. The Fig.

2 illustrates some elements of this circuitry in the frontal cortex.
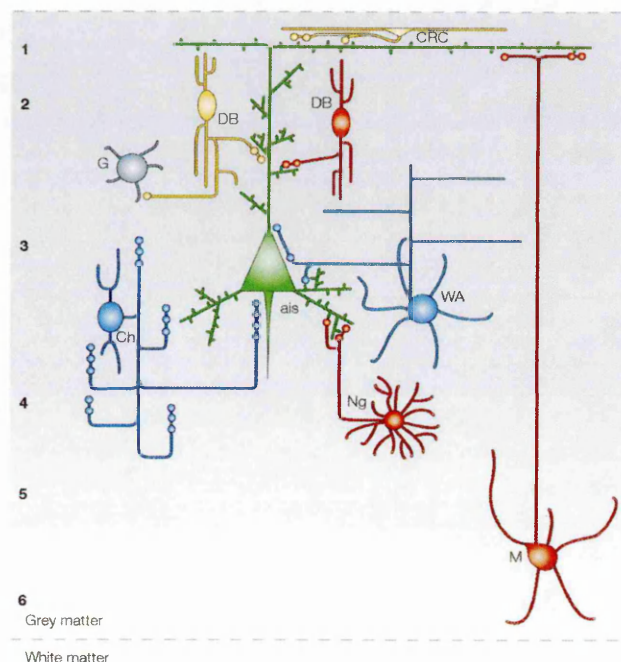
Figure 2. The diagram illustrates the calcium-binding proteins — parvalbumin (blue), calbindin (red) and calretinin (yellow) — and the locations of inhibitory synaptic inputs to a pyramidal neuron (green) by different morphological classes of cortical GABA(•-aminobutyric acid) neurons. The chandelier (Ch) and wide arbor (WA) or basket neurons provide inhibitory input to the axon initial segment (ais) and the cell body proximal dendrites, respectively, of pyramidal neurons. By contrast, the calbindin-expressing double bouquet (red DB), neurogliaform (Ng) and Martinotti (M) neurons tend to provide inhibitory inputs to the distal dendrites of pyramidal neurons. Finally, calretinin-expressing (yellow) DB and Cajal–Retzius cell (CRC) appear to target both pyramidal cell distal dendrites and other GABA (G) neurons. 1–6, layers of dorsolateral prefrontal cortex. (from (Lewis et al., 2005)).

Certain of these cells receive direct thalamic inputs (large basket cells) and are in position to control these inputs to the pyramidal cells and apical and basal dendrites while others (chandelier cells) forming "cartridges" surrounding the initial portion of the axon control the output of pyramidal cells (Somogyi and Cowey, 1981). Additional control of pyramidal cells by cortico-cortical inputs may be exerted through GABA interneurons forming contacts with side branches of apical and basal dendrites (double bouquet cells) (Benes and Berretta, 2001). Thus integration of input signals in the pyramidal neurons is exerted at various cellular levels, with a key role played by the large apical dendrites. Other cellular elements control the generation of action impulses along the pyramidal axons, critical for influencing the activity of output pathways.

Glutamate serves as the major excitatory neurotransmitter in the CNS. Given the multiplicity of receptor subtypes that have been described for glutamate, a particular neuron's response to this excitatory neurotransmitter will be determined by the presence and organization of diverse receptors types; the ionotropic NMDA, AMPA and kainate receptors and the metabotropic mGLU receptors are present on various cellular domains of neurons and glial cells. The predominant role of glutamate receptors of NMDA type in synaptic excitatory neurotransmission and their crucial role in diverse functions such as developmental processes, transmission of sensory information, synaptic plasticity, learning and memory and neurotoxicity has been firmly established. In the layers II/III and V/VI of the cerebral cortex the NMDA receptors are particularly enriched and preferentially localized in dendritic spines of pyramidal neurons but also in excitatory and inhibitory axon terminals (Conti et al., 1997). Abnormal functioning of the glutamate system has consequences for other neurotransmitter system locally (for example GABA) and distally (for example, in

areas such as VTA and DR nucleus). Blockade of NMDA receptors decreases GABA activity (Olney et al., 1989). The decreased stimulation of NMDA receptors on GABA neurons leads to decreased release of GABA and a disinhibition of glutamate neurons under control of GABA receptors. These disinhibited neurons proceed to release high levels of glutamate (Olney et al., 1991). This mechanism has been implicated in the increase in glutamate release induced by NMDA receptor antagonists (Olney et al., 1991; Konradi and Heckers, 2003). That the increased glutamate release in the PFC induced by NMDA receptor antagonists might have functional consequences probably through stimulation of AMPA receptors has been shown in some neurochemical and behavioural experiments (Moghaddam et al., 1997). In fact, mGlu2/3 receptor agonists have been shown to decrease both glutamate release and behavioural impairments induced by NMDA antagonists in rats (Moghaddam and Adams, 1998). There is some suggestive evidence of similar effects in man (Krystal et al., 2005). Additionally, preventing the stimulation of AMPA receptors by antagonists reversed both the behavioural and the effects on DA release by NMDA receptor antagonists (Moghaddam et al., 1997).

The level of functioning of the PFC is highly dependent upon the 5-HT, NA, DA and ACh inputs arising from subcortical areas. A most important aspect of the relationship of PFC with these chemically defined systems is that these are in turn innervated by fibres from the PFC and in particular the orbital and medial PFC in primates and medial PFC in rats. This bi-directional connection of PFC with cholinergic and monoaminergic systems, indicate that PFC may influence its own cholinergic and monoaminergic neurotransmission (Groenewegen and Uylings, 2000). Interestingly, the NMDA receptor antagonists have been reported to profoundly affect neurotransmission in these systems. Genetic deletion of NMDA

receptor (Miyamoto et al. 2001) and phencyclidine (PCP) and ketamine (two non-competitive NMDA receptor antagonists) increased 5-HT (Martin et al., 1998a; Miyamoto et al., 2001), DA (Miyamoto et al., 2001; Lorrain et al., 2003a) and NA release (Lorrain et al., 2003b) in the PFC. The exact mechanisms responsible for these effects are unclear. Some indications that increased release of 5-HT may be a consequence of increased glutamate release is suggested by observation that stimulation of AMPA receptors may increase 5-HT release in the mPFC (Martin-Ruiz et al., 2001). That the enhanced 5-HT and DA tone may not contribute to the behavioural effects of NMDA receptor antagonists is suggested by findings showing that in animals in which either 5-HT or DA levels were drastically decreased by paracholophenylalanine (PCPA) and α-methylparatyrosine (α-MPT) respectively, PCP was still able to increase hyperactivity. These data strongly suggest that the effects on behaviour were not due to changes in 5-HT or DA release in the PFC (Swanson and Schoepp, 2002).

Systemic and intracortical NMDA receptor antagonists have been shown to cause hyperactivity (O'Neill and Liebman, 1987; Jentsch et al., 1998), impairments in sensori-motor gating (Jentsch and Roth, 1999; Yee et al., 2004) and deficits reminiscent of frontal lobe dysfunction. These include deficits in working memory in T-maze (Wesierska et al., 1990; Verma and Moghaddam, 1996; Moghaddam et al., 1997; Moghaddam and Adams, 1998; Romanides et al., 1999) and in a Delayed Matching-to-Position task (DMPT) (Aura and Riekkinen, 1999), impairments in attentional functioning (Le Pen et al., 2003) and inhibitory response control in rats performing a 5-CSRT task (Higgins et al., 2003b) and in attentional set shifting (Egerton et al., 2005; Stefani and Moghaddam, 2005). Both excitotoxic lesions of the IL and blockade of NMDA receptors in the same area disrupted response

inhibition as shown by increased anticipatory responding (Chudasama et al., 2003; Murphy et al., 2005). The NMDA receptor antagonist, CPP, injected into IL or PrL impaired attentional functioning in a 5-CSRT task (Murphy et al., 2005). Injections of NMDA receptor antagonists into the orbitofrontal cortex impaired reversal learning (Bohn et al., 2003).

The functions of ACh, DA, NE and 5-HT systems have been studied extensively using electrophysiological, neurochemical, pharmacological and behavioural methodologies. Manipulations of these systems can modulate excitatory and/or inhibitory neurotransmission within the PFC (for a review see Aghajanian and Marek (Aghajanian and Marek, 2000) and (Svensson, 2000). Numerous evidence suggest that these neurochemical systems although all implicated in functions associated with PFC mediate different forms of neuromodulation, which is shown by their distinct contribution to various aspects of PFC functions such as working memory, vigilance, decision-making, reversal learning, attentional set shifting, sustained and selective attention and executive response control, (Aston-Jones et al., 1996; Goldman-Rakic, 1998; Robbins, 2000b; Sarter et al., 2001). Interestingly, distinct contributions to attentional functioning and inhibitory response control have been revealed when the behavioural effects of manipulation of these chemically defined systems were examined systematically on a common behavioural task. The task used in these studies was the 5-CSRT task (Carli et al., 1983; Robbins, 2002), derived from a 5-CSRT test for human subjects (Wilkinson, 1963) and analogous to the Continuous Performance Test used to test attentional functioning after drugs and stressors in normal volunteers and to probe attention in schizophrenic patients (Orzack and Kornetsky, 1966; Kurtz et al., 2001). A 5-CSRT task by its various largely independent measures of performance is able to measure the attentional control over

performance. The main variables are accuracy, anticipatory and perseverative responses and latencies to make a correct response and to collect the food reinforcement. In order to better define distinct profiles of deficits, variations of various task parameters can be made such as making the stimuli unpredictable in time, introducing distracting stimuli, or varying the duration or brightness of visual stimuli. Thus, using a 5-CSRT task and variations in parameters in order to better define distinct profiles of deficits, it was revealed an attentional deficit in rats with impaired noradrenergic transmission, particularly when they were aroused by interpolation of bursts of white noise and when the stimuli were presented unpredictably in time (Carli et al., 1983). Excitotoxic or immunotoxic lesions of cholinergic basal forebrain nucleus impaired accuracy at baseline task performance (Muir et al., 1994; McGaughy et al., 2002). By contrast, depletion of forebrain 5-HT affected impulsivity but had no effect on accuracy (Harrison et al., 1997a). Manipulation of various DA systems had distinct effects. The DA depletion in the NAcc decreased the overall probability of responding and speed but had no effect on other measures; mesostriatal DA depletion impaired accuracy and enhanced perseverative responding; DA depletion in the mPFC had some effect on accuracy but only when the stimuli were presented unpredictably in time (Robbins et al., 1998). These studies have been extended and include studies examining the selective manipulations of specific receptors in particular brain areas and more conventional pharmacological studies (see (Robbins, 2002)), but due to amount of data that have been gathered they cannot be reviewed here. Some of the studies examining the effects of 5-HT manipulations will be reviewed in a section dealing with behavioural functions of the 5-HT system.

As has been shown in this very short review, the 5-CSRT task was able to reveal separable roles of monoaminergic, cholinergic and of neural systems centred on prefrontal, cingulated and parietal cortex in different aspects of performance. This task may be therefore particularly useful for systematically comparing the effects of selective pharmacological manipulations of various 5-HT receptors and so it has been employed in these experiments.

Section 3.   Functions of the 5-HT system: anatomy, physiology of 5-HT system, and behavioural functions.

Serotonin plays a wide range of roles in the brain. Compared with other monoamines is anatomically more widespread (Fig.3) and behaviourally much more diverse. The activity of 5-HT cells has been examined mostly during arousal-waking sleep cycle. The findings of these studies have been put into an interpretative framework were the brain 5-HT system coordinate autonomic and neuroendocrine function with the present motor demands, and concurrently inhibits information processing in various sensory pathways (Jacobs and Fornal, 1999). Nevertheless, based largely on the effects of drugs and neurochemically selective lesions there has been some attempts to suggest general theories for 5-HT system functions. One is its involvement in behavioural inhibition, (Soubrié, 1986) the other its involvement in aversion and punishment (Deakin and Greaff, 1991). The 5-HT system has also been implicated in cognitive functions, but the experimental evidence is sparse and often not consistent across studies.

In the brain serotonin (5-HT) is synthesized from amino acid tryptophan in a two step process catalysed by the enzyme tryptophan hydroxylase-2 forming 5-hydroxytryptophan (5-HTP) which is then rapidly decarboxylated to form 5-HT. Tryptophan hydroxylation is the rate-limiting step in the synthesis and is regulated by a number of factors including neuronal firing and substrate availability. 5-HT is stored in vesicles that accumulate in the varicosities of 5-HT containing nerve fibres and is usually released through a vesicular exocytotic mechanism but drugs such as fenfluramine and parachloroamphetamine (PCA) may release it from the cytoplasmic compartment. 5-HT release may be inhibited by 5-HT_{1A} somatodendritic

autoreceptors that suppress 5-HT cell firing and by 5-HT$_{IB}$ or 5-HT$_{ID}$ (human) presynaptic autoreceptors that act locally on the releasing mechanism. 5-HT in the synaptic cleft is transported back into the nerve terminal by a re-uptake mechanism, which is inhibited by some antidepressants. Degradation occurs through mitochondrial enzyme monoamine oxidase (MAO) to form 5-hydroxyindoleacetaldehyde, which is then rapidly converted by aldehyde-dehydrogenase to 5-hydroxyindoleacetic acid (5-HIA).

The 5-HT release occurs from varicosities but the extent to which these varicosities form conventional synaptic connections with dendrites or soma of post-synaptic neurons is not clear. Early studies have found only occasional post-synaptic densities or other types of membrane specializations whereas more recently a much higher incidence of synaptic junctions with 5-HT terminals has been described (Parnavelas and Papadopoulos, 1989; Maley et al., 1990). However, this issue has not been resolved and it could not be excluded that the primary mode of 5-HT to operate is in a paracrine manner via activation of its many receptors.

- Serotonergic pathways in the brain

Serotonin cell bodies that give rise to the ascending and descending axonal pathways innervating large areas of the brain and spinal cord are found primarily in the raphé nuclei and reticular region of the lower brain stem. Dahlstrom and Fuxe, (Dahlstrom and Fuxe, 1964) in their pioneering studies described nine 5-HT- containing cell groups, which they labelled B1-B9 (Fig. 3).

New procedures including autoradiography following *in-vivo* application of [^{3}H]5-HT, immunohistochemistry for tryptophan hydroxylase (5-HTP), and for 5-HT have led to similar conclusions regarding the location of 5-HT-containing cell bodies. The

larger 5-HT cell cluster is found in the dorsal raphé nucleus and it has been estimated to contain about 24,000 cells in cat and 165,000 in man (Tork, 1990). The 5-HT cell clusters identified histochemically do not necessarily match the raphe nuclei but may extend beyond the boundaries of the corresponding raphé nucleus. In most of the raphè nuclei, the majority of the cells are non-serotonergic (Nieuwenhuys, 1985).

The 5-HT neurons of the brainstem are divided into a caudal and a rostral system. The cell groups B1-B4 of the caudal system are located in the median and paramediamn regions of the medulla and caudal pons. The axons of these cells descend to the dorsal and ventral horns of spinal cord along several pathways. From nucleus raphè magnus (B3 cell group) the 5-HT fibres travel via dorsolateral funiculus to the lamina I and II of the dorsal horn. These pathways largely mediate the 5-HT roles in sensory, motor and autonomic functioning.

The rostral B5-B9 cell groups system comprise the raphé nuclei of the rostral pons and mesencephalon as well as caudal linear nucleus, the nucleus pontis oralis, and the supralemniscal region. The 5-HT cells in the dorsal and median raphé account for 80% of the forebrain 5-HT terminals (Azmitia and Segal, 1978) and give raise to two distinct ascending projection; ventral and dorsal pathways (Nieuwenhuys, 1985). The ventral pathway originates primarily in the B6-B8 cell groups. The 5-HT fibers pass through the midbrain and innervate substantia nigra, ventral tegmental area, and interpeduncular nucleus. The major part of this pathway enter the medial forebrain bundle and afterwards branch off in several directions. Terminal regions of this projection are presented pictorially in Fig. 3.
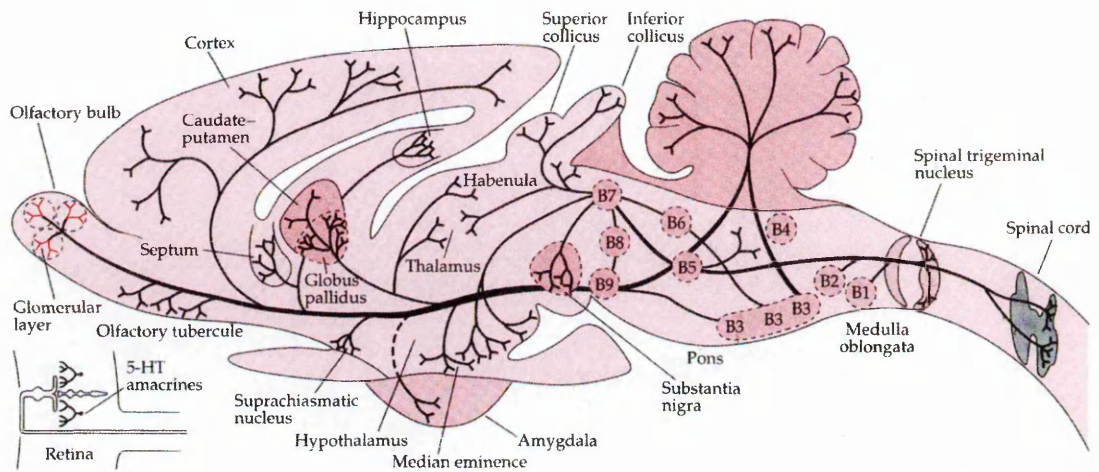
Figure 3. Schematic representation of main areas of 5-HT projections and nuclei of origin. Adapted from Consolazione and Cuello, 1982).

The dorsal pathway originates mainly in B7-B8 and sends fibers to the mesencephalic gray as well as inferior and superior colliculi. The majority of axons of this pathway enter the medial forebrain bundle where it combines with the ventral pathway to form an ascending projection system (Fig. 3). The fibres originating from mostly B2, B3 and B5 cell groups innervate the cerebellar cortex and deep cerebellar nuclei. Locus ceruleus, dorsal tegmental nucleus, inferior olivary nucleus, nucleus solitarius, romboencephalic reticular formation and cranial nerve nuclei receive 5-HT fibres. Connections between some raphé nuclei have been described.
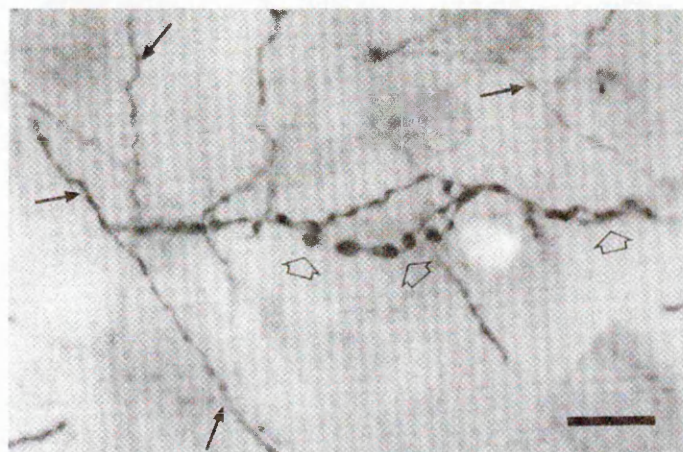
Figure 4. The 5-HT-immunoreactive fibres in the forebrain (Molliver, 1987).

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Light microscopic examination of 5-HT-immunoreactive fibres in the forebrain has revealed the existence of two fibre types. Some bear small fusiform varicosities whereas other fibres display much larger varicosities that give them a beaded appearance. The fine fibres with small varicosities appear to originate in the dorsal raphè (DR) while the thick beaded originate in the median raphè (MR), thereby forming the basis for two distinct projection systems. Although both systems are extensively represented in most areas of the neocortex, the predominance of one over the other in some brain areas has been described. The 5-HT fibres originating in the DR innervate primarily frontal cortex and the striatum while hippocampal formation and septum receive fibres from MR (Molliver, 1987; Tork, 1990).

5-HT projections to the neocortex

The 5-HT fibre terminals are found in all cortical regions where they display a non-uniform laminar distribution. In some cases such as in the primate visual cortex high density of 5-HT terminals in layer 4 seem to be complementary to noradrenergic system which is sparse in this layer (Morrison et al., 1982). In the cortex the fine 5-HT axons terminals are widely distributed throughout all cortical layers although variations in density and laminar distribution have been observed. The beaded 5-HT axons terminals were found primarily in the outer cortical layers (Kosofsky and Molliver, 1987). In the primate prefrontal cortex, 5-HT axon terminals were found primarily in association with the smooth dendrites of putative interneurons (Smiley and Goldman-Rakic, 1996). Although it could not be generalized to other brain regions, the 5-HT innervation of the frontal cortex arising from the DR nucleus seems to follow the laminar distribution of $5-HT_2$ binding sites (Blue et al., 1988).

The retrograde tracing experiments combined with extracellular recordings, have shown rather selective and strong bilateral projections from the infralimbic and dorsal peduncular cortices to the DR (Hajos et al., 1998). Other anterograde tracing studies found strong innervation of raphè nuclei also from medial and prelimbic PFC (Sesack et al., 1989; Vertes, 2004). The input from the ventral portions (IL) of the mPFC to the DR appears to be excitatory and its main target is a GABA neurone (Varga et al., 2001). It is well established that GABA neurons in the DR synapse with 5-HT neurons (Magoul et al., 1986; Wang et al., 1992). Thus, the same GABA neurons receiving inputs from IL subregion of mPFC may form inhibitory synapses with 5-HT neurons. However, specific dendritic domains of 5-HT and non-5-HT neurons appear to be innervated by different population of glutamate-containing axons (Commons et al., 2005). The DR receives excitatory inputs also from sub-cortical sites (Lee et al., 2003). Together, this reciprocal innervation between raphe nuclei and mPFC infralimbic and prelimbic regions may provide the anatomical substrate for the observed modulation of a broad range of functions.

5-HT receptors

The pleiotropic functions of 5-HT are afforded by the concerted actions of multiple serotonin receptor subtypes. The 14 sub-types of 5-HT receptors have been classified into seven receptor families 5-HT_{1-7} on the basis of their structural, functional and pharmacological characteristics. However, a more complex picture may emerge in the future due to evidence that specific 5-HT receptor subtypes (5-HT_{2C}, 5-HT_3, 5-HT_4 and 5-HT_7) can occur as multiple isoforms due to gene splicing, post-transcriptional RNA editing and polymorphic variants. Almost all 5-HT sub-types belong to the seven transmembrane spanning G-protein-coupled super-family of

metabotropic receptors linked to multiple signal transduction mechanisms. The exception being 5-HT$_3$ receptor, which is a ligand-gated ion channel. The five members (1A, 1B, 1D, 1E and 1F) of the 5-HT$_1$ family couple via G$_i$-G$_o$ proteins negatively to adenylate cyclase (AD) which leads to opening of K$^+$ channels whereas all three (2A, 2B and 2C) receptor subtypes belonging to the 5-HT$_2$ receptor family are positively coupled to phospholipase C (PLC) and results in increased accumulation of inositol phosphates (IP) and intracellular Ca^{2+} (Barnes and Sharp, 1999). While the 5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ receptor families are coupled positively via G$_s$-proteins to AD the 5-HT$_5$ has been negatively linked to AD. Unlike other 5-HT receptors, 5-HT$_3$ receptor exhibits structural and functional similarities with nicotinic cholinergic receptors; a pentameric cation channel selectively permeable to Na$^+$, K$^+$ and Ca^{2+} ions mediating rapid excitatory responses.

As revealed by autoradiographic, immunocytochemical and in-situ hybridization studies the 5-HT receptors are present in all areas receiving 5-HT cell afferents. However, the subtypes and their relative density in any brain area show a highly distinctive pattern of distribution. The 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_3$ and 5-HT$_4$ receptors are located postsynaptically, modulate ion flux and cause neuronal depolarisation while 5-HT$_{1A}$ receptors cause hyperpolarisation. Certain 5-HT receptors are located at somatodendritic (5-HT$_{1A}$) or the axon terminals (5-HT$_{1B}$) of 5-HT neurons and serve as autoreceptors regulating the release of 5-HT. The 5-HT$_{1B}$, 5-HT$_{2A,2C}$, 5-HT$_3$ and 5-HT$_4$ have also been found on nerve terminals of non-5-HT neurons where they may function as heteroceptors regulating neurotransmitter release. There is considerable evidence that different sub-types may interact with one another in mediating the various effects of 5-HT (Glennon et al., 1991).

In the following sections neuroanatomical distribution of $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors as well as their physiological responses will be reviewed. The review of behavioural responses mediated by 5-HT mechanisms will focus on the effects of selective 5-HT lesions and various agonists and antagonists at $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors in cognitive functions associated with the PFC.

- Anatomical organization and functions of $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors

$5\text{-}HT_{1A}$ receptors   Studies of cellular localisation and the effects of neuronal lesions show that $5\text{-}HT_{1A}$ receptors are located both postsynaptic in the forebrain areas but also on the soma and dendrites of 5-HT neurones in the DR and MR (Azmitia et al., 1996). There is some evidence that in hippocampus $5\text{-}HT_{1A}$ receptors are present at synaptic membranes but also extrasynaptically (Kia et al., 1996a) and on glial cells (Azmitia et al., 1996) but also in the ACh cells in the medial septum (Kia et al., 1996b). The $5\text{-}HT_{1A}$ receptors are barely detectable in the basal ganglia and cerebellum.

A high density of $5\text{-}HT_{1A}$ binding sites mapped by receptor autoradiography using a range of ligands was found in all cortical areas. Distribution of mRNA encoding the $5\text{-}HT_{1A}$ receptor is almost identical to that of binding sites (Pompeiano et al., 1992) and similar across species although the laminar organisation in cortical and hippocampal regions of humans differ from that in rat (Burnet et al., 1995). In the monkey and human cortical layers II and V the $5\text{-}HT_{1A}$ receptor immunoreactivity has been observed in the axon hillock (Azmitia et al., 1996) and proximal to inputs of GABAergic chandelier cells (DeFelipe et al., 2001). In primates $5\text{-}HT_{1A}$ immunoreactive pyramidal axons are more abundant in layer II/III than in layer V/VI

and qualitatively similar findings were reported in rats; the 5-HT$_{1A}$-immunopositivity was predominantly on proximal portions of pyramidal axons close to the parvalbumin-positive surroundings of the cell bodies of pyramidal neurons (Czyrak et al., 2003). While parvalbumin-immunoreactive chandelier cell terminals outlined pyramidal axons, the 5-HT-immunoreactive axons were not close enough to the pyramidal axon for synaptic contact. Thus, the pyramidal axon may be under dual control of 5-HT in a paracrine and GABA in a synaptic manner (DeFelipe et al., 2001). Recent *in-situ* hybridization studies showed that in ACg, PrL, IL subregions of mPFC and in deep layers (layer VIa) of rat PFC the 5-HT$_{1A}$ receptor mRNA transcript is present in the 40 to 60% of glutamatergic (vGluT1 mRNA positive) pyramidal as well as in the 21 - 28% GABA (GAD mRNA positive) neurons (Santana et al., 2004). The 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors are highly co-localized (80%), but in some layers such as layer VI there was a prevalence of 5-HT$_{1A}$ receptor mRNA and the co-localization was significantly lower (38 %) (Amargos-Bosch et al., 2004).

Electrophysiological experiments have established that 5-HT exerts its inhibitory effects in the brain by activation of 5-HT$_{1A}$ receptors. In fact, activation of 5-HT$_{1A}$ receptors causes neuronal hyperpolarisation through the G-protein-coupled opening of the K$^+$ channels without the involvement of diffusible intracellular messenger such as cAMP (for review see (Nicoll et al., 1990; Aghajanian, 1995). One study has reported that low doses of 8-OH-DPAT increased whereas high doses inhibited the firing of PFC neurons. The effect of low doses was not prevented by destruction of 5-HT containing neurons suggesting that the disinhibitory effects of 8-OH-DPAT were not due to stimulation of presynaptic 5-HT neuronal system (Borsini et al. 1995). In addition a recent report has suggested that low doses of a 5-HT1A receptor

agonist BAY3702 preferentially activate 5-HT1A receptors in GABA interneurons (Diaz-Mataix et al. 2005). However, *in-vitro* experiments have shown that $GABA_A$ receptor currents in pyramidal neurons were not affected by the $5\text{-}HT_{1A}$ receptor agonist 8-OH-DPAT (Feng et al., 2001). Thus, the functional role of $5\text{-}HT_{1A}$ receptors located in the GABA neurons is not clear.

$5\text{-}HT_{2A}$ receptors   Converging evidence from *in-situ* hybridisation, receptor autoradiography and immunohistochemistry suggest that the $5\text{-}HT_{2A}$ receptor are present in various cortical areas where they are particularly enriched in layer V. The 5-HT innervation of the frontal cortex arising from the DR seems to follow the laminar distribution of $5\text{-}HT_2$ binding sites (Blue et al., 1988). In the monkeys prefrontal cortex the $5\text{-}HT_{2A}$ receptors have been demonstrated on apical dendrites of pyramidal neurons as well as in large and medium-sized calbindin and parvalbumine-positive neurons (Willins et al., 1997; Jakab and Goldman-Rakic, 1998, 2000). A recent study of their ultrastructural localization show that in the rat prelimbic PFC most $5\text{-}HT_{2A}$ receptors are postsynaptic predominantly on proximal and distal dendritic shafts. Presynaptically, they are present mainly in thin, unmyelinated axons and varicosities and only rarely in terminals forming asymmetric synapses (Miner et al., 2003). An *in-situ* hybridisation study showed recently that in pyriform cortex, secondary motor cortex, ACg and PrL areas of the rat PFC $5\text{-}HT_{2A}$ receptors mRNA transcripts were found in about 50-66% of glutamate and about 22-34% GABA neurons. However, in ventral parts of infralimbic and in deep layer (VIa) of PFC the $5\text{-}HT_{2A}$ receptors were expressed in about 12 and 26% of glutamate cells respectively (Santana et al., 2004).

The 5-HT$_{2A}$ receptors have been found in some sub-nuclei of midbrain ventral tegmental area (VTA) co-localized with about 20% of DA cells (Nocjar et al., 2002) and in other sub-cortical regions (Pompeiano et al., 1994; Wright et al., 1995). In the DR nuclei they have been found on GABAergic interneurons

The 5-HT$_{2A}$ receptor couples positively to phospholipase C and leads to increased accumulation of inositol phosphate (IP) and intracellular Ca^{2+} (reviewed by Sanders-Bush and Canton (Sanders-Bush and Canton, 1995)). Under certain conditions, 5-HT$_{2A}$ receptors are constitutively active and some of the antagonists may act as inverse agonists. Clear evidence for a 5-HT$_{2A}$ receptor-mediated excitation of pyramidal neurons in PFC come from the work of G. Aghajanian and his collaborators (Aghajanian and Marek, 2000). Inhibitory actions of 5-HT$_{2A}$ receptors on the activity of pyramidal neurons have also been reported (Tanaka and North, 1993; Arvanov et al., 1999; Zhou and Hablitz, 1999).

5-HT$_{2C}$ receptors   The highest density of 5-HT$_{2C}$ binding sites was found in the choroid plexus. Within the brain tissue the 5-HT$_{2C}$ receptors mRNA and binding sites are widely distributed in neocortical areas, hippocampus, nucleus accumbens, amygdala, dorsal striatum, SN, VTA, LC and DR nucleus (Pompeiano et al., 1994; Eberle-Wang et al., 1997; Clemett et al., 2000). In monkey brain overlapping distribution of 5-HT$_{2C}$ mRNA and 5-HT$_{2C}$ binding sites was observed in the majority of brain regions suggesting a somatodendritic localization (Lopez-Gimenez et al., 2001). Similar distribution has been obtained in autopsy samples of human brain (Pasqualetti et al., 1999). However, compared to the 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors the cellular localisation of 5-HT$_{2C}$ receptors in the PFC has not been examined in

detail.   In the areas of monoaminergic cell bodies the 5-HT$_{2C}$ mRNA was found on GABA cells intrinsic to these areas (Serrats et al., 2005).

Activation of 5-HT$_{2C}$ receptors increases phospholipase C activity via a G-protein coupled mechanism. They have been shown to be constitutively active and certain antagonists may act as inverse agonists (Barnes and Sharp, 1999). There is evidence that activation of 5-HT$_{2C}$-receptor excites neurons in various brain regions and appears to depolarise pyramidal neurons in pyriform cortex (Sheldon and Aghajanian, 1991). The physiology of cortical 5-HT$_{2C}$ receptors is not clear.

Prefrontal cortex - raphé nuclei control of 5-HT system   The reciprocal control by raphé nuclei and mPFC on cortical 5-HT neurotransmission was recently demonstrated by a series of *in-vivo* electrophysiological and microdialysis experiments.

The somatodendritic 5-HT$_{1A}$ receptors in the DR and MR nuclei have been considered a major determinant of the output of the 5-HT neuronal system. However, recent studies have shown that in these nuclei the 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, which are present in GABA cells (Serrats et al., 2005) control the activity of 5-HT system (Liu et al., 2000). Activation of 5-HT neurons in the DR and MR nuclei by electrical stimulation has been shown to inhibit or excite pyramidal neurons in the PFC through the activation of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors, respectively (Hajos et al., 2003; Amargos-Bosch et al., 2004).

A substantial number of ventral mPFC neurons project monosynaptically to the 5-HT cells in DR (Hajos et al., 1998; Hajos et al., 2003). The activity of 5-HT cells in the DR and MR nuclei is under control by projections from pyramidal neurons of the mPFC (Hajos et al., 1998; Hajos et al., 1999; Celada et al., 2001). Presynaptic (in

the DR) and postsynaptic (in mPFC) $5\text{-}HT_{1A}$ receptors, as well as $GABA_A$ and ionotropic glutamate receptors in the DR are involved in this feedback control (Celada et al., 2001). The afferents from mPFC have been shown to excite or inhibit the 5-HT neurons in the DR and thus differentially drive the activity of 5-HT neurons projecting to different forebrain structures (Hajos et al., 1998; Celada et al., 2001; Varga et al., 2001). Stimulation of $5\text{-}HT_{2A}$ receptors in mPFC by DOI increased the firing of a subgroup of 5-HT neurons in the DR and increased 5-HT release in mPFC. Systemic administration of DOI markedly reduced the 5-HT output in the mPFC (Martin-Ruiz et al., 2001) by stimulation of $5\text{-}HT_{2A}$ receptors on GABA neurons in the DR (Liu et al., 2000) inasmuch as the activation of $5\text{-}HT_{2A}$ receptors in the mPFC has opposite effects. These effects appear discordant with the reported inhibitory influence of electrical stimulation of the mPFC on the DR 5-HT neurons by the activation of a local network of GABA neurons (Varga et al., 2001). However, in the study of Varga et al. (Varga et al., 2001), electrical stimulation was made in IL region of the mPFC, which preferentially innervated the GABA neurons in DR whereas in the study by (Amargos-Bosch et al., 2004) stimulation was made in the PrL region of mPFC. Interestingly, the IL region shows an apparent prevalence of $5\text{-}HT_{1A}$ over $5\text{-}HT_{2A}$ receptors compared to other regions of mPFC where both receptors are present in similar concentrations (Santana et al., 2004). Additionally, it was shown that the response evoked in a given pyramidal neuron of the mPFC (inhibition, excitation or no-effect) depended on the site of stimulation in the DR or MR (Amargos-Bosch et al., 2004).
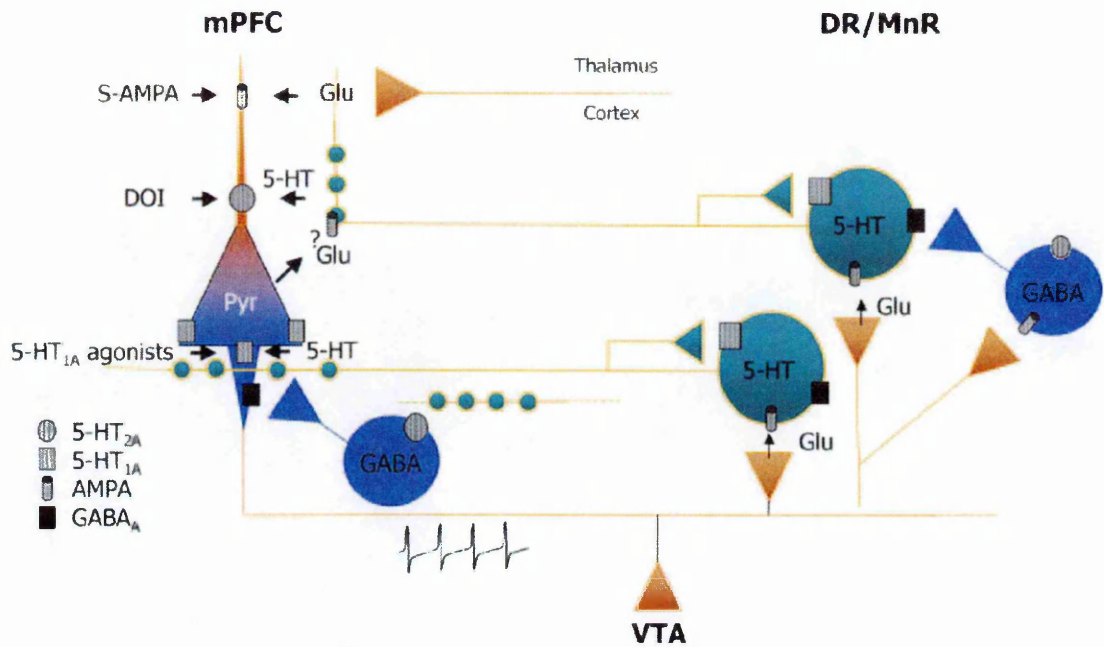
Figure 4. Schematic representation of the relationship between the mPFC and DR involving 5-HT_{1A} and 5-HT_{2A} receptors based on the electrophysiological and neurochemical effects of drugs selective for these 5-HT receptors. Taken from (Amargos-Bosch et al. 2004).

Therefore, these studies suggest that the overall result of this feedback control depends on various factors, such as the specific clusters of neurons in the raphè nuclei which may be activated or inhibited by afferents from the mPFC, the relative proportion of pyramidal neurons that express one or the other 5-HT receptor and the affinity of 5-HT for 5-HT_{1A} and 5-HT_{2A} receptors. However, other mechanism (at the moment unknown) may also co-operate to determine the overall result.

Modulation of monoamines and acetylcholine neurotransmission by 5-HT receptors

Numerous biochemical and behavioural studies have suggested that the 5-HT system may also affect PFC function by its modulation of NA, DA and ACh neurotransmission. The selective 5-HT_{1A} receptor agonist 8-OH-DPAT increases the release of acetylcholine in frontal cortex (Consolo et al., 1996) and hippocampus (Izumi et al., 1994) and noradrenaline in brain areas such as hypothalamus, frontal cortex, hippocampus and ventral tegmental area (VTA) (Done and Sharp, 1994;

Chen and Reith, 1995; Suzuki et al., 1995). It appear that these effects involve postsynaptic 5-HT$_{1A}$ receptors since they are still present in rats pretreated with either a 5-HT neurotoxin or a 5-HT synthesis inhibitor (Chen and Reith, 1995; Suzuki et al., 1995; Consolo et al., 1996; Hajos et al., 1999). However, the exact location of these postsynaptic 5-HT$_{1A}$ receptors is not entirely clear. Electrophysiological and microdialysis experiments show that 8-OH-DPAT increases the firing of DA cells and stimulates DA release in the VTA through stimulation of presynaptic 5-HT$_{1A}$ (Prisco et al., 1994; Chen and Reith, 1995) whereas stimulation of postsynaptic 5-HT$_{1A}$ receptors in the PFC increases the release of DA in the mPFC (Sakaue et al., 2000; Ago et al., 2003). Activation of 5-HT$_{1A}$ receptors increased the effects of the DA D2 antagonist l-sulpiride on DA release in the PFC while the effects of clozapine on DA release in the PFC were significantly reduced by a 5-HT$_{1A}$ receptor antagonist WAY100635 (Ichikawa et al., 2001).

Receptors of the 5-HT$_{2A}$ and 5-HT$_{2C}$ sub-type are prominent in the DA-rich areas and it is not surprising that they are involved in the modulation of DA neurotransmission. Although the selective 5-HT$_{2A}$ antagonist M100907 does not affect the spontaneous firing of DA neurons or alter the basal levels of DA release (Kehne et al., 1996; Pehek et al., 2001), it reverses the effects of amphetamine on the firing of VTA neurons (Sorensen et al., 1993) and attenuates K$^+$-stimulated DA release (Pehek et al., 2001) or release induced by methylenedioxymethamphetamine (MDMA) (Schmidt et al., 1994). By contrast, stimulation of 5-HT$_{2A}$ receptors by DOI increases the firing rate of DA cells in the VTA (Pessia et al., 1994) and increases the release of DA and NA in PFC (Gobert and Millan, 1999). The 5-HT$_{2A}$ receptors have been shown to modulate the activity of DA neurons differently in different regions of the brain such as PFC and ventral striatum. In fact blockade of 5-HT$_{2A}$ receptors

increased the effects of DA D_2 antagonists such as haloperidol on DA release in the mPFC but decreased it in subcortical areas such as NAcc (Meltzer et al., 2003).

Acting through the 5-HT$_{2C}$ receptors 5-HT exerts both tonic and phasic control on mesocorticolimbic DA release (Di Matteo et al., 2001). Activation of 5-HT$_{2C}$ receptors inhibits the firing rate of DA neurons in the VTA through activation of GABA interneurons (Di Matteo et al., 2000; Di Matteo et al., 2002) and decreases DA release in the NAcc (Di Matteo et al., 2002) whereas blockade of 5-HT$_{2C}$ receptors by SB242084 increases DA release in the PFC and NAcc (Millan et al., 1998; Gobert et al., 2000; Pozzi et al., 2002). In microdialysis studies a selective 5-HT$_{2C}$ receptor agonist Ro60-0175 slightly reduced basal extracellular DA in the NAcc, striatum and PFC (Di Matteo et al., 1998; Millan et al., 1998; Di Matteo et al., 2000; Gobert et al., 2000). Additionally, Ro60-0175 inhibits DA release in the PFC induced by immobilization stress (Pozzi et al., 2002). The role played by 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors in DA neurotransmission appears opposite and might explain the opposite behavioural profile.

These studies demonstrate a functionally important interaction of 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor activities and DA neurotransmission in the PFC and might contribute to some effects on cognitive functions associated to PFC. This complex 5-HT - DA interaction was also suggested to confer beneficial properties on cognitive dysfunction and to explain the fewer side effects of atypical antipsychotics (Meltzer et al., 2003).

- 5-HT receptors and glutamate transmission in the PFC

Activation of 5-HT$_{1A}$ receptors inhibits the induction of long term potentiation (LTP) by inhibiting NMDA receptor-mediated synaptic excitation in the rat visual cortex

(Edagawa et al., 1998) and suppresses glutamatergic signalling in the PFC by reducing glutamate NMDA and AMPA receptors currents through a mechanism involving protein-kinase A (PKA) inhibition (Cai et al., 2002). In *in-vitro* studies the NMDA-evoked glutamate release or cyclic GMP elevation was reduced by activation of 5-HT_{1A} receptors whereas NMDA-evoked glutamate release was enhanced by blockade of these receptors by a 5-HT_{1A} receptor antagonist NAN-190 (Matsuyama et al., 1996; Maura et al., 2000). Although in *in-vivo* studies PFC application of 8-OH-DPAT did not affect the NMDA-evoked glutamate release, the 5-HT_{1A} receptor antagonist, WAY100135 enhanced basal and NMDA-evoked release of glutamate in the striatum of rats (Dijk et al., 1995) suggesting that 5-HT_{1A} receptors may modulate glutamatergic neurotransmission.

The interaction between 5-HT_{1A} and NMDA receptor mechanisms has been extensively studied in learning and memory processes associated with hippocampal functions. The findings indicate that decreasing 5-HT system function by stimulation of 5-HT_{1A} somatodendritic autoreceptors or blockade of postsynaptic 5-HT_{1A} receptors in the hippocampus remediate the spatial learning deficits induced by blockade of NMDA transmission in the hippocampus (Carli et al., 1999). Additionally, the 5-HT_{1A} receptor partial agonists and full antagonists attenuated working memory deficits as well as psychotomimetic effects induced by NMDA receptor antagonists (Harder and Ridley, 2000; Wedzony et al., 2000). It is not known whether 5-HT_{1A} agonists or antagonists may reverse the NMDA receptor antagonists induced deficits in attentional performance.

Using *in-vitro* techniques of intracellular recordings in interneurons of PFC layer V, Aghajanian and Marek (Aghajanian and Marek, 1997) showed that 5-HT induces EPSP and EPSC by stimulation of 5-HT_{2A} receptors. In this system the 5-HT-

evoked EPSC appears to involve presynaptic impulse-flow-independent release of glutamate mediated by 5-HT$_{2A}$ receptors (Aghajanian and Marek, 2000). The "hot-spot" for this induction being the apical dendrites of neocortical layer V pyramidal cells (Aghajanian and Marek, 1999) and correspond to the laminae that are rich in 5-HT terminals and 5-HT$_{2A}$ receptors (Blue et al., 1988; Aghajanian and Marek, 1997). On the basis of lesion studies, the 5-HT$_{2A}$ receptors involved in these effects were suggested to be located on the thalamo-cortical afferents (Marek et al., 2001). Thalamic lesions prevented also the increase in *c-fos* expression induced by systemic administration of a 5-HT$_{2A}$ agonist, DOI (Scruggs et al., 2000). However, *in-vivo* studies found that the 5-HT$_{2A/2C}$ receptor agonist DOI enhanced the firing rate of pyramidal neurons and 5-HT release in the mPFC by an action on postsynaptic 5-HT$_{2A}$ receptors not related to thalamocortical afferents (Puig et al., 2003). The DOI-induced 5-HT release depended on activation of glutamate AMPA receptor in the mPFC (Martin-Ruiz et al., 2001). Interstingly, DOI increased extracellular glutamate levels in the somatosensory cortex (Scruggs et al., 2003). Blockade of 5-HT$_{2A}$ receptors reduces *fos* expression induced by NMDA receptor antagonists (Habara et al., 2001).

A reduction of GABAergic inhibition has been suggested as a likely mechanism for the excitatory action of 5-HT$_{2A}$ receptors in the PFC. The vicinity of 5-HT$_{2A}$ receptors with postsynaptic domains of GABA$_A$ receptor on pyramidal neurons (Nusser et al., 1996) may be a possible anatomical substrate for the 5-HT$_{2A}$ receptor modulation of GABA$_A$ receptor currents (Feng et al., 2001). That in the PFC 5-HT$_{2A}$ receptor functionally interacts with GABA mechanism is further supported by in-vivo studies showing that DOI administered locally increased extracellular GABA levels (Abi-Saab et al., 1999).

Several studies have demonstrated a functional interaction between 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor systems and behaviours related to NMDA receptor blockade. Notably, the selective 5-HT$_{2A}$ receptor antagonist M100907 reduces the elevated locomotor activity induced by NMDA receptor antagonists (Gleason and Shannon, 1997; Martin et al., 1997). Similar effects have been reported with the 5-HT$_{2A/2C}$ antagonist ketanserin (Swanson and Schoepp, 2002). Forced swimming immobility and deficits in pre-pulse inhibition (PPI) induced by NMDA antagonists were also reduced by M100907 (Corbett et al., 1999; Varty et al., 1999). In rats performing a 5-CSRT task, systemic administration of M100907 but not a 5-HT$_{2C}$ antagonist SB242084 reduced the impulsivity induced by dizocilpine and NMDA-R2B receptor antagonist Ro63-1908 (Higgins et al., 2003a). Interestingly, in the same study M100907 had no effect on NMDA antagonist-induced increase in "compulsive" perseveration. However, Adams and Moghaddam (Adams and Moghaddam, 2001) found that blockade of 5-HT$_{2A}$ receptors by systemic M100907 did not antagonize the increase of cortical extracellular glutamate induced by systemically administered NMDA receptor antagonist PCP. Since behavioural effects of NMDA receptor antagonists have been suggested to depend on increased glutamate levels in the PFC, the results of Higgins et al. (Higgins et al., 2003a) and Adams and Moghaddam (Adams and Moghaddam, 2001) together suggest that they may be independent.

The 5-HT$_{2C}$ receptors may also be involved in glutamatergic signalling since their activation inhibits the cyclic GMP response in human neocortical slices and rat cerebellum (Marcoli et al., 1997; Maura et al., 2000).

These findings suggest that 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors may modulate glutamatergic neurotransmission and provide a basis for examining the effects of

agonists and antagonists at these receptors on behavioural responses induced by blockade of glutamate NMDA receptors in the PFC.

- Behavioural function of 5-HT system

This section will focus on reviewing converging evidence for the involvement of 5-HT mechanisms in cognitive processes such as attention, working memory, attentional set shifting, reversal learning and inhibitory control associated with medial PFC functions. A comparison with other monoamines and acetylcholine will be made.

Attention The vast majority of studies concerned with the role of 5-HT mechanisms in attention tested the effects of 5-HT selective lesions and drugs on attentional functioning (measured by accuracy) of rats performing a 5-CSRT task. Lesion studies do not clearly suggest an important involvement of 5-HT system in attentional functioning. In fact, global 5-HT depletion by intracerebroventricular injection of 5,7-dihydroxytryptamine (5,7-DHT) had no effect on accuracy in a 5-CSRT task. Different results were found when 5-HT was depleted by parachlorophenylalanin (PCPA) (Jakala et al., 1992). Similarly to the effects induced by depletion of hippocampal and cortical NA levels by 6-OHDA lesion of dorsal noradrenergic bundle (DNAB) in the 5-CSRT task (Carli et al., 1983), PCPA reduced accuracy when the stimuli were presented at a faster than normal rate or at low stimulus intensity. By contrast, accuracy of discrimination was transiently enhanced after 5,7-DHT lesions of the DR nucleus (Harrison et al., 1997b). This finding is consistent with negative correlation between accuracy and 5-HT utilization in the left PFC (Puumala and Sirvio, 1998). However, increasing 5-HT function by a

5-HT releasing drug such as d-fenfluramine has no effect on accuracy (Carli and Samanin, 1992).

The functions of 5-HT are afforded by the concerted actions of multiple serotonin receptor subtypes and as shown repeatedly 5-HT through these receptor subtypes may exert diverse, often antagonistic action on the same behavioural response. Several pharmacological studies have been performed in an attempt to define the role of various 5-HT receptor subtypes in different aspects of attentional performance in the 5-CSRT task. The findings of these studies suggest that 5-HT_{1A} receptors are involved in accuracy. However, the direction of the behavioural response depends on whether the 5-HT_{1A} somatodendritic autoreceptors or postsynaptic receptors are activated. In fact, systemic 8-OH-DPAT impaired accuracy and this effect was reversed by 5,7-DHT lesion or blockade of 5-HT_{1A} receptors in the DR nucleus by a 5-HT_{1A} antagonist WAY100635 (Carli and Samanin, 2000). In contrast 8-OH-DPAT injected into the mPFC improved accuracy (Winstanley et al., 2003b). However, in contrast to the study by Carli and Samanin ((Carli and Samanin, 2000)), Winstanley et al. ((Winstanley et al., 2003b)) found a facilitation of accuracy with systemic 8-OH-DPAT. Although these results are difficult to reconcile since similar doses of 8-OH-DPAT (0.1 mg/kg) were used in both studies, it is likely that different routes of drug administration employed in these studies might have contributed to the observed differences. The role of 5-HT_{2A} receptors in attentional functioning are not clear. Systemic $5\text{-HT}_{2A/2C}$ receptor agonists LSD and quipazine impaired accuracy (Carli and Samanin, 1992). The effects of LSD were partially antagonized by the $5\text{-HT}_{2A/2C}$ receptor antagonist ritanserin whereas those of quipazine were completely antagonized suggesting that in addition to the $5\text{-HT}_{2A/2C}$ receptors LSD activated other possibly 5-HT_{1A} receptors (Carli and Samanin, 1992). A follow up study with

the 5-HT$_{2A/2C}$ receptor agonist DOI, however, led to only mild impairments in accuracy in one study (Koskinen et al., 2000) and none at all in another under a range of test conditions (Koskinen et al., 2000b). Administered into the NAcc or into the cingulated cortex, DOI had no effect on accuracy (Koskinen et al., 2000b; Koskinen and Sirvio, 2001). DOI was not injected into the mPFC but a selective 5-HT$_{2A}$ antagonist M100907 injected into this cortical area facilitated accuracy (Winstanley et al., 2003b). Stimulation of 5-HT$_{2C}$ receptors by mCPP or their blockade by SB242084 had no effect on accuracy (Carli and Samanin, 1992; Higgins et al., 2003a). Other 5-HT receptor subtypes have been studied much less extensively, if at all.

In contrast to the effects of forebrain 5-HT depletion, degeneration of cholinergic cortical projection by the excitotoxin AMPA or the immunotoxin 192-IgG-saporin consistently impaired attentional functioning as shown by reduced accuracy in the 5-CSRT task (Muir et al., 1994; McGaughy et al., 2002). However, studies measuring ACh release in the mPFC during performance of a 5-CSRT task have suggested that the cortical ACh system may be involved in optimizing the sustained performance of the task rather than its attentional requirements (Passetti et al., 2000b; Dalley et al., 2001). Various studies have revealed an important interaction between ACh and 5-HT systems in cognitive functions (Steckler and Sahgal, 1995). This interaction appears to importantly contribute to attentional functioning in the 5-CSRT task. In a recent study, the 5-HT$_{1A}$ receptor antagonist WAY100635 reversed the accuracy deficit induced by AMPA lesions of the basal forebrain nucleus. In the same study the increased anticipatory responses in AMPA-lesioned rats were reduced by WAY100635 (Balducci et al., 2003). However, the increase in anticipatory

responses could not be specifically attributed to disruption of cortical ACh transmission (McGaughy et al., 2002).

The studies dealing with the role of mesocorticolimbic DA system in the performance of a 5-CSRT task have suggested that mPFC DA transmission may be involved in processes by which performance reaches a high level of accuracy with the DA D_1 receptor providing the necessary activity (Cole and Robbins, 1987; Robbins et al., 1998; Granon et al., 2000). Interestingly, 8-OH-DPAT increased ACh release in the mPFC through stimulation of postsynaptic $5-HT_{1A}$ receptors and indirect involvement of DA D_1 receptors (Consolo et al., 1996) suggesting that interactions with ACh and DA mechanisms may have contributed to its facilitation of attentional functioning.

Inhibitory response control (executive function) The ability to inhibit inappropriate responses in complex situations is a fundamental aspect of executive control. As reviewed in previous sections of this Introduction converging evidence implicate the PFC in executive attentional processes that enable accurate selection of appropriate responses under high attentional demands or in condition of interference. The loss of inhibitory response control may lead to "impulsive" behaviour (actions that occur without foresight, usually disadvantageously) perhaps akin to the risky behaviour seen in patients with ventromedial lesions in decision-making tasks or to "compulsive" perseveration (aimless repetition of responding) observed in humans and in animals with orbitofrontal lesions.

Impulsivity Impulsivity is probably made up of several independent factors, psychological and neurobiological. This is evident when the effects of pharmacological interventions are compared across tasks proposed to measure different aspects of impulsivity (Evenden, 1999a). For example, it is evident

comparing "impulsivity" induced by a drug in tests measuring the intolerance of the delay of reward (procedure for "decision-making" in rodents; i.e. impulsive choice), persistence, rapid decision making, timing, switching and anticipatory responding in reaction time tasks. Dissociations in different forms of impulsivity such as impulsive choice and anticipatory responses in a 5-CSRT task have been shown after lesions to different brain structures; nucleus accumbens, basolateral amygdala and orbitofrontal cortex being particularly involved in impulsive choice; mPFC and in particular IL region of mPFC controlling the impulsive responding in a 5-CSRT task (Cardinal et al., 2001; Chudasama et al., 2003; Christakou et al., 2004; Winstanley et al., 2004a).

Although the role of 5-HT in controlling impulsivity has been accepted its role may be more complex than previously envisaged and 5-HT may influence different aspects of impulsivity in different ways depending upon the importance of various behavioural factors, receptors sub-types and brain areas involved. This view receives support from various findings. In behavioural procedures explicitly designed to measure timing, selective 5-HT lesions affected impulsivity by impairing the ability of rats to regulate the control of behaviour in time probably by increasing the rate of switching between response alternatives but had no effect on the temporal discrimination (Ho et al., 1995; al-Zahrani et al., 1996). In tests measuring the intolerance of the delay of reward (impulsive choice) no effect (Winstanley et al., 2003a) or increased impulsive behaviour has been reported after forebrain 5-HT depletion by 5,7-DHT (Wogar et al., 1993; Mobini et al., 2000). Similar 5-HT depletion impaired the acquisition as well as the performance (although to a lesser extent) of a symmetrically reinforced go-no go conditional discrimination (Harrison et al., 1999), asymmetrical go-no go visual discrimination (Fletcher, 1993) and

resistance to extinction (Fletcher, 1993). These findings suggest that behavioural disinhibition induced by a reduction in 5-HT neurotransmission has pervasive behavioural effects and cannot be easily accounted for by a single theoretical construct.

Contrasting effects on impulsivity were reported for various 5-HT receptor ligands on different tests of impulsivity. For example, the 5-HT_{1A} agonists 8-OH-DPAT and buspirone increased impulsive choice (Liu et al., 2004; Winstanley et al., 2005) but 8-OH-DPAT and the 5-HT_{2A} receptor agonist DOI reduced "reflection impulsivity" where responses need to be withheld until accurate information is available such as in unreliable visual discrimination (Evenden, 1999). Opposite effects on impulsivity by these agonists have also been reported in the DRL-72 schedule or when tendency to terminate the chains of responses was measured (Evenden, 1998, 1999a). Blockade of 5-HT_{1A} and 5-HT_{2A} receptors by WAY100635 and ritanserin respectively increased and decreased impulsivity in paced fixed consecutive number schedule and unreliable visual discrimination (Evenden, 1999a).

In reaction time tasks such as a 5-CSRT task, impulsivity may take the form of increased anticipatory responding. Although some evidence suggests that there is a definite relationship between 5-HT function and the control of anticipatory responses on the 5-CSRT task, the precise nature of this is unclear. In fact, global 5-HT depletion as well as more selective pattern of depletion induced by 5,7-DHT lesions of the DR nucleus substantially and permanently increase anticipatory responding (Harrison et al., 1997b, 1999). These findings may appear to be contradicted by findings showing a positive correlation between 5-HT utilization (Puumala and Sirvio, 1998) or extracellular 5-HT levels in the mPFC (Dalley et al., 2002) and anticipatory responding of rats performing a 5-CSRT task.

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However, by acting on distinct receptor subtypes in diverse brain areas 5-HT may exert differential control on impulsivity in a 5-CSRT task. Several studies have shown that 5-HT$_{2A}$ receptors in the mPFC may be involved in the control of anticipatory responding in this task. Systemic or intra-mPFC administration of 5-HT$_{2A}$ receptor agonist increased while antagonists reduced anticipatory responding (Koskinen et al., 2000; Winstanley et al., 2003b; Passetti et al., 2003a). Anticipatory responding is increased by systemic 8-OH-DPAT through stimulation of somatodendritic 5-HT$_{1A}$ autoreceptors in the DR nucleus (Carli and Samanin, 2000) but not by stimulation of postsynaptic 5-HT$_{1A}$ receptors in the mPFC (Winstanley et al., 2003b). The findings by Carli and Samanin (Carli and Samanin, 2000) are consistent with those suggesting that diminished 5-HT function leads to increased impulsivity. Recent findings show that 5-HT might exert a tonic inhibition on impulsivity by stimulation of 5-HT$_{2C}$ receptors since blockade of this receptor by a selective antagonist SB242084 greatly increased anticipatory responding (Higgins et al., 2003a; Winstanley et al., 2004b). The 5-HT$_{1B}$ receptors appear to play some role in impulsivity as shown by impairments in DRL-36 performance by 5-HT$_{1B}$ knock-out mice (Pattij et al., 2003).

Dopaminergic mechanisms are also importantly involved in impulsivity. D-amphetamine (Robbins, 2002) and dorsostriatal DA depletion increase anticipatory responding in a 5-CSRT task (Baunez and Robbins, 1999). Interestingly, DA D$_1$ antagonist SCH23390 reduced anticipatory responses induced by 5-HT depletion while the effects of d-amphetamine on anticipatory responding were less pronounced in 5-HT depleted rats (Harrison et al., 1997a, 1997b). However, the brain area critical for this 5-HT-DA interaction is unknown. Recently, has been shown that 5-

HT-DA interactions within the NAcc affects the regulation of impulsive choice (Winstanley et al., 2005).

Perseveration and reversal learning   Perseveration represents a form of behavioural inflexibly and is commonly observed in non-human primates with lesions to the ventral PFC and orbitofrontal cortex when performing reversal-learning tasks (Fuster, 1989).   In rats performing a 5-CSRT task increased perseverative responses were found after lesions to the orbitofrontal cortex (Chudasama et al., 2003), the mPFC (Muir et al., 1996) but also when the lesions were confined to the PrL region of mPFC (Chudasama and Muir, 2001; Passetti et al., 2002).

The involvement of 5-HT mechanisms in perseveration is not clear.   No effects (Harrison et al., 1997a; Carli and Samanin, 2000) or increased perseverative responding on a 5-CSRT task has been reported after forebrain depletion of 5-HT (Winstanley et al., 2004b).   Systemic 8-OH-DPAT had no effects on perseverative responses (Carli and Samanin, 2000). The key difference between these studies appears to be that in the study by Winstanley et al. (Winstanley et al., 2004b) perseverative responses had no programmed consequences.   Reversal learning is significantly impaired after selective PFC 5-HT lesions in monkeys probably due to enhanced perseverative tendencies (Clarke et al., 2004; Clarke et al., 2005). Similarly, systemic 8-OH-DPAT impaired serial reversal learning by increasing perseveration; the effects were reversed by WAY100635 (Clarke et al., 2003). Deficits were found in 5-HT deficient rats (by tryptophan deficient diet) and monkeys with high doses of 5-HT$_3$ antagonist ondansetron (Barnes et al., 1990; Domeney et al., 1991). However, low doses of ondansetron (Domeney et al., 1991)

and LSD improved reversal learning (King et al., 1974). These effects were not specific to reversal learning but extended to learning the initial discrimination.

In studies with healthy human volunteers suppression of 5-HT by acute tryptophan depletion impaired reversal learning on the intradimensional/extradimensional (ID/ED) shift task (Park et al., 1994; Rogers et al., 1999a) but not ED shifting (Rogers et al., 1999a). Similarly to the studies in animals the effects of 5-HT manipulations generalised to the initial visual discrimination acquisition. A recent study found that in acute trypthophan depleted human subjects the task-related BOLD response increased in the dorsomedial but not ventrolateral PFC during probabilistic reversal learning (Evers et al., 2005).

Reversal learning is also significantly affected by manipulation of DA system. Manipulation of DA system by DA D_2 receptor antagonists as well as the indirect catecholamine agonist methylphenidate in young healthy volunteers reduced the BOLD response in left ventrolateral but not dorsolateral PFC during reversal learning (Clark et al., 2004). Additionally, bromocriptine a DA D_2 receptor agonist impaired performance of human subjects on the probabilistic reversal-learning task whilst improving performance on spatial memory task (Mehta et al., 2001). In animals stimulation of DA system by d-amphetamine induce perseverative responding on a reversal task and this effect was abolished by DA D_2 antagonist haloperidol (Ridley et al., 1981). However, 6-OHDA lesions of the NAcc impaired reversal learning in rats (Taghzouti et al., 1985). However, mesocortical DA system exerts a complex control of PFC functions and both excessive and insufficient stimulation impair cognitive function, including reversal learning (Ridley et al., 1981; Arnsten, 1997; Zahrt et al., 1997).

Attentional set shifting   In attentional set shifting, instead of shifting

responding between specific exemplars, such as in reversal learning, subjects are

required to shift attention between distinct perceptual dimensions of complex stimuli

such as shapes and lines in monkeys (Dias et al., 1996) or texture and odours in rats

(Birrell and Brown, 2000) to track stimulus reward associations.   Different

neuroanatomical structures in the PFC mediate these two types of shifts, lateral PFC

in monkeys (Dias et al., 1996) and mPFC in rats (Birrell and Brown, 2000) in the

case of attentional set shifting and orbitofrontal cortex in the case of reversal learning

in monkey and rats (Dias et al., 1996; McAlonan and Brown, 2003). Attentional set

shifting in marmosets is apparently facilitated by mesofrontal DA depletion (Roberts

et al., 1994) while PFC 5-HT depletion has no effect (Clarke et al., 2005). Several

recent studies have reported that treatments that increase DA release in the mPFC

improve rather than impair set shifting (Tunbridge et al. 2004). Blockade of D2

receptors in the mPFC impaired attentional set-shifting by increasing perseverative;

D2 receptor agonists had no effect (Ragozzino et al. 2002; Floresco et al. 2005).

Similarly Metha et al. (2004) using human volunteers showed a trend for attentional

set shifting to be impaired after l-sulpiride. In rats the $5-HT_6$ receptor antagonists

SB399885-T and SB271046-A improved attentional set shifting by reducing ID/ED

shift (Hatcher et al., 2005). The role of other 5-HT receptors has not been tested in

this paradigm.

Working memory Serotonergic lesions with 5,7-DHT do not impair rats' working

memory in tests such as delayed non-matching to position (DNMTP) (Sahgal and

Keith, 1993) and continuous non-matching to sample (CNMTS) (Sakurai and Wenk,

1990) although T-maze delayed alternation was impaired following raphé 5,7-DHT

lesions but only if the cognitive burden was high (Wenk et al., 1987). However, 5-

HT interaction with cholinergic system at hippocampal but not cortical level appears to mediate working memory (see (Steckler and Sahgal, 1995)). The 5-HT$_{1A}$ receptor agonists administered systemically have been shown to have no effect but also to improve at low doses and impair at high doses DNMTP performance (Cole et al., 1994; Warburton et al., 1997). In a spatial working memory test 8-OH-DPAT injected into the MR nucleus improved performance accuracy independently of delay whilst injected into the DR nucleus had no effect (Warburton et al., 1997). Increasing 5-HT levels by 5-hydroxy-trypthophan and a decarboxylase inhibitor Ro4-4602 in cats performing a delayed response had no effect on the performance at 0 delay but significantly increased the errors during delay trials (Roberge et al., 1980). Recently, a role for 5-HT$_{2A}$ receptors in the working memory processes in monkeys performing an oculomotor delayed-response (ODR) task has been claimed (Williams et al., 2002)]. Iontophoresis of 5-HT$_{2A}$ antagonists onto the putative pyramidal cells produced a reduction in delay activity for preferred target locations, stimulation of 5-HT$_{2A}$ receptors by 5-HT had opposite effects. From these findings it would be expected that increased 5-HT might benefit working memory performance but no behavioural data clearly support this case. In fact, LSD (a 5-HT$_{2A/2C}$ agonist) impaired monkeys' performance of a delayed alternation task (Jarvik and Chorover, 1960) and visual discrimination (Fuster, 1959), but improved accuracy of visual discrimination in pigeons (Becker et al., 1967). In human subject, supranormal levels of 5-HT may be deleterious for spatial working memory (Luciana et al., 1998). By contrast, the mesofrontal DA system has seemingly a specific role in working memory processes. 6-OHDA lesions of the PFC impaired accuracy of performance on a delayed response type test and the deficits were remediated by DA agents (Brozoski et al., 1979). Applications of DA D$_1$ antagonists to the principal sulcus of

the PFC impaired performance of a delayed saccade task. However, iontophoretic application of DA D_1 antagonists enhanced the tuning of PFC pyramidal cells by directly boosting the strength of their memory fields and reducing activity in the opponent memory field during the delay period in monkeys performing an ODR task (Williams and Goldman-Rakic, 1995). In rats activation of PFC DA mechanism is associated with poor performance in tests of working memory such as delayed alternation (Murphy et al., 1996; Zahrt et al., 1997).

In conclusion, drugs and manipulation of 5-HT system affect specifically certain tasks sensitive to PFC dysfunction. Some aspects of performance of these tasks may be differentially affected by the activity of different 5-HT receptor subtypes. This is particularly evident in aspects of performance that are sensitive to changes in inhibitory response control and attention. An important aspect is the opponent partnership with dopaminergic mechanisms on different behavioural responses associated with functions of the PFC.

- Summary: Statement of the problem

The fundamental goal of the work described in this thesis was to provide a better understanding of role played by serotonergic mechanisms in cognitive functions of the prefrontal cortex and their involvement in the psychophatology of schizophrenia. The preceding pages have set out the general background to this work. This has included material from psychophatology, neuropsychology and behavioural pharmacology. With such a diverse background it is essential to frame specific questions to which experiments will be addressed. The general aim has already been expressed as examining the role of 5-HT mechanism in attentional and executive functions. It has also been stated that the project would adopt a methodological approach of concentrating on the functional consequences of the interaction between 5-HT mechanisms dependent on activity of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors and the glutamate NMDA receptor mechanisms in the mPFC. The experimental methods have concentrated on behavioural and neurochemical techniques.

This methodological approach is well suited to provide information in two main areas: first, to investigate the involvement of 5-HT mechanisms in cognitive processes associated with the functions of the PFC, and second to investigate the underlying neurochemical mechanisms. By concentrating on a task in which attentional functioning and inhibitory response control are regulated by relatively independent factors a specific contribution to different aspects of attention and response control may be more precisely delineated. *In-vivo* neurochemistry has attempted to provide some insight into neurochemical mechanisms associated with behavioural changes.

The work has four main parts; 1) establishing a model of attentional performance deficit based on the blockade of NMDA transmission in the mPFC, 2) testing the

hypothesis that NMDA antagonist-induced increase in glutamate efflux in the PFC may have functional consequences; 3) investigation of the behavioural and neurochemical effects of 5-HT receptor ligands and 4) testing the effects of DA D2 receptor antagonists.

1) As reviewed in the Introduction the role of specific regions of PFC in different aspects of attention and executive functions is well established in human and non-human primates and in rodents. NMDA receptor antagonists have been shown to induce behavioural impairments in task sensitive to PFC functions and these effects have been attributed to changes in glutamate release in the mPFC. However, in the vast majority of experiments non-competitive NMDA antagonists were used and they were administered systemically. Thus, the role of glutamatergic mechanisms within the PFC in cognitive processes associated with PFC functions is still poorly understood. I studied whether and how blockade of NMDA receptors in the mPFC by a competitive antagonist such as CPP affected various aspects of attentional performance in the 5-CSRT task. I also examined whether blockade of NMDA receptors by CPP in the mPFC increased extracellular glutamate concentrations in this area. The work helped to establish a model of attentional performance deficit based on disregulation of glutamate transmission in the mPFC. This model has been used throughout this thesis to investigate the modulatory role of some 5-HT and DA D_2 receptor mechanisms in attentional processes and inhibitory response control.

2) The NMDA receptor antagonists induce glutamate release in the PFC, which may play a pivotal role in behavioural abnormalities. Several findings suggested that increased glutamate release induced by NMDA receptor antagonists and a consequent over-stimulation of AMPA receptors may be responsible for their behavioural effects. This hypothesis is still debated. Thus, I investigated; 1)

whether $mGlu_{2/3}$ agonist that inhibits the release of glutamate can reduce the attentional deficits caused by intracortical NMDA receptor antagonists; 2) whether the behavioural effects of blockade of NMDA receptors could be accounted for by enhanced stimulation of AMPA receptors in the mPFC.

3) The 5-HT mechanisms have been suggested to modulate various aspects of cognitive functions of PFC but their exact role is far from clear. Several studies have demonstrated a functional interaction between $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptor mechanisms and behaviours related to NMDA receptor blockade, as reviewed in the Introduction. However, the role played by the interaction of these 5-HT-ergic and glutamatergic mechanisms in the PFC in different aspects of attentional and executive function is largely unknown. The questions these investigations were designed to answer were whether a systematic analysis of the effects of $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ ligands on behavioural and neurochemical changes induced by NMDA receptor blockade in the mPFC can provide informations on the specificity of various 5-HT receptor mechanisms to aspects of attention and inhibitory response control. This investigation was designed to provide some information as to whether changes in glutamatergic transmission in the PFC may be associated with specific aspects of attentional performance. The experiments attempted to answer questions such as; 1) which aspects of attentional performance deficit were sensitive to blockade of $5\text{-}HT_{2A}$ receptors, 2) if activation of $5\text{-}HT_{2C}$ receptor would result in effects similar to those found by blockade of $5\text{-}HT_{2A}$ receptors, 3) what was the contribution of $5\text{-}HT_{2A}$ receptors in the mPFC, 4) whether activation of $5\text{-}HT_{1A}$ receptor in the mPFC had the same effects as blockade of $5\text{-}HT_{2A}$ receptor or if they could be dissociated. Additional neurochemical experiments were performed to determine whether $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ antagonists could modify the rise in

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extracellular glutamate efflux induced by blockade of NMDA receptors in the mPFC.

4) Numerous lines of evidence suggest that the 5-HT and DA systems, although both implicated in functions associated with PFC, mediate different forms of neuromodulation, which is shown by their distinct contribution to various aspects of PFC functions. As reviewed in the General Introduction, distinct contributions of these chemically defined systems to attention and inhibitory response control have been revealed when the behavioural effects of their manipulation by drugs or selective lesions were examined systematically on a common behavioural task such as the 5-CSRT task. An additional aim of the present work was to compare the relative contribution of 5-HT and DA mechanisms to different aspects of attentional performance deficits induced by blockade of NMDA receptors in the mPFC. The present investigation was restricted to the role played by DA D_2 receptor mechanisms in the mPFC. Furthermore the contribution of D_2 and "non-D_2" receptor mechanisms to the effects of atypical antipsichotics was also investigated.

In conclusion, it was hoped that these pharmacological, behavioural and neurochemical studies could provide new information on the physiological mechanisms involved in the control of attention and executive functions, possibly relevant to cognitive deficits associated with neuropsychiatric disorders such as schizophrenia.

CHAPTER 2. GENERAL METHODS

In order to define the causal relationship between a particular behaviour and the underlying neural substrate it is necessary to study the effects of selective manipulation of defined neural or neurochemical systems in ways that necessitate the use of experimental animals. Furthermore, in animals, is possible to study the involvement of a specific brain area by administering locally selective receptor agents. Although, it is problematic to extrapolate the findings in animals to humans, the use of comparable cross-species tests of cognitive function may increase the likelihood that similar cognitive functions are studied in each species. The relative ease with which brain neurotransmitter and neural systems can be manipulated in the rat makes this species an obvious choice.

This chapter contains a description of the general methods and techniques used in this thesis to examine the role of serotonin receptors in attention and executive function in condition of disrupted glutamate neurotransmission in the prefrontal cortex. The methodological approach has concentrated primarily on examining the behavioural effects of selective pharmacological manipulation by agonists or antagonists at serotonin $5-HT_{1A}$, $5-HT_{2A}$ and $5-HT_{2C}$ receptors, glutamate mGlu2/3 and AMPA receptors and dopamine D_2 receptors on the attentional performance deficits in the 5-CSRT task induced by selective blockade of glutamate NMDA receptors in the medial prefrontal cortex (mPFC). Attempts have been made to associate the behavioural effects of pharmacological manipulations of $5-HT_{1A}$ and $5-HT_{2A}$ receptors to changes induced in glutamate and serotonin release in the mPFC using *in vivo* microdialysis techniques.

The first section contains a description of general methods employed to assess the behavioural effects of various pharmacological manipulation such as animals used

and their husbandry, stereotaxic surgery, microinfusion and histological techniques, and test apparatus and behavioural procedures. Some methodological problems inherent to the intracerebral microinfusion technique are also discussed. Section 2, contains a description of microdialysis methodology and techniques for biochemical determination of glutamate and serotonin concentrations in dialysates. The methodological problems of measuring in vivo changes in glutamate and serotonin release by microdialysis are discussed. The experimental design and general statistical methods are presented in Section 3. The specific procedures and methods that are unique to, or deviate from, these general methods are described in the appropriate experimental chapters. A flow diagram outlining the basic experimental plan of behavioural experiments is schematically presented in Fig. A.

Time-line of a typical experiment
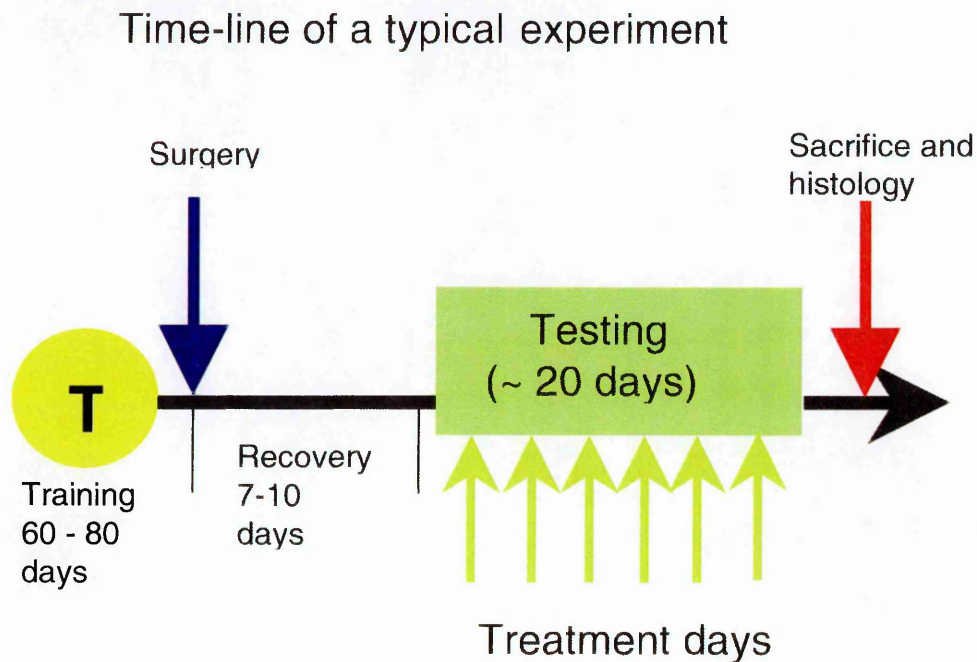
Figure A. Time plan of behavioural experiments.

Section 1: Pharmacological manipulation of discrete brain areas and effects on behaviour

- Methodological issues

The pharmacological manipulation of defined neurochemical projection systems or of intrinsic neuronal population in restricted brain regions, by intracerebral microinfusions techniques for understanding the neurochemical basis of behaviour has many advantages and are extensively employed. A major advantage is the reversibility of the effects that could not be found with other techniques such as neurotoxic or neurochemically selective lesions. However, these procedures to provide reliable results, must met several important criteria of specificity: anatomical, physiological, pharmacological and behavioural.

Anatomical specificity and damage   The anatomical specificity is fraught with problems raised by the intracellular diffusion and extracellular spread of the chemicals to adjacent and possibly also to distant site. The major factors affecting spread and diffusion are the chemical nature (liposolubility, catabolic breakdown and neuronal uptake rates) and volume of the infused substance, the rate of infusion and the morphological characteristics of the tissue at the infusion site (Routtenberg, 1972; Myers, 1974). Thus a precise area of spread and diffusion is extremely difficult to estimate. Some studies dealing with the problems of spread have shown that the substance spreads along the path of least resistance, that is, it refluxes along the cannulae shaft. Preventing this to happen by allowing some time to elapse before removing the infusion cannulae, as well as limiting the volume and rate of infusion may help to confine the substance to the intended area.

Therefore the infused volume was 1 µL (an aqueous solution has been estimated to occupy 1.1 mm^3) at a rate of 0.5 µL/min and 2 min were allowed before the injection cannulae were withdrawn.

Another major criticism of microinfusion technique is that they produce non-specific tissue damage, in various ways, for example by the permanent implantation of the guide cannulae, the mechanical insertion of the infusion cannulae, because of the volume and the chemical characteristics such as pH of the infused solutions, or through the cell loss due to brain oedema or infection.

Whenever, the cannulae are inserted or permanently implanted they cause gliosis and necrosis in the surrounding cells as well as proliferation of astrocytes (Routtenberg, 1972). Therefore, very small cannulae (0.6 mm) with sharpened, filed-smooth tips were fashioned to penetrate tissue as cleanly as possible. The permanent guide cannulae were implanted 2.0 mm above the intended site so that infusions would not be made into a damaged area.

To decrease the probability of non-selective damage it has been suggested that only one microinfusion per animal be given. This is somewhat impractical from the point of view of complex behavioural experiments such as those used in this study. Behavioural evidence that animals reverted to a high level of performance in the intervening days (when no injection were given) similar to that of pre-implantation of guide cannulae suggest that valid results can be obtained after an animal had repeated infusions. To prevent the scar tissue to develop and encapsulate the locus of injection (Myers, 1974) only sufficient time for recovery from drug effects was allowed.

Evidence of brain infection or oedema was rarely observed. However, some rats in the studies here did developed infection. Dramatic weight loss, anorexia, and

catalepsy were observed. These animals were immediately removed from the experiment and sacrificed for humane reasons. Histological examination of brains revealed severe oedema and infection at the infusion site and surrounding area.

In any consideration of the problem of non-specific damage, histological assessment of each rat brain and site of injection is crucial. Standard histological procedures (Wolf, 1971) employing cresyl violet stain of Nissl substance within cell bodies were used. The guide cannulae track was distinctly visible, and beneath it was a darkly stained area of gliosis at the infusion tip.

Physiological specificity There is some evidence to suggest that the behavioural effects observed with central infusions in this study are produced physiologically. The majority of the experiments reported here show dose related effects, which is strong evidence for specificity.

Pharmacological specificity The criteria for pharmacological specificity were met by the use of highly selective agonists and antagonists at various receptors. Details of the receptor binding profile of each drug used in the present study will be given in the appropriate experimental chapter.

Behavioural specificity Behavioural specificity of intracerebral infusions can be determined studying their effects in different behavioural tasks or showing that task conditions may be strong determinants of drug effects. Here, identical drug infusions into the same cortical area in animals subjected to different task conditions show contrasting behavioural effects. In addition, since changes in motor activity may interfere with the performance of a complex task such as 5-CSRT the effects of some pharmacological treatments on motor activity have been examined. The behavioural procedures are further discussed in the Introduction and relevant experimental chapters.

- General experimental techniques and methods

Animals and their care

In all behavioural experiments male Lister Hooded rats (Charles River, Italy) weighing between 300 and 350 g before surgery were used. Animals used to examine the effects of various treatments on the performance of the 5-CSRT task were food deprived. They were housed in pairs until surgery and then singly in a temperature-controlled room (21°C) with a day/night cycle (7 am-7 pm). Water was available *ad libitum*. Limited access to food (about 15 g/rat of Altromin pellets) at the end of each day's testing kept the animals at 85-90% of their initial free-feeding weight.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with the national (D.L. n. 116, G.U., suppl., 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Surgical techniques

Implantation of guide cannulae   The implantation of guide cannulae to animals used in behavioural experiments was made in rats previously trained to a stable level of performance. Rats were anaesthetized by an intraperitoneal (IP) injection (2mL/kg) containing 40 mg/mL ketamine and 5 mg/mL xylazine. All animals received IP injections of 0.1 mg/kg atropine sulphate. After, the rat head was shaved it was secured by blunt ear-bars (45°) in a stereotaxic frame (Kopf Ins. U.S.A.).

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Next the scalp was incised on the midline of the occipito-frontal plane, the underlying tissue reflected and the fascia scraped from the surface of the cranium. Bilateral 23-gauge, stainless steel guide cannulae (Cooper's Needles, U.K.), placed on a specially constructed cannulae carrier, were implanted in the medial region of the prefrontal cortex through drilled burr holes and secured to the skull using three bone screws and dental cement. Care was taken throughout to keep the instruments sterile (by keeping them in a UV chamber and cleaning them with a solution of antiseptic). To prevent post-surgical infection and to keep the cannulae shaft clear and free from obstruction a 30-gauge stainless steel stylets flush with the end of the guide cannulae was inserted. Finally, the wound was dusted with antibacterial powder.

Coordinates   The co-ordinates used were: anterior-posterior +3.8 mm from bregma, lateral ±0.8 mm from midline and dorsal-ventral -3.2 from dura (Paxinos and Watson, 1982). The incisor bar was set at –3.3 mm relative to the inter-aural line.

Recovery and post-operative care   After surgery rats were housed singly. The rats that were food deprived during the experiments were given free access to food and water for two days after surgery. Afterwards they were kept food-deprived as before surgery and were re-trained on the 5-CSRT task to re-establish a pre-surgery level of baseline performance. This took between 7 to 10 days. During this period the animals were habituated to be hand held for a few minutes and the cannulae stylettes that had been pulled out were replaced. Checks were made for any weight loss or bloated gastrointestinal tract. Occasional rats showing gastrointestinal bloating or discomfort were excluded from experiments. Rats are relatively resistant to infection and only rarely were brain edema or infection of the flesh surrounding the cap observed.

Drugs and injection procedures

Drugs Various drugs were microinfused into the mPFC or administered peripherally in these experiments. The drugs were the following: 3-(R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Tocris, U.K); R-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol (M100907) (Aventis, USA); 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT)(Sigma, Italy); ((S)-2-chloro-5-fluoro-indol-1-yl)-1-metyl ethylamine fumarate (Ro60-0175) (Roche, CH); (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) (Eli Lilly, USA); 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f) quinaxaline (NBQX) (Tocris, UK), clozapine (Sandoz, CH); haloperidol (Lusofarmaco, Italy), l-sulpiride (Sinthlabo, France). All drug doses were calculated in terms of their salts and dissolved in an appropriate vehicle.

Injections Peripheral drug injections were given via the peritoneum (IP) or subcutaneously (SC). The volume of 2 mL/kg was used in all experiments. For each substance employed, details of solution preparation, doses, volume of injections, route of administration and pre-treatment time will be given in the corresponding experimental chapters.

Microinfusion procedures On testing days, while the rat was held, the stylets were removed and two injection units terminating 2 mm below the tip of the guides were inserted. A volume of 1 µL per hemisphere of various doses of drugs or appropriate vehicle was delivered at a rate of 0.5 µL/min by a 10 µL Hamilton syringe mounted in a CMA/100 infusion pump (CMA Microdialysis, Sweden), connected by PP10 tubing to the injection units. Injection units were left in place for 2 min to allow for diffusion.

The rats only occasionally showed signs of agitation or discomfort during microinfusion, and they never showed the strong reaction to the lowering of the cannulae that has been observed with certain placements (Personal observations).

Histology

After completion of the behavioral testing, rats were killed by a lethal dose of Equithesin and perfused transcardially with phosphate buffer saline followed by 4% formalin solution. Brains were removed and post-fixed in 4% formalin solution. Before being cut, the brains were transferred to 20% sucrose in 0.2 M phosphate buffer saline. Coronal sections were cut at 30 μm in a Cryo-cut and stained with cresyl violet. Inspection of the stained slides under the light microscope and the trajectory of gliosis produced by the cannula allowed its location and tip to be estimated and mapped on figures of the atlas (Paxinos and Watson, 1982) (Fig. 1.1). Only data from rats in which the cannulae were located in the desired area were included in the results.

The grey areas in Fig. 1.1 depict the location of the injector tips of rats included in the results. The majority of injections were confined to the pre-limbic area between bregma +4.2 and +3.7. In some rats the tips were between bregma +3.7 and +3.2. However, we did not observe any difference in the behavioral results of rats with injection tips confined to the more anterior (bregma 4.2 - 3.7) or the posterior (bregma 3.7 - 3.2) area of mPFC.
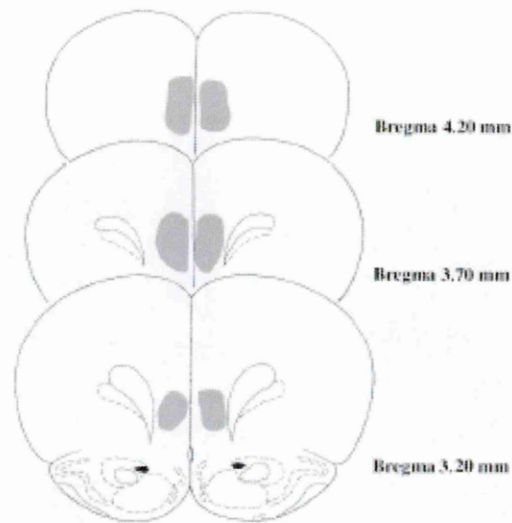
Figure 1.1
Schematic representation of the injection sites in the mPFC. The gray area indicates the location of the injection tips.

Examination of stained coronal sections of the brains showed that multiple injections into the mPFC produced limited tissue damage as shown by the extents of gliosis along the cannulae tract and at the tip of injection cannulae, and Fig. 1.2 consist of a number of representative microphotographs of histological sections of rats brains from various experiments. Additionally, in the between-injections days rats returned to perform at very high level of accuracy (80-90%), making less than 20% omissions and only a small number of anticipatory and perseverative responses. Thus, the various treatments and multiple injections did not have a permanent effect on performance.
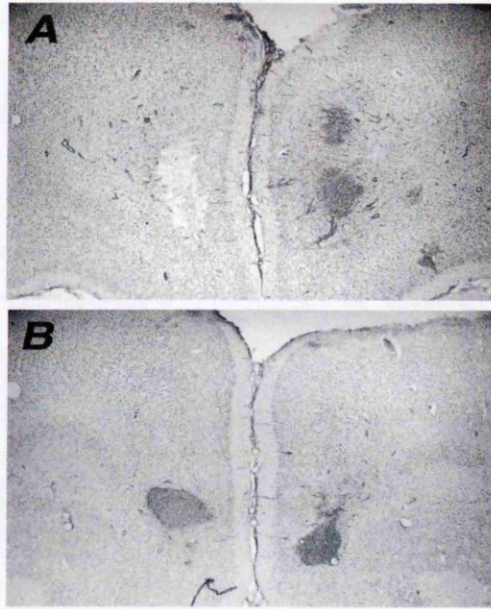
Figure 1.2
Representative photographs of the histological sections stained with cresyl violet. Examples from several rat brains are shown. The histological sections show the extent of gliosis along the cannula tracks and at the infusion sites in rats from different experimen
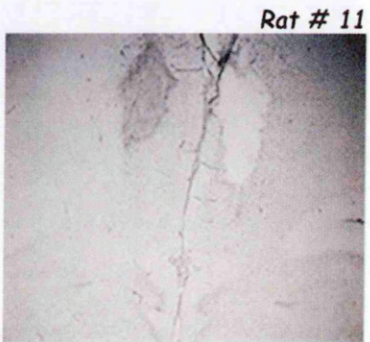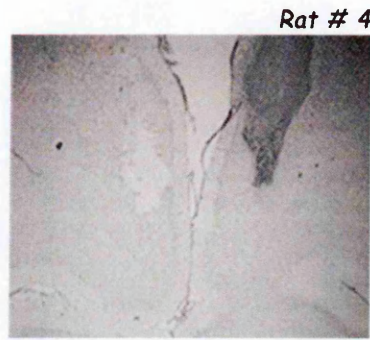
Figure 1.3

Representative photographs of the histological sections stained with cresyl violet. Examples from several rat brains are shown. The histological sections show the extent of gliosis along the cannula tracks and at the infusion sites in rats from different experiments.
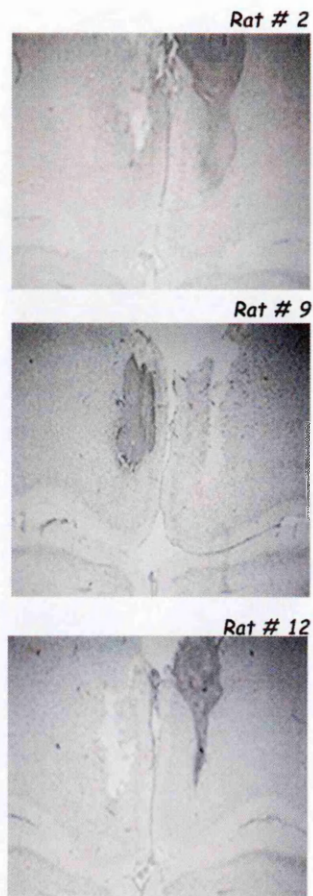
Figure 1.4

Representative photographs of the histological sections stained with cresyl violet. Examples from several rat brains are shown. The histological sections show the extent of gliosis along the cannula tracks and at the infusion sites in rats from different experiments.

- Behavioural experimental techniques and methods

The behavioural paradigm employed in the present study to examine the effects of glutamatergic, 5-HT and DA manipulation on attention and executive functions is the 5-CSRT task which has been extensively used to assess the effects of cortical lesions and manipulations of monoamines and ACh system on these processes. Due to its characteristics it has provided results relevant for a more precise definition of the role played by various cortical areas and neurochemical systems in various aspects of attentional performance (for a review see Robbins, 2002).
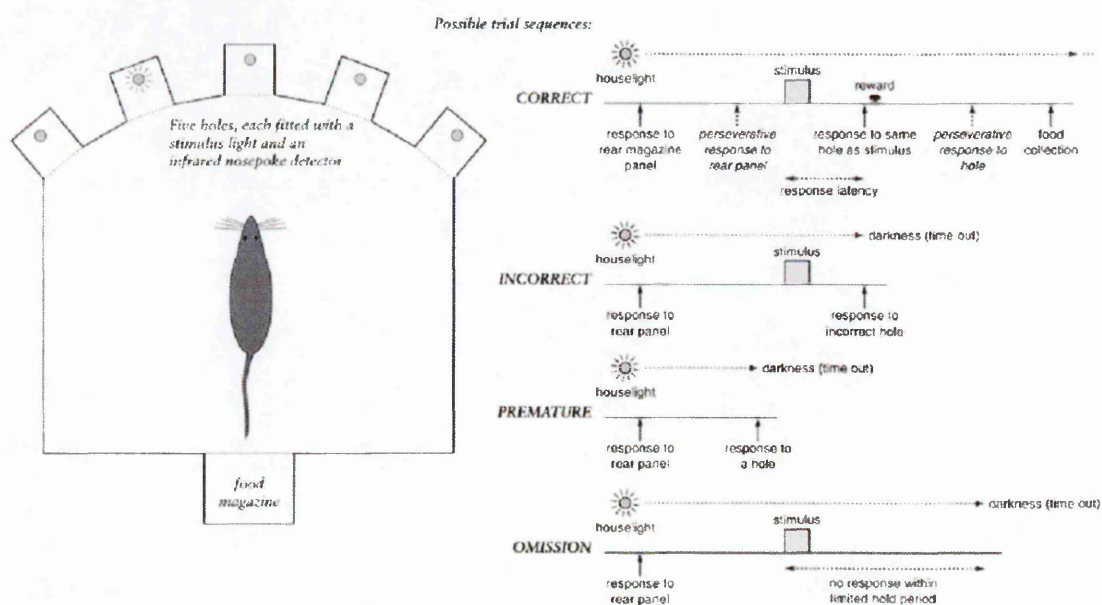
Figure 2.1. Schematic representation of the apparatus (left) and the sequence of scheduled events and possible outcomes (right) (from (Dalley et al., 2004)).

The 5-CSRT task essentially taxes the capacity of rats to sustain spatial attention divided among a number of locations to detect a brief visual target over a large

amount of trials in addition to inhibitory response control or executive functioning. Attentional capacity is measured by accuracy (proportion of correct responses over the total number of correct plus incorrect responses) expressed by the percentage of correct responses. Changes in accuracy could not be explained as a simple motor effect since both responses have equivalent motor requirements. Two types of inhibitory response control can be indexed. First, anticipatory responses occur inappropriately when the rats is waiting for the target stimulus (during the inter-trial-interval) and an increase in these responses might signal that the inhibitory control over highly prepotent responses is lost, and, as such, might represent a form of impulsivity (Evenden, 1999a). Second, perseverative responses by which rats continue to respond in the holes after a correct response has been made and might represent a different form of deficit in inhibitory response control. This deficit has been suggested to be more akin to "compulsive" rather than "impulsive" behaviour, in which rats continue to respond in the holes in spite of signals that food is available in the magazine (Chudasama et al., 2003). Omissions, not included in the accuracy are expresses as the percentage (number of omissions over the total number of trials completed). An increase in omissions may reflect gross impairments in attention, motivation or motor ability. Similar interpretation may be made for correct response latency that may reflect decision time but also motor and motivational factors. These different interpretations may be disambiguated by observing the overall pattern of effects on omissions and other measures such as latency to make a correct response and latency to collect the food. The great advantage of this task is that it provides largely independent measures of accuracy, speed, "impulsivity", "compulsive" perseveration and motivation (see Robbins (Robbins, 2002)).

The difficulty of the 5-CSRT task can be varied by reducing or increasing the duration or brightness of the visual target. This manipulation provides a mean of decreasing or facilitating attentional performance and may be useful to characterize the factors that may contribute to the effects of various manipulations on accuracy. The visual stimuli can be made temporally unpredictable, a manipulation that is equivalent to the non-paced version of the continuous performance task (versus the standard, or paced, version in which the stimuli come at predictable intervals). This means that the rat cannot rely on automatic processing to control orientation to the location of the stimuli at a particular time and it has to monitor its readiness to respond on a continuous basis. These manipulations may probe other aspects of attention or more general performance factors.

One problem with this type of paradigm particularly with pharmacological manipulation that may affect satiety is that changes in various measures of attention and performance may be due to simple effects on satiety. Prefeeding the rat with food pellets had no effect on accuracy but reduced the speed of responding and to collect the reinforcement. It also increased the proportion of omissions (Carli and Samanin, 1992). These effects of prefeeding suggest that the measures of omissions and latencies may be affected by motivational factors.

5-CSRT task apparatus and procedures

Apparatus   The test apparatus consisted of two 25 x 25 cm aluminum chambers built in the Dept. of Experimental Psychology, University of Cambridge. The rear wall of each box was concavely curved, and had set into its full arc nine square holes, 4 cm deep and 2 cm above floor level. Each hole had an infrared beam crossing the entrance vertically and illuminating a photoelectric cell. A standard 3W

bulb at the rear of each hole provided illumination. Food pellets (Sandown Scientific, UK) were delivered to a tray at the front of the box. A hinged panel blocked the entrance to the tray. A 3W house-light was installed centrally in the box roof. Each apparatus was controlled on-line and data were collected by a Control Universal Cube microcomputer system (Cambridge, U.K.), with software written in ONLIBASIC.

Behavioral procedures   Animals were trained on the 5-CSRT task to a stable performance as previously described (Carli et al., 1983). The start of the session was signaled by illumination of the house-light and the delivery of a single food pellet. Opening the panel to collect the pellet began the first trial. After a fixed delay (the inter-trial interval, ITI), the light at the rear of one of the holes came on for a short period. The light stimulus was presented in each hole for an equal number of times during the course of a complete session, with the order of presentation randomized by the computer. While the light was on, and for a short period afterwards (the limited hold), responses in the hole that was illuminated (correct response) resulted in the delivery of a food pellet. Responses in the holes that had not been illuminated (incorrect responses) or failure to respond within the limited hold (omissions) caused the houselights to be turned off for a short period (time out). Responses made in the holes while the house-light was off restarted the time out.

After the delivery of food, or at the end of time out, the rat started the next trial by opening the panel at the front of the chamber. Responses made in the holes after a correct response (perseverative responses), or after the end of time out before opening the panel, resulted in a period of time out. Responses made in the holes during the ITI (anticipatory responses) also resulted in a period of time out. After anticipatory responses, however, opening the panel restarted the current trial.

Each daily session consisted of 100 trials or 30 min of testing, whichever was completed sooner, after which all lights were turned off and further responses had no effect.

In the first session of the test schedule the stimulus and limited hold each lasted 1 min and, depending on individual performances, they were progressively reduced to 0.5 sec. The ITI and time out both lasted 2 sec during the first session and the ITI was raised to 5 sec in subsequent sessions; time out was not changed. When the rats reached a stable performance with a mean of 80% correct responses and no more than 15% omissions, they were allocated to different treatment schedules. Each rat had only one session on the 5-CSRT task per day throughout the experiments.

Measurements of spontaneous motor activity

Spontaneous motor activity was assessed in activity cages (40 x 25 x 18 cm) equipped with infrared photocell beams running horizontally along the axis of the cage (6 cm from the cage-end and 1 cm above the floor). The apparatus was controlled and data were collected by an Acorn computer system equipped with SPIDER extension (Paul Fray, Cambridge, UK).

Section 2: Pharmacological manipulation of discrete brain areas and changes in glutamate and serotonin concentrations measured by in vivo microdialysis

- Methodological issues

Brain microdialysis is a technique that samples over time substances, including neurotransmitters and their metabolites present in the extracellular fluid in brain tissues of living animals. A tubular dialysis membrane is introduced into the tissue. The tube is perfused with a liquid which equilibrate with the fluid outside the tube by diffusion in both direction. Thus, by mimicking the passive proprieties of a blood vessel the microdialysis probe can collect all the substances present in the extracellular fluid (provided that they pass the membrane) and at the same time deliver substances to the tissue. The substances that are collected through the microdialysis probe are thus accessible to conventional analytical techniques. This distinguishes the technique from *in-vivo* sensor techniques such as implantable biosensors and *in-vivo* voltammetry (Westerink and Justice, 1991). This technique makes possible to compare the effects of drug on the brain when applied directly through the probe or via the systemic route. The major advantages of this technique are that it can be performed in "intact" tissue of awake and freely moving animals and that by sampling continuously for hours in a single animal it decreases the number of animals needed in an experiment.

Microdialysis in principle is a very simple technique. The complexity of the technique comes from interactions between the dialysis tube, the perfusion liquid and the living tissue. Thus, factors that concur to determine the results of a microdialysis experiment are many (Benveniste and Hansen, 1991; Ungerstedt, 1991): tissue

disturbances and damage induced by probe implantation, composition of the perfusion medium and perfusion speed and dimensions and geometry of the probe. The physico-chemical proprieties of the dialysis fiber membrane have been shown to influence diffusion dynamic of some neurotransmitters and membranes should be as inert as possible to prevent interference with the passage of molecules. The biocompatibility with the surrounding tissue is also an important feature that should not be disregarded since particular chemical characteristics of fiber membranes could cause tissue disturbances.

Microdialysis experiments may be performed acutely on anaesthetized animals or on awake animals with chronically implated probes and guide cannulae. The advantage of performing microdialysis experiments on awake animals is that of avoiding the interference of anaesthesia. The use of anaesthetized or awake animals seems critical in studies measuring glutamate release. As shown by (Herrera-Marschitz et al., 1996) the difference in extracellular levels of glutamate in neostriatum of awake or anaesthetized rats is particularly high (+2,210 % of anaesthetized). However, the use of awake animals is not without problems since during the microdialysis experiments animals are susceptible to all kind of influences ranging from the conceivable pain of implantation and the restraint by tubing and wires, to reaction to various environmental stimuli. Thus extreme care should be taken to minimize all the external interference. For example, animals should be habituated to handling and to the isolated environment of the test cage; the experiments should be performed in an environment as neutral as possible and during the same period of the day to minimize the changes in diurnal rhythm.

- Application of brain microdialysis to measurements of neurotransmitter release

A central question in applying this technique to the study of drugs effects on neurotransmitter release is whether the recovered neurotrasmitters reflect "true" synaptic release. The size difference between the microdialysis probe (0.3 – 0.8 mm) and the nerve terminals (0.2 – 0.5 µm) indicates that the brain microdialysis is unable to estimate neurotrasmitter concentrations at release site. However, a series of microdialysis studies measuring monoamine (dopamine, noradrenalin and serotonin) and acetylcholine extracellular levels under conditions known to stimulate or inhibit the exocytotic release have demonstrated that, the basal concentrations of these neurotransmitters in dialysates were dependent on physiological activity of the neurons and could be thus ascribed to exocytotic release processes (reviewed by (Di Chiara, 1991; Ungerstedt, 1991)). Therefore, to the extent that the synaptic compartment where release take place is in equilibrium with the extracellular space surrounding the microdialysis probe, changes in the amount of neurotrasmitter released by nerve terminals are expected to be reflected by changes in the amount of neurotransmitter recovered in dialysates. In spite of qualitative similarity, the quantitative relationship between the concentration of neurotransmitters in dialysates and that at release site is unknown. Given that a neurotransmitter released from a nerve ending before it reaches the probe is likely to be subjected to catabolic, uptake and binding processes of unknown kinetic constants, the changes in neurotrasmitter concentration observed in dialysates will not be linearly related to its concentrations at release sites.

As advocated by (Di Chiara, 1991; Ungerstedt, 1991; Westerink and Justice, 1991) and many others the application of microdialysis for measurements of in-vivo

neurotransmitter release should fulfill a series of criteria. The proposed criteria involve the application of conditions, which reduce neurotransmitter output as a result of an interference with specific steps of the release process. In practical terms it should be demonstrated Ca^{2+}-dependence, TTX-sensitivity and impulse-dependency of basal as well as induced neurotransmitter release.

Brain microdialysis of 5-HT   Numerous studies have shown that changes in basal and stimulated release of 5-HT measured by microdialysis in different brain regions are to a large extent Ca^{2+}-dependent and inhibited by TTX (Kalen et al., 1988; Carboni et al., 1989; Sharp et al., 1990). Thus, the amount of 5-HT released from nerve terminals is likely to be reflected in corresponding changes of 5-HT in dialysates.

However, measurements of 5-HT levels by microdialysis raise some technical problems. Care should be taken in choosing the appropriate fibre membrane since delayed recovery of 5-HT in dialysates has been reported with some fiber membranes such as AN-69.

Brain microdialysis of glutamate   Application of brain dialysis to glutamate is debated. Several authors have questioned the neuronal origin of glutamate in dialysates (Herrera-Marschitz et al., 1996; Timmerman and Westerink, 1997) because of evidence that basal extracellular levels of glutamate appears to be impulse-independent; probably derived from non-neuronal pool that might reflect general and glial metabolism. Moreover, a recent study has shown that basal glutamate levels do not reflect neuronal release but mainly depend on the activity of cystine-glutamate exchanger (Baker et al., 2002).

Electrical or chemical stimulation of neurons cell bodies have been shown to increase extracellular levels of glutamate in terminal areas that were TTX- and

calcium-dependent. These approaches appear to be a more straightforward method for obtaining extracellular levels that are related to neuronal release upon basal, largely TTX-independent levels. In addition nicotine (Toth et al., 1993; Gioanni et al., 1999) and other pharmacological agents (as reviewed in (Timmerman and Westerink, 1997) after systemic administration or local infusion raised extracellular levels of glutamate in various brain areas in a TTX- and calcium-dependent way. Increases in glutamate release after nicotine, were confirmed by elecrophysiological studies measuring excitatory postsynaptic currents (EPSCs) in prefrontal cortical slices (Lambe et al., 2003) and [H^3]-l-glutamate overflow from tissue slices (Reno et al., 2004). Therefore, under certain circumstances the rise in extracellular levels of glutamate may be related to neurotransmission. However, as discussed by (Timmerman and Westerink, 1997), there is no absolute proof that in these experiments the extracellular glutamate was released from neurons rather than from glial cells. Assuming that pharmacological proprieties of neuronally released glutamate can be studied, the neuronal origin and specificity of increased extracellular level of glutamate should be verified in every experimental condition.

In the present study various criteria were applied to show that it was possible to measure increases in extracellular concentration of glutamate dependent on neuronal activity.

1.   Stimulation of exocytotic release by KCl should increase extracellular glutamate levels.

2.   Locally infused nicotine should increase extracellular glutamate.

3.   Pharmacologically induced increases in extracellular glutamate should be reversed by the sodium-channel blocking agent TTX, thus showing that

neuronal activity is required for these manipulations to stimulate glutamate release.

• Experimental techniques and methods

Animals Male rats (CD-COBS, Charles River, Italy) with free access to food and water were used in microdialysis experiments. They weighted about 250-300 g and were housed at constant room temperature (21±1°C) and relative humidity (60±5%) on a 12 h light/dark cycle (light on 7:00 A.M.).

Surgery, microdialysis and histology The probes were implanted in rats anesthetized with Equithesin (9.7 mg/mL sodium pentobarbital in saline + 42.6 mg/mL chloral hydrate in propylenglycol + 21.2 mg/mL Mg_2SO_4 in ethanol; 3.0 ml/kg intraperitoneally, i.p.). Since the microdialysis experiments were performed in awake animals the probes were fixed to the skull with stainless steel screws and acrylic cement according to the standard stereotaxic surgical procedure as those described in section 1. In all rats the microdialysis probe were implanted mono-laterally into the right mPFC and 24 h were allowed for recovery from anesthesia. Concentric dialysis probes were prepared with AN-69 and cuprophan hollow fibers (310 μm outer diameter; Hospal, Bologna, Italy), essentially as described by (Robinson and Whishaw, 1988). Since diffusion of 5-HT through AN-69 membrane is markedly delayed (Tao and Hjorth, 1992) we used a Cuprophan membrane (216 μm outer diameter, Sorin Biomedica, Italy) for measurements of 5-HT. The exposed membrane was 4 mm long. Stereotaxic coordinates for the mPFC (referring to the probe tip) were determined according to the Paxinos and Watson atlas (Paxinos and Watson, 1982): AP +3.7 and L ±0.7 mm from bregma and V -4.8 from dura surface.

While the rat was freely moving in an individual transparent cage the probe was perfused with artificial cerebrospinal fluid (CSF composition in mM: NaCl 140, $CaCl_2$ 1.26, KCl 3, $MgCl_2$ 1, Na_2HPO_4 1.2, glucose 7.2, pH 7.4 with a few drops of 0.6 M NaH_2PO_4) at 1 μL/min. Samples of dialysate were collected every 20 min and stored at 4°C.

At the end of the experiment, rats were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and killed by decapitation. Their brains were immediately removed and frozen on dry ice. The correct placement of the probes was checked by visual inspection of the probe tracks on 40-μm coronal sections from the mPFC of each rat. Only rats with correct probe placement were considered in the results.

- Biochemical analysis

Glutamate   The concentrations of glutamate in dialysate samples were determined by high-performance liquid chromatography (HPLC) with fluorometric detection after pre-column derivatization with o-phthaldialdehyde (OPA)/β-mercaptoethanol reagent according to Donzanti and Yamamoto (Donzanti and Yamamoto, 1988). Briefly, the derivatizing reagent was prepared by dissolving 27 mg OPA (Sigma-Aldrich, Milan, Italy) in 1 mL methanol followed by 5 μL of β-mercaptoethanol and 9 mL of 0.1 M sodium tetraborate buffer (pH 9.3). This stock solution was stored for about five days at room temperature in a sealed vial darkened with aluminum film. It was diluted 1:4 with 0.1 M sodium tetraborate buffer 24 h before use. Twenty-five μL of the diluted reagent were added to 5 or 10 μL sample and the reaction was allowed to proceed for 2 min at room temperature before injection onto the chromatograph. Glutamate was separated with a reverse phase column (HR-80, 80x4.6 mm, 3 μm packing; ESA Inc., MA) protected with a guard column (New

Guard RP-18, 7 μm, 15 x 3.2 mm; Perkin Elmer, CT). The mobile phase consisted of 0.1 M Na_2HPO_4, 0.13 mM Na_2EDTA, 28% CH_3OH, adjusted to pH 6.4 with 85% H_3PO_4, pumped at 1 mL/min with a LC-10ADvp pump (Shimadzu, Milan, Italy). Glutamate was detected by a scanning fluorescence detector (model 470; Waters S.p.A., Milan, Italy), using an excitation wavelength of 330 nm and an emission wavelength of 450 nm. The assay was calibrated daily with 20 pmol/10 μL glutamate standard made up in CSF.

Serotonin   5-HT was measured as described elsewhere (Invernizzi et al., 1992). Briefly, 5-HT was separated by a reverse phase column (Supelcosil LC18-DB 3 μm, 150 x 4.6 mm; Supelchem, Italy) and a mobile phase consisting of citric acid 9 mM, sodium acetate trihydrate 48 mM, Na_2EDTA 0.1 mM, 100 μL/L triethylamine and 40 mL/L acetonitrile, pumped at 1 mL/min. 5-HT was measured by a Coulochem II electrochemical detector equipped with a 5011 analytical cell (ESA Inc., Chelmsford, MA) at the following potentials (E1 +50 mV, E2 +180 mV. 5-HT was read as the second electrode output signal.

Pharmacological treatments Substances were administered either directly through the microdialysis probe or peripherally. The experimental design, the concentrations and doses of drugs as well as treatment procedures employed in these experiments will be given in the appropriate experimental chapter.

Section 3: Experimental design and statistical analysis

- Experimental design of behavioural experiments

The 5-CSRT task   Repeated measurements of rats performance under different pharmacological treatments were done in all experiments. Experiments using repeated measurements have several advantages. The measurements obtained from different treatments can be highly correlated, since they are from the same subject, and thereby the variance is reduced. Another clear advantage, especially when several months are needed to train the animals to perform a behavioural task, is the economic one of reducing the number of subjects in each experiment. A potential disadvantage in pharmacological experiments of repeated measurements, if not properly controlled for, is that of so-called carry-over effects. An animal's performance under prior treatment or task condition may affect its subsequent performance. For example, the saline scores may be "confounded" with drug scores if the preceding test involved a drug condition. However, different experimental design may be used to control for any disadvantageous carry-over effects that may be caused by the order of task condition or drug injections and by the residual effects of a drug. Residual effects of drugs, were minimized, by allowing a sufficient number of between treatments days. In addition any residual effect of a drug was determined by behavioural testing of the animals on these intervening days. To control for the carry-over effects caused by the order of task conditions or drug injections, a randomized repeated measurements design was used. The procedure that was chosen as appropriate was the Latin-square design.

The main dependent variables of the 5-CSRT task selected for analysis were: (a) the percentage of correct responses (total correct responses/total correct + total incorrect

responses x 100); (b) percentage of omissions (total omissions/ total number of trials x 100); (c) mean correct response latency (measured to the nearest 0.01 s); (d) the number of anticipatory responses in the holes during the ITI and (e) the number of perseverative responses in the holes after a correct response.

We also measured and analyzed the mean latency to make an incorrect response, mean latency to collect the earned food pellet (both measured to the nearest 0.01 s) and the number of panel-pushes during ITI.

Motor activity A completely randomized between subjects experimental design was used to determine the effects of a treatment in this experiment. The pharmacological treatments were administered to different groups of rats. The motor activity data recorded in 5-min time bins were summed over the first 30 min of the test period.

- Experimental design of microdialysis experiments

A split-plot (between-within subject) experimental design was used in these experiments. This allowed estimating the effect of treatment (between subjects), time (within subjects) and the interaction between treatment and time. Various pharmacological treatments or doses of drugs were administered to different groups of rats and the time-course of their effects determined. Levels of the assayed glutamate and 5-HT were expressed as the concentrations found in the dialysates.

- Statistical analysis

Data of each variable measured in 5-CSRT task experiments were analysed by a parametric multifactorial analysis of variance (ANOVA). Analysis was performed on the "Mario Negri" Institute mainframe Micro VAX 3500 computer (Digital, USA). The SAS version 8 statistical software package (SAS Institute Inc., USA) was

used that could analyse multifactorial experiments with more than one within-subject factor. Statistical analysis of data from some behavioural and from all microdialysis experiments, were performed by ANOVAs using StatView statistical package run on iMAC (Apple, USA) microcomputer.

Use of ANOVA requires that several conditions be met, such as normal distribution and homogeneity of the variance (Winer, 1971). Some data from the 5-CSRT task such as correct responses and omissions, that were expressed as percentages, were transformed according to the formula 2arcsin(SQRT (%X/100)). The mean latencies to respond correctly and to collect the earned food pellet were transformed by log10. The normality of distribution and homogeneity of variance were not tested. However, the ANOVA model of F distribution is robust and tolerates large deviations from normality (Winer, 1971).

Since the repeated measure experimental design was employed in the 5-CSRT task experiments data were analysed by randomized block 2-way (within-subject) ANOVA. The orthogonal factors were various treatments or task conditions. Given that the overall ANOVA showed statistically significant ($P<0.05$) main effects a number of post hoc tests were preformed to statistically establish the differences between various treatments group means. The most common tests were the analysis of simple effects by one-way ANOVAs followed by Tukey's test for unconfounded means, which compares orthogonal treatment (or task conditions) group means. In each experimental chapter further details and rationale of statistical analysis are given.

CHAPTER 3. BLOCKADE OF GLUTAMATE NMDA RECEPTORS IN THE MEDIAL PREFRONTAL CORTEX (MPFC): BEHAVIOURAL AND NEUROCHEMICAL EFFECTS.

Section 1: Effects of CPP microinjections into the mPFC on the performance of a 5-CSRT task and on motor activity

In experimental animals pharmacological manipulation of glutamate system by NMDA antagonists produce a range of behavioural deficits reminiscent of frontal-lobe dysfunction. Peripheral administration of NMDA receptor antagonists causes deficits in sensory-motor gating, hyperactivity, deficits in attentional set shifting, working memory and response inhibition (Verma and Moghaddam, 1996; Jentsch and Roth, 1999; Egerton et al., 2005; Rodefer et al., 2005). Relevant to this study are findings that blockade of glutamate NMDA receptors by peripherally administered phencyclidine or dizocilpine, or the selective NMDA-R2B receptor antagonist Ro 63-1908, cause deficits in attentional performance in the 5-CSRT task (Higgins et al., 2003b; Yasuno et al., 2003). However, since in these studies drugs were administered peripherally the role played by glutamate NMDA receptors in the PFC in these behavioural deficits is undetermined.

The distribution of NMDA receptors in the rat brain is heterogeneous, with particularly high densities found in medial prefrontal cortex (mPFC) (Cotman and Monaghan, 1986). The competitive glutamate NMDA receptor antagonist CPP, selectively displaces NMDA-sensitive glutamate binding, shows no significant effects at kainate or quisqualate receptors and competitively antagonizes NMDA-induced release of acetylcholine from striatal slices (Lehmann et al., 1987). Microinjected into the mPFC, CPP caused deficits in working memory (Aura and Riekkinen, 1999; Romanides et al., 1999) and hyperactivity (O'Neill and Liebman, 1987).

As discussed in the introduction there is evidence that mPFC is specifically involved in the performance of the 5-CSRT task. In fact, excitotoxic lesions of the mPFC but not cingulate, anterior-lateral or parietal cortex in rats impair attentional functioning and cause a loss of inhibitory response control leading to impulsivity and compulsive perseveration in the 5-CSRT task (Muir et al., 1996; Passetti et al., 2002; Passetti et al., 2003a; Passetti et al., 2003b)).

At the time of these experiments it was unclear whether it was sufficient to block the glutamate NMDA receptors in the PFC to cause attentional performance deficits in the 5-CSRT task. Therefore, the putative role of glutamate NMDA receptors in the mPFC in attention and executive response control were examined by studying the effects of various doses of CPP infused bilaterally into the mPFC on rats' performance in the 5-CSRT task. To characterise further the effects of CPP on attentional performance its effects were also examined in task conditions of decreased or increased attentional load by introducing "challenge" session with longer or shorter stimulus duration. Since CPP may cause changes in motor activity (O'Neill and Liebman, 1987), which may interfere with the performance of a complex task such as the a 5-CSRT task, the effects of various doses of intra-mPFC CPP on motor activity were tested.

MATERIALS AND METHODS

Animals, food deprivation, 5-CSRT task and behavioural procedures, surgery and CPP microinjections into the mPFC, and histology were as described previously (General methods).

- Drugs and experimental design

Baseline condition   Twelve male rats were used to examine the effects of various doses of CPP on the performance of a 5-CSRT task. Various doses of CPP were dissolved in 1 μL saline and injected bilaterally into the mPFC 10 minutes before the test session. Each rat received saline (1 μL) or 1, 10 and 50 ng/μL CPP. On each test day saline or various doses of CPP were administered according to a Latin-square design. At least two days were left between test days. Rats were always tested on these "free" days to re-establish the baseline and check the lasting effects of drugs. One rat was excluded from the data analysis due to signs of infections at the injection sites. Two rats developed generalized seizures and were excluded from results. Only data from 9 rats were statistically analysed and are included in the results.

Changes in stimulus duration A different group of 10 rats were used to examine the effects of CPP in condition of increased or decreased stimulus duration. To examine the effects of CPP in condition of increased stimulus duration each rat received 1 μL saline or 50 ng/μL CPP injected into the mPFC and was exposed to sessions of a 5-CSRT task in which stimulus duration was 0.5 or 1.0 s. Similarly, the effects of CPP in condition of decreased stimulus duration were examined by administering 1 μL saline or 20 ng/μL CPP into the mPFC. Rats were exposed to sessions of a 5-CSRT task in which stimulus duration was 0.5 or 0.25 s. CPP and stimulus durations were

administered according to a Latin-square design. At least two days were left between test days. Rats were always tested on these "free" days on baseline task with stimulus duration of 0.5 s to re-establish the baseline and check the lasting effects of drugs. Data from 9 rats were statistically analysed and are included in the results.

- Effects of CPP on spontaneous motor activity

Animals　　The effects of various doses of CPP on motor activity were assessed using 24 experimentally naïve male Lister Hooded rats weighing between 250 and 300 g before surgery. Throughout the experiment they had free access to food and water (for details see General methods).

After the rats were implanted with cannulae in the mPFC they had seven days of recovery. On the testing day the animals were habituated to the activity cages for 1 h after which they were taken out and injected bilaterally with 1, 10 and 50 ng/μL CPP or 1 μL vehicle and placed in the activity cages, and the photocell beam interruptions were recorded over a 2 h period in 5-min bins.

Statistical analysis

The 5-CSRT task　　The variables selected for analysis were: (a) the percentage of correct responses; (b) percentage of omissions; (c) the mean correct response latency; (d) the number of anticipatory responses and (e) the number of perseverative responses and (f) mean incorrect response latency.

Baseline task condition　　Results of CPP dose-response study were analysed by within-subjects one-way ANOVA, and the means of individual treatments were compared with saline by Dunnett's t-test.

Changes in stimulus duration Results of the experiments testing the effects of 20 and 50 ng/μL CPP in combination with short (0.5 versus 0.25) and long (0.5 versus 1.0 s) stimulus durations were analysed by within-subjects two-way ANOVA. The means of the individual treatment combinations were compared by Tukey's HDS test.

Spontaneous motor activity The motor activity data recorded in 5-min time bins were summed over the first 30 min of the test period and analyzed by between-subjects one-way ANOVA. The means of individual treatments were compared with controls using Dunnett's t-test.

RESULTS

- *Blockade of NMDA receptors in the mPFC by CPP impairs accuracy and increases anticipatory and perseverative responses*

Figure 1.1A shows how CPP impaired rats' discriminative accuracy. Overall, CPP (1, 10 and 50 ng/μL per side) dose-dependently lowered the percentage of correct responses ($F_{(3,21)}$=6.4, P=0.003). However, despite the apparent decrease with 10 ng/μL (P>0.05, Tukey's test), the percentage was only significantly lower after 50 ng/μL (P<0.05, Tukey's test).

In addition, CPP particularly impaired the rats' ability to withhold inappropriate nose-poke responses, as shown by increased number of anticipatory ($F_{(3,21)}$=5.3, P=0.007) (Fig. 1.1B) and perseverative responses ($F_{(3,21)}$=3.95, P=0.02) (Fig. 1.1C). These increases were already maximal at 10 ng/μL and did not rise further with 50 ng/μL (both Tukey's test p<0.05). The dose of 1 ng/μL had no effect on any measure of attentional performance.
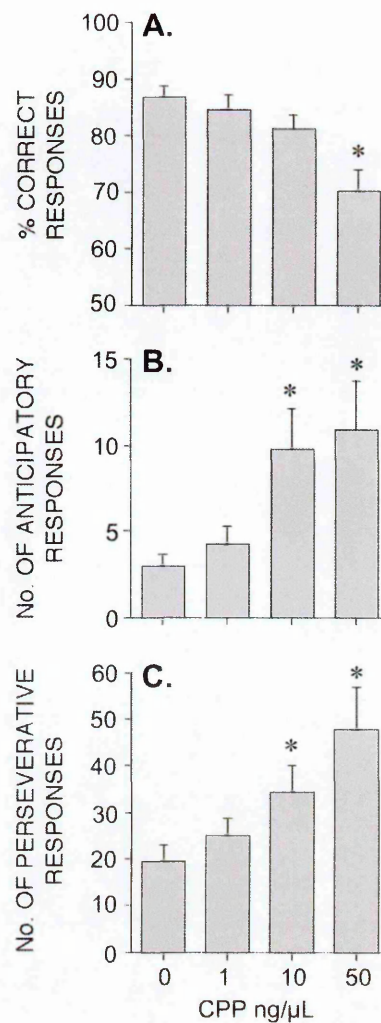
Figure 1.1
The effects of 1, 10 and 50 ng/μL of CPP or 1 μL vehicle injected into the mPFC 10 min before the test session on correct responses (A), anticipatory responses (B) and perseverative responses (C). CPP and vehicle were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 8 rats.
   *P<0.05 vs. 0 (Tukey's test)

In parallel to the deleterious effects on accuracy, Table 1.1 shows that CPP increased omissions ($F_{(3,21)}$=10.59, P=0.0002) and correct response latencies ($F_{(3,21)}$=5.6, P=0.005). The omissions were significantly increased by 50 ng/μL (P<0.05, Tukey's test) while both 10 and 50 ng/μL increased correct response latencies (both P<0.05,

Tukey's test). Incorrect response latencies were not significantly affected by CPP ($F_{(3,21)}$=2.6, P=0.08).

Table 1.1 Effect of various doses of CPP on omissions and correct and incorrect response latency

DOSE (ng/µL)	OMISSIONS (%)	CORRECT LATENCY (s)	INCORRECT LATENCY (s)
VEHICLE	16.6 ± 2.1	0.58 ± 0.02	1.78 ± 0.14
CPP 1	15.1 ± 1.9	0.65 ± 0.05	1.89 ± 0.18
CPP 10	25.3 ± 5.0	0.78 ± 0.05 *	2.14 ± 0.27
CPP 50	31.2 ± 5.7 *	0.73 ± 0.03 *	1.98 ± 0.20

Each value is the mean ± SEM of 8 rats. CPP was injected into the mPFC 10 min before the test session. Doses of 1, 10 and 50 ng/µL of CPP or vehicle (1 µL) (VEHICLE) were injected bilaterally, at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEHICLE (Dunnett's t test).

- *The CPP-induced deficit in accuracy was abolished by increasing stimulus duration; anticipatory and perseverative over-responding was not affected*

The performance accuracy is shown in Fig. 1.2A. Increasing stimulus duration improved the accuracy of discrimination of rats. The accuracy deficits of rats performing the task at the standard stimulus duration of 0.5 s and injected with 50 ng/µL CPP were abolished when the stimulus duration was increased to 1.0 s (% correct responses: stimulus x CPP, $F_{(1,24)}$=6.7, P=0.016; CPP, $F_{(1,24)}$=12.2, P=0.002; stimulus, $F_{(1,24)}$=32.1, P=0.0001).

Increasing the stimulus duration reduced the number of anticipatory (Fig. 1.2B) and perseverative (Fig. 1.2C) responses in controls and CPP treated rats ($F_{(1,24)}$=4.2, P=0.05; $F_{(1,24)}$=17.5, P=0.002; respectively). However, these nose-pokes were increased proportionally by CPP at both stimulus durations (anticipatory; stimulus x CPP, $F_{(1,24)}$=1.5, P>0.05; CPP, $F_{(1,24)}$=22.7, P=0.0001 and perseverative; stimulus x CPP, $F_{(1,24)}$=0.8, P>0.05; CPP, $F_{(1,24)}$=27.7, P=0.0003).
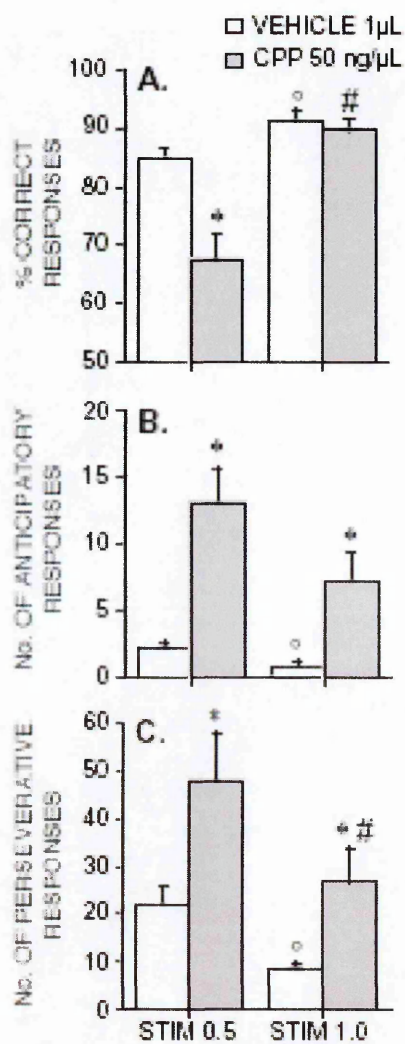
Figure 1.2

Effects of lengthening the stimulus from 0.5 to 1.0 s on correct responses (A), anticipatory responses (B) and perseverative responses (C) of rats injected with 50 ng/µL CPP or 1 µL vehicle into the mPFC 10 min before the test session. CPP or vehicle were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 9 rats. * $P<0.05$ vs. VEHICLE; ° $P<0.05$ vs. VEHICLE (STIM 0.5); # $P<0.05$ vs. CPP (STIM 0.5) (Tukey's test)

Table 1.2 shows that CPP-induced increase in the percentage of omissions was also abolished by increasing the stimulus duration from the standard 0.5 s to 1.0 s (stimulus x CPP, $F(1,24)=6.8$, $P=0.01$; CPP, $F_{(1,24)}=9.46$, $P=0.005$; stimulus, $F_{(1,24)}=1.3$, $P>0.05$). The CPP injections in rats performing at the 0.5 s stimulus increased correct response latencies. Increasing stimulus duration also increased correct response latency. However, these effects did not sum up when increased stimulus length and CPP treatment were combined. CPP did not increase correct response latency when the rats performed the task with the 1.0 s stimulus (stimulus x CPP, $F_{(1,24)}=6.9$, $P=0.01$; CPP, $F_{(1,24)}=10.1$, $P=0.004$; stimulus, $F_{(1,24)}=1.5$, $P>0.05$)(Table 1.2). Incorrect response latencies were decreased by CPP, but only when the stimulus duration was 1.0 s (stimulus x CPP, $F_{(1,24)}=5.3$, $P=0.03$; CPP, $F_{(1,24)}=0.12$, $P>0.05$; stimulus, $F_{(1,24)}=5.1$, $P=0.03$)(data not shown).

Table 1.2 Effects of increasing stimulus duration and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)		CORRECT RESPONSE LATENCY(s)	
	ST 0.5 s	ST 1.0 s	ST 0.5 s	ST 1.0 s
VEHICLE	8.0 ± 1.7	11.1 ± 2.7	0.51 ± 0.03	0.65 ± 0.04#
CPP 50 ng/µL	22.8 ± 4.1*	12.7 ± 2.8#	0.68 ± 0.05*	0.67 ± 0.04

Each value is the mean ± S.E.M. of 9 rats. Fifty ng/µL and 20 ng/µL CPP or 1 µL vehicle were injected bilaterally into the mPFC 10 min before the test session. Various stimulus durations (ST) plus CPP were administered at least 48 h apart, according to a Latin-square design.
* P<0.05 vs. VEHICLE ; # P<0.05 vs. ST 0.5 s (Tukey's test)

- *Decreasing stimulus duration had no interactive effects with CPP on accuracy or anticipatory and perseverative responding*

Fig. 1.3 shows how decreasing stimulus duration from 0.5 to 0.25 s affected the accuracy of discrimination (Fig. 1.3a), and anticipatory (Fig. 1.3b) and perseverative

(Fig. 1.4c) responding. Overall, rats tested under control conditions (1 μL vehicle injected into the mPFC) had worse accuracy when performing the task at 0.25 s than at 0.5 s stimulus duration. Twenty ng/μL CPP further decreased the accuracy, however, the effect of CPP was independent of stimulus duration (stimulus x CPP, $F_{(1,27)}=0.35$, P>0.05; stimulus $F_{(1,27)}=136.2$, P=0.0001; CPP, $F_{(1,27)}=18.9$, P=0.0002).
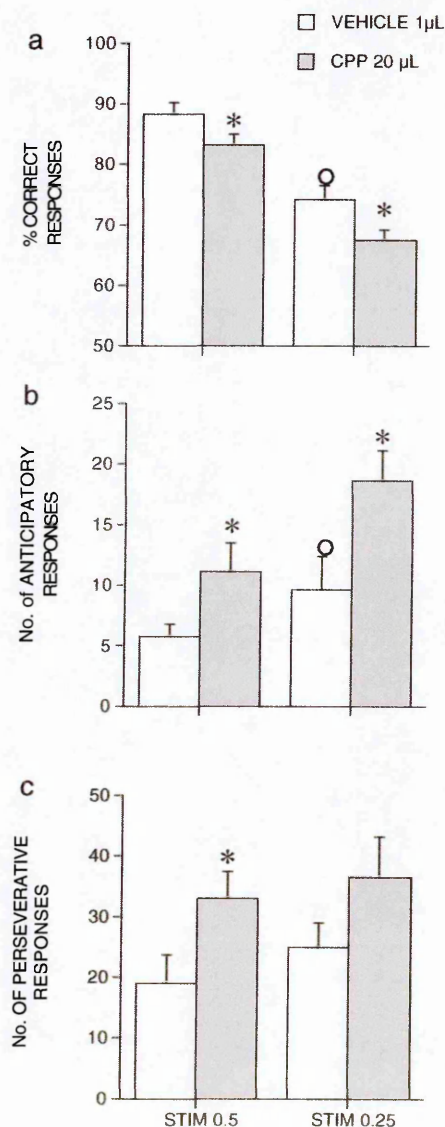
Figure 1.3
Effects of decreasing the stimulus from 0.5 to 0.25 s on correct responses (A), anticipatory responses (B) and perseverative responses (C) of rats injected bilaterally with 20 ng/μL CPP or 1 μL vehicle into the mPFC 10 min before the test session. CPP or vehicle were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 9 rats.
* P<0.05 vs. VEHICLE; ° P<0.05 vs. VEHICLE (STIM 0.5); (Tukey's test)

The statistical analysis of anticipatory and perseverative responses by ANOVA revealed that shortening the stimulus (stimulus $F_{(1,27)}=10.6$ P=0.003) or injecting CPP into the mPFC increased the number of anticipatory responses ($F_{(1,27)}=12.0$, P=0.0018). However, the effects of stimulus duration and CPP were not interactive (stimulus x CPP; $F_{(1,27)}=1.1$, P>0.05). Thus, regardless of stimulus conditions, CPP increased the number of anticipatory responses to the same extent (P<0.05, Tukey's test) (Fig. 1.3B). The number of perseverative responses did not increase on shortening the stimulus (stimulus, $F_{(1,27)}=1.5$, P>0.05) but was raised by 20 ng/μL CPP regardless of stimulus conditions (stimulus x CPP, $F_{(1,27)}=0.6$, P>0.05; CPP, $F_{(1,27)}=14.7$, P=0.007). This increase was only significant at the stimulus duration of 0.5 s (P<0.05, Tukey's test) (Fig. 1.3C).

Table 1.3 Effects of CPP and stimulus duration on omissions and correct response latency

TREATMENT	OMISSIONS (%)		CORRECT RESPONSE LATENCY(s)	
	ST 0.5 s	ST 0.25 s	ST 0.5 s	ST 0.25 s
VEHICLE	12.2 ± 1.7	10.9 ± 2.3	0.49 ± 0.02	0.69 ± 0.05#
CPP 20 ng/μL	12.9 ± 2.7	10.0 ± 1.5	0.56 ± 0.06	0.57 ± 0.04*

Each value is the mean ± S.E.M. of 9 rats. Twently ng/μL CPP or 1 μL vehicle were injected bilaterally into the mPFC 10 min before the test session. Various stimulus durations (ST) plus CPP were administered at least 48 h apart, according to a Latin-square design.
* P<0.05 vs. VEHICLE ; # P<0.05 vs. ST 0.5 s (Tukey's test)

As shown in Table 1.3 the percentage of omissions was not affected by the stimulus duration or by CPP (stimulus x CPP, $F_{(1,27)}=0.6$, P>0.05; CPP, $F_{(1,27)}=0.35$, P>0.05; stimulus, $F_{(1,27)}=0.34$, P>0.05). At stimulus duration of 0.25 s rats were slower to make a correct response and 20 ng/μL of CPP made them significantly faster (stimulus x CPP, $F_{(1,27)}=6.2$, P=0.02; CPP, $F_{(1,27)}=0.53$, P>0.05; stimulus, $F_{(1,27)}=8.5$,

P=0.007). Multiple comparison between treatment group means showed that animals performing in control condition (Vehicle) took longer to make a correct response at short stimulus duration (Tukey's test, P<0.05). CPP decreased the latency to make a correct response at short stimulus duration (Tukey's test, P<0.05) but had no effect when the stimuli were 0.5 s long. Incorrect responses were faster when the stimuli were short and were not affected by CPP (stimulus x CPP, $F_{(1,27)}$=0.74, P=0.39; CPP, $F_{(1,27)}$=0.41, P>0.05; stimulus, $F_{(1,27)}$=5.8, P=0.02)(data not shown).

- *CPP elicited motor hyperactivity but only at the highest dose.*

Table 1.4 Effects of CPP on motor activity

DOSE (ng/µL)	ACTIVITY COUNTS
VEHICLE	127 ± 17
CPP 1	122 ± 17
CPP 10	137 ± 31
CPP 50	205 ± 32*

Each value is the mean ± S.E.M. of 6 rats per group. Animals were habituated to activity cages for 1 h after which they were taken-out and injected bilaterally with 1, 10 and 50 ng/µL CPP or 1 µL vehicle into the mPFC. Ten min later they were put back into the cages and their activity recorded over a 2 h period in 5 min bins. Motor activity is expressed as the total number of activity counts measured in the first 30 min of testing.
* P<0.05 vs. VEHICLE (Dunnett's t-test)

DISCUSSION

The selective and competitive glutamate NMDA receptor antagonist CPP, (Lehmann et al., 1987), injected into the mPFC, had profound effects on rats' performance on the 5-CSRT task. At 10 ng/µL it enhanced anticipatory and perseverative responding and increased the correct response latencies while 50 ng/µL impaired accuracy and omissions and increased motor activity. The deficits observed in this study were grossly comparable in nature and magnitude to the deficits observed in the same task after either systemic administration of NMDA antagonists (Higgins et al., 2003b; Le Pen et al., 2003) or after excitotoxic lesions of the mPFC (Muir et al., 1996; Passetti et al., 2002). Therefore, they provide strong evidence for the involvement of glutamate NMDA receptor mechanism in the mPFC in aspects of visual attentional function and executive control.

This task requires that at the beginning of each trial rats push the magazine panel located on the front wall of the apparatus and then turn around to scan the stimuli array of the rear wall in order to detect the presentation of a brief flash of light in one of the 5 locations. An additional characteristic of the task is the imposed time interval of 5 sec between panel push and stimulus presentation. Various studies have shown that after extensive training animals learn to temporally schedule their behaviour so that at the end of this interval they are oriented towards the stimulus array and ready to respond as quickly as possible (Robbins, 2002). The analysis of temporal distribution of the responses made by Passetti et al. (Passetti et al., 2002) showed that naïve rats are very consistent across trials, turning around quickly after food collection and responding as soon as stimulus appears. About 40 % of responses, almost all correct (>90 %), are made when the stimulus is still on (between 0 and 0.4 s). Additional 40 % of responses still about 90% correct are

made in the successive time bin (0.4 – 0.8 s). This indicates that naïve rats are able to hold "on-line" mental representation of planned responses after the stimulus offset (Passetti et al., 2002). Although attentional impairments may almost certainly account for accuracy deficits observed in this task a major contribution to the accuracy deficit could be from either an impairment in timing the interval between the panel push and the presentation of the stimulus or difficulties in scheduling behaviour during that time. Indeed, evidence from the literature suggests that lesion to areas of the frontal cortex can result in deficits in temporal discrimination (Olton et al., 1988), and theories of prefrontal cortex function have emphasised its role in temporal organization of behaviour (Fuster, 1989). The results of the present study suggest that timing problems could at least in part account for the accuracy deficit resulting from CPP injections into the mPFC. Similarly to findings with mPFC lesions (Passetti et al., 2002) CPP-injected rats were slower to make a correct response and made more omissions. This suggest that rats were slower in initiating a response after they have oriented and detected the stimulus in time or that they were engaging in some other behaviour during this interval thus missing the stimulus presentation. This interpretation is in line with findings showing that the accuracy deficit induced by the high dose of CPP was completely abolished by prolonging the stimulus duration. Because the frequency of stimulus presentation was regular relative to each trial initiation (5 sec), when the stimulus duration was increased, the position of the visual target in both space and time was emphasised, thus facilitating accurate responses.

However, when measures of timing and temporal organisation of behaviour were dissociated from measures of response selection and attention, the impairments in timing behaviour were insufficient to explain the attentional deficits of mPFC

lesioned rats (Passetti et al., 2000a). A commission error in the 5-CSRT task may be the result of a faulty decisional process, distraction or of inability to hold "on-line" the planned response. Thus, it could not be excluded that additional deficits in response selection, increased distractability and working memory may account for attentional impairments after CPP. The impaired response selection is an important component of attentional deficit and the correct response latency may reflect the operations of decisional processes involved in stimulus detection and response selection or both. Since correct and incorrect responses in this task have the same motor requirements the slowing of correct but not incorrect responses after CPP might suggest dysfunctional mechanisms of stimulus detection most likely due to distraction. Thus, it could be argued that on occasions when the animals were able to overcome distraction and respond correctly they were doing it at the cost of slower responding. This indicates that animals injected with CPP when they correctly detected the visual stimulus could hold "on-line" mental representation of planned responses well after the visual stimulus has disappeared. In line with this suggestion are observations that control animals will compensate for the decreased salience of the visual stimulus by increasing the correct response latencies (present results). That deficits in working memory may not completely account for accuracy impairments induced by CPP may also be suggested by recent findings of Chudasama et al. (Chudasama et al., 2005) who using an attentional-working memory combined task have shown that rats with PFC lesions were impaired on the attentional but not on the working memory component of the task.

Omissions, which occur when the subject does not orient its attention on the stimulus presentation array in time, might reflect some motor or motivational factors. Again, prolonging the stimulus abolished CPP-induced increases in omissions. These

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findings rule out the possibility that the CPP-induced impairments in accuracy and omissions were a consequence of hyperactivity, poor motivation or a failure to make associations or remember the general rules of the task.

CPP did not affect accuracy differentially when the stimulus duration was decreased, suggesting that visual perception was not a critical determinant of the accuracy deficit. This finding is in keeping with the results of other experiments, in which the accuracy impairments resulting from bilateral mPFC or disconnection lesions disrupting the mPFC-dorsal-striatal circuit were not differentially sensitive to reductions of stimulus duration (Passetti et al., 2000a; Christakou et al., 2001).

The ability to inhibit inappropriate responses in complex situations is a fundamental aspect of executive control. The 5-CSRT task indexes two different aspects of inhibitory response control. First, disturbances in a preparatory response mechanism measured by anticipatory responses, which occur in anticipation of the visual stimulus and are generally interpreted as a form of impulsive behaviour. Second, failure to withhold from an apparently aimless repetition of responding by which rats continue to respond in stimulus holes after a correct response has been made, akin to "compulsive" behaviour exemplified by the effects of frontal lesions patients (Milner, 1964; Luria, 1966/1980; Fuster, 1989). These response repetitions are measured by perseverative responses.

Impulsivity and perseveration are both intimately related to executive attentional processes that enable accurate response selection in the face of distraction and interference (Shallice, 1982; Robbins, 1996)). In fact, increasing the duration of the target stimulus reduced while decreasing it increased anticipatory and perseverative responses of rats tested under control conditions (vehicle in the mPFC) thus suggesting that premature and perseverative responses may be under attentional

control. However, the CPP-induced increase in anticipatory and perseverative responses persisted even when the longer stimulus helped alleviate the accuracy and omissions deficits. CPP caused a failure in executive functions at 10 ng/μL a dose that did not affect motor activity or accuracy. Together these data suggest that the CPP-induced deficits in anticipatory and perseverative responses were relatively independent of processes involved in stimulus detection and motor activation.

It may be argued that there was a primary deficit of response inhibition making the animals "impulsive" and "compulsive". In fact, despite having oriented to the stimulus array, the animals were unable to wait for the stimulus presentation. Increased impulsivity in this task has been reported after highly arousing stimuli such as brief presentation of loud white noise during the waiting period (ITI) (Carli et al., 1983). The inverted U-shaped function linking arousal and performance (Yerkes and Dodson, 1908) has been shown in human subjects performing the 5-CSRT task under conditions of elevated arousal (Wilkinson, 1963). In accordance, findings in rats show that elevated arousal may under conditions of impaired noradrenergic function cause attentional performance deficit in a 5-CSRT task (Carli et al., 1983). Thus the behavioural profile of CPP injected rats showing accuracy deficit and impulsivity may be reconcilable with the hypotheses of CPP injected animals being in a state of hyper-arousal.

Previous studies have strongly implicated the anterior cingulate cortex extending posterior to the genu of the corpus callosum (Cg1, Cg2 and Fr2 according to the nomenclature of (Zilles, 1985) in the control of anticipatory responses (Muir et al., 1996). However, it is unlikely that diffusion of CPP into the anterior cingulate cortex contributed in some major way to the increased anticipatory responding. Doses of CPP (10 and 50 ng/μL) similar to those used in the present study had to be

injected into the cingulate cortex to induce anticipatory over-responding (M. Carli and M. Baviera, unpublished observation).

However, not only were CPP-injected rats more "impulsive", they were also unable to suppress irrelevant "compulsive" perseverative responses and shift their attention to the next relevant response in a well-learned sequence. The increased perseveration, which is in line with that reported after excitotoxic lesions of the mPFC (Muir et al., 1996) could be the result of CPP preventing the suppression of responses once effective for obtaining reward. The preseverative deficit was not general; it was specifically directed to the stimulus array holes and not the panel of the food magazine (data not shown). This observation suggests that the deficit is related to aspects of response processing, such as selection of an adequate response in a long sequence leading to reinforcement and its optimal integration, rather than motivational aspects of performance. The enhanced tendency to perseverate, presumably an expression of behavioural inflexibility, appears to be a distinctive trait of the frontal-lesioned animals (Mishkin, 1964; Muir et al., 1996; Dias et al., 1997; Ragozzino et al., 1999a; Killcross and Coutureau, 2003), and of frontal-lobe patients when required to inhibit previously reinforced responses (Owen et al., 1993). In addition, schizophrenic patients show increased perseverative responding in a two-choice visual task (Lyon and Gerlach, 1988) and in the Wisconsin Card Sorting Test, a task sensitive to prefronto-cortical dysfunction (Goldberg and Weinberger, 1994). The anatomical selectivity of these attentional and executive control processes has been reported after excitotoxic lesions or CPP injections into the ventral sub-regions of rat PFC (Chudasama et al., 2003; Murphy et al., 2005)). The prelimbic (PRL) and orbito-frontal (OFC) sub-regions of PFC are particularly involved in perseverative responding (Passetti et al., 2002; Chudasama et al., 2003)) whereas lesions or

blockade of NMDA receptors of infralimbic (IL) sub-region mainly affected anticipatory "impulsive" responding (Chudasama et al., 2003; Murphy et al., 2005). Other evidence for functional heterogeneity of rat PFC were obtained by Passetti et al. (Passetti et al., 2002) and Chudasama et al. (Chudasama et al., 2003) who showed that impairments in attentional accuracy on a 5-CSRT task after lesions to the mPFC (Muir et al., 1996; Passetti et al., 2002)) are mainly reproduced by lesions confined to more dorsal (Cg1) aspects of PFC and sparing more ventral PRL and IL sub-regions. However, accuracy deficits induced by CPP injections separately to PRL and IL sub-regions of PFC were less well localised (Murphy et al., 2005). These findings in rat are grossly comparable in nature to those reported in primates and human subjects (Fuster, 1989; Goldman-Rakic, 1998) showing that on-line maintenance and selection of responses under high attentional demands and inhibitory response control may be divided along a dorsal-ventral axis within the PFC (Morgan and LeDoux, 1995; Seamans et al., 1995; Kesner et al., 1996; Ragozzino et al., 1998; Brown and Bowman, 2002). Thus the pattern of deficits observed in this study after CPP injections into a single site in the mPFC may be the sum of somewhat "independent" deficits in more "dorsal" attentional and "ventral" inhibitory response control.

Section 2. Effects of CPP infused into the mPFC on glutamate and serotonin release: Microdialysis studies

The results presented in Section 1 of this chapter show that intra-mPFC administration of the competitive NMDA receptor antagonist, CPP, impaired processes dependent on the prefrontal cortex such as attentional functioning and executive response control.

Behavioral deficits induced by acutely administered NMDA antagonists have been associated with enhanced glutamate release in the mPFC (Moghaddam et al., 1997; Moghaddam and Adams, 1998). In addition, the finding that PCP or genetic deletion of the NMDA receptor raised extracellular 5-HT in the mPFC (Martin et al., 1998a; Miyamoto et al., 2001) suggests that enhanced 5-HT tone in the mPFC may contribute to the effects of NMDA receptor hypofunction.

When these studies were started it was not known whether CPP like non-competitive NMDA receptor antagonists can increase cortical extracellular glutamate and 5-HT efflux. It was also unclear whether blockade of NMDA receptors in the PFC was sufficient to cause increases in glutamate release in this cortical region. Therefore, using *in vivo* microdialysis in the conscious rat we investigated whether CPP injected intraperitoneally or infused intracortically through the probe raised extracellular concentration of glutamate and 5-HT in the mPFC.

In view of the poor relation between the basal extracellular concentration of glutamate and neuronal activity (Herrera-Marschitz et al., 1996; Timmerman and Westerink, 1997; Del Arco and Mora, 2002), in one experiment we used the sodium channel blocker tetrodotoxin (TTX) to study the action potential-dependent effect of CPP on extracellular glutamate. The activation of presynaptic acetylcholine

heteroreceptors, with nicotine stimulates the TTX- and calcium-dependent neuronal release of glutamate (Gioanni et al., 1999; Lambe et al., 2003; Reno et al., 2004) and other neurotransmitters (Wonnacott, 1997). Thus, to examine whether under the present experimental conditions, stimulation of the release of glutamate may be detected in the dialysates 60 mM KCl or nicotine at 100 μM and 1 mM was given through the probe.

MATERIALS AND METHODS

Animals, surgery and microdialysis were performed as described previously (General methods).

Drugs and reagents   CPP and the D-isomer of the 2-amino-7-phosphono heptanoic acid (AP7) were purchased from Tocris Cookson Inc. (Ellisville, MO, USA); nicotine and TTX were from Sigma-RBI (Milan, Italy). Drugs were dissolved in aCSF and infused through the probe or injected IP. Control rats were perfused with normal CSF or injected with saline or CSF. All drugs or vehicle were infused during the phase of stable glutamate output defined as three consecutive baseline samples not differing by more than 20%. Chemical reagents were of analytical grade and were purchased from Merck (Bracco, Milan, Italy) or Sigma-Aldrich (Milan, Italy).

Neuronal origin of GLU   Rats were perfused through the probe with 1 mM TTX to verify the neuronal origin of the basal extracellular GLU. TTX was perfused throughout the 2-h experiment. Other rats were perfused with 60 mM KCl through the probe for 20 min to stimulate exocytotic release. In this case, NaCl concentration was reduced to 83 mM to maintain osmolarity. Another group of rats was given nicotine (100 µM and 1 mM) through the probe to assess the ability of microdialysis to reveal changes in extracellular GLU presumably dependent on neuronal activity. Each rat was perfused with 100 µM nicotine in CSF for 60 min. Thereafter perfusion medium was manually switched to a solution containing 1 mM nicotine for 60 min.

Extracellular glutamate: effect of CPP and AP7   A group of rats received CPP (10 and 20 mg/kg) dissolved in aCSF or vehicle intraperitoneally. Other rats were given 30, 100 and 300 µM CPP or 1 mM AP7 through the probe. After baseline stabilization, the perfusion fluid was manually switched to a solution containing CPP

or AP7 in CSF. Each concentration of CPP or 1 mM AP7 were perfused for 60 min. A separate group of rats was co-infused with 1 µM TTX and 100 µM CPP to assess the contribution of the action potential-dependent effect of CPP on extracellular glutamate. TTX infusion started 20 min before CPP and lasted 140 min.

Extracellular 5-HT in response to CPP   A group of rats was implanted with Cuprophan probes and given 10 and 20 mg/kg CPP or saline intraperitoneally or infused with the drug (30 and 100 µM) through the probe. Dialysate was collected every 20 min and splitted for the simultaneous measurement of 5-HT (15 µL) and glutamate (5 µL).

Statistical analysis

Extracellular levels of glutamate and 5-HT, not corrected for *in vitro* recovery, shown in the result section were expressed as percentages of basal values. Values missing because of occasional problems in sample collection or analysis were replaced by the mean of the samples immediately before and after. Statistical analyses were done on raw data. Basal values of glutamate in different experiments were compared by one-way ANOVA. All time-course data were analyzed by a split-plot ANOVA as described previously (General Methods).

RESULTS

- *Basal concentrations of glutamate: effect of TTX, elevated KCl, and nicotine*

Glutamate levels were stable about 2h after the start of the experiment. Mean basal GLU concentrations in the mPFC of rats implanted with AN-69 probes, obtained by pooling basal values of different experimental groups, were 14.2 ± 0.7 pmol/20 μL (n=85). No significant differences were found among basal GLU levels in the different experimental groups ($F_{15,69} = 0.6$, P>0.05).

Fig. 2.1 shows that 1 μM TTX had no significant effect on cortical extracellular GLU ($F_{5,5}= 0.8$, P>0.05; one-way ANOVA) whereas the 20-min perfusion with 60 mM KCl increased extracellular GLU by about 2.5-fold ($F_{3,3} = 24.1$, P<0.05; one-way ANOVA). Nicotine significantly increased extracellular GLU ($F_{4,8}=11.8$, P< 0.01). Extracellular GLU reached 284% of basal levels after 1 mM nicotine whereas 0.1 mM nicotine had no significant effect (132% of basal levels) (Fig. 2.1).
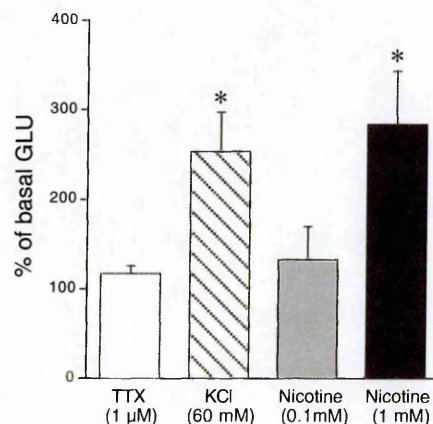
Figure 2.1

Effect of the perfusion of 1 μM tetrodotoxin (TTX), 60 mM KCl and 0.1 and 1 mM nicotine though the probe on basal extracellular glutamate (GLU) in the mPFC. Results are mean $\pm$SEM and are expressed as percentage of basal levels. Basal levels of glutamate in pmol/60 μL were: TTX, 41.8 $\pm$ 6.8 (n=6); KCl, 35.7 $\pm$ 8.4 (n=4); Nicotine, 33.5 $\pm$ 5.7 (n=5). *P<0.05 vs. baseline levels (Dunnett's test).

- *Increase of extracellular glutamate in response to CPP and effect of TTX*

Basal levels of glutamate in pmol/20 μL were: CSF, 17.9 ± 4.6 (n=5); CPP 30 μM, 18.6 ± 3.7 (n=4); CPP 100 μM, 13.8 ± 3.1 (n=9); TTX+CPP, 14.5 ± 1.3 (n=4). *$P<0.05$ vs. CSF; #$P<0.05$ vs. CPP 100 μM (Tukey-Kramer's test).

Fig. 2.2A shows that intraperitoneal CPP significantly raised extracellular GLU in the mPFC ($F_{2,10}=4.9$, $P<0.05$). Post-hoc analysis showed that extracellular GLU in rats given 20 mg/kg CPP was significantly higher than in those receiving saline. Extracellular GLU rose by about 50% during the first hour after the injection, reached 195% at 80 min and was still increased at 2 h (190% of basal levels). Ten mg/kg CPP had no significant effects.

The infusion of CPP through the probe significantly raised extracellular GLU in the mPFC ($F_{2,15}=23.9$, $P<0.0001$) (Fig. 2.2B). Post-hoc analysis showed that extracellular GLU in rats given 100 μM CPP was significantly higher than in rats infused with CSF ($P<0.05$; Tukey-Kramer's test). Extracellular GLU rose by about 225% in response to 100 μM CPP at 60 min. Surprisingly, the maximal increase was reached at 80 min (350%), i.e. 20 min after the end of CPP infusion. Then, extracellular GLU gradually returned to baseline. Infusion of 300 μM CPP did not cause any greater increase in extracellular GLU (n=2; data not shown). The concentration of 30 μM CPP had no significant effect on extracellular GLU (Fig. 2B; $P>0.05$, Tukey-Kramer's test). The infusion of 1 μM TTX completely prevented the effect of 100 μM CPP on extracellular GLU ($F_{1,12}=29.6$, $P<0.001$) (Fig. 2.2B).
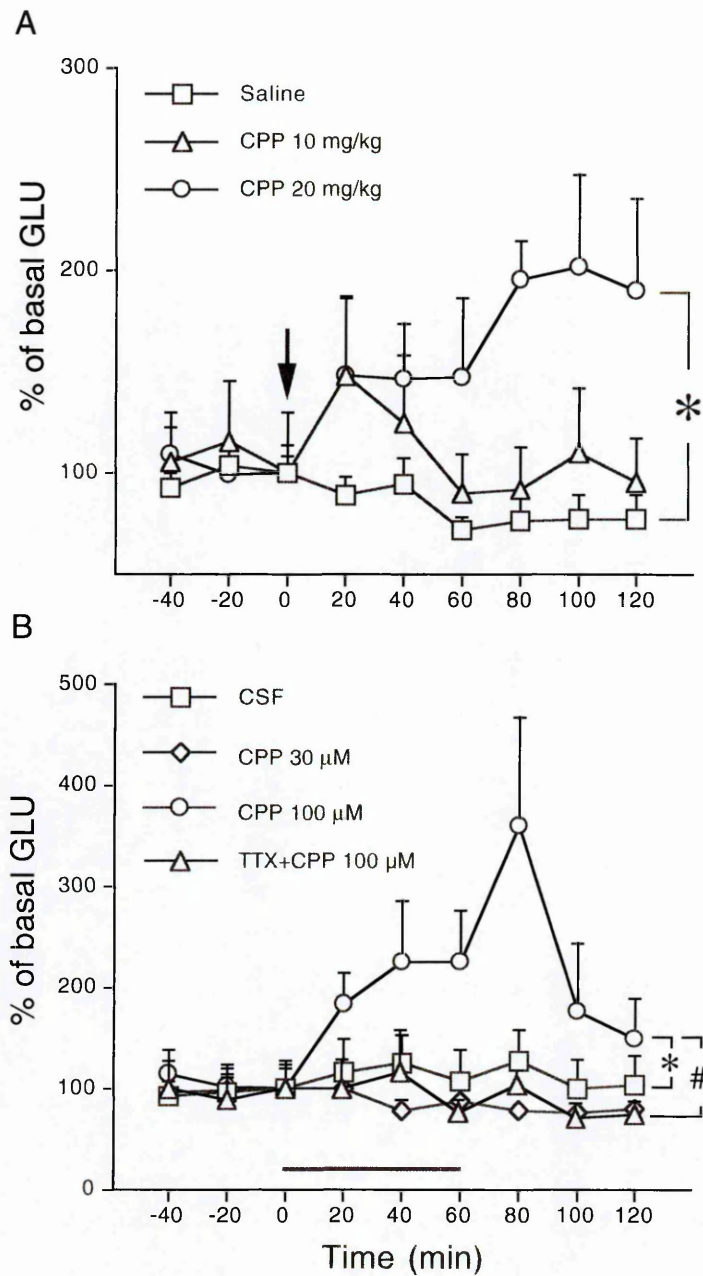
Figure 2.2

Panel A shows the effect of CPP 10 and 20 mg/kg (IP) or saline on extracellular glutamate (GLU). Arrow indicates the injection of saline or CPP. Basal levels of glutamate in pmol/20 μL were: saline, 16.0 ± 1.3 (n=4); CPP 10 mg/kg, 12.7 ± 3.8 (n=5); CPP 20 mg/kg, 13.9 ± 1.8 (n=4). Panel B shows the effect of 30 and 100 μM CPP on extracellular GLU in rats perfused with normal CSF and the effect of 100 μM CPP in rats co-perfused with 1 μM TTX. TTX perfusion started 20 min before CPP and continued for the rest of the experiment. Each concentration of CPP was perfused through the probe for 1 h (horizontal bar). Results are mean $\pm$ SEM. *P<0.05 vs. saline (Tukey-Kramer's test)

In another experiment we examined the effect of the competitive NMDA receptor

antagonist AP7 on extracellular GLU. We found that the infusion of 1 mM AP7 for

60 min almost doubled extracellular GLU levels (Basal 30.8 – 7.2, AP7 61.8 –13.8 pmol/h; n=8; $F_{1,7}$= 7.2, P<0.05).

- ***CPP increases extracellular 5-HT levels***

Fig 2.3A shows that extracellular levels of 5-HT in the mPFC of rats given intraperitoneal CPP were significantly higher than in control rats (CPP, $F_{2,10}$=9.4, P<0.01; time, $F_{6,60}$= 1.5, P>0.05; CPP x time, $F_{12,60}$ = 1.0, P>0.05). Ten mg/kg CPP increased extracellular 5-HT by 140% of basal levels at 40 min whereas 20 mg/kg had no significant effect.

The infusion of CPP through the probe significantly increased extracellular 5-HT (CPP, $F_{2,12}$=5.1, P<0.05; time, $F_{6,72}$=1.6, P>0.05; CPP x time, $F_{12,72}$= 6.7, P<0.0001). Extracellular 5-HT in rats infused with 100 µM CPP reached about 150% of baseline levels at 60 min and had fallen back at 100 min (Fig. 2.3B). Extracellular levels of 5-HT in rats infused with 30 µM CPP were slightly lower than in those infused with CSF but the difference were not significant.
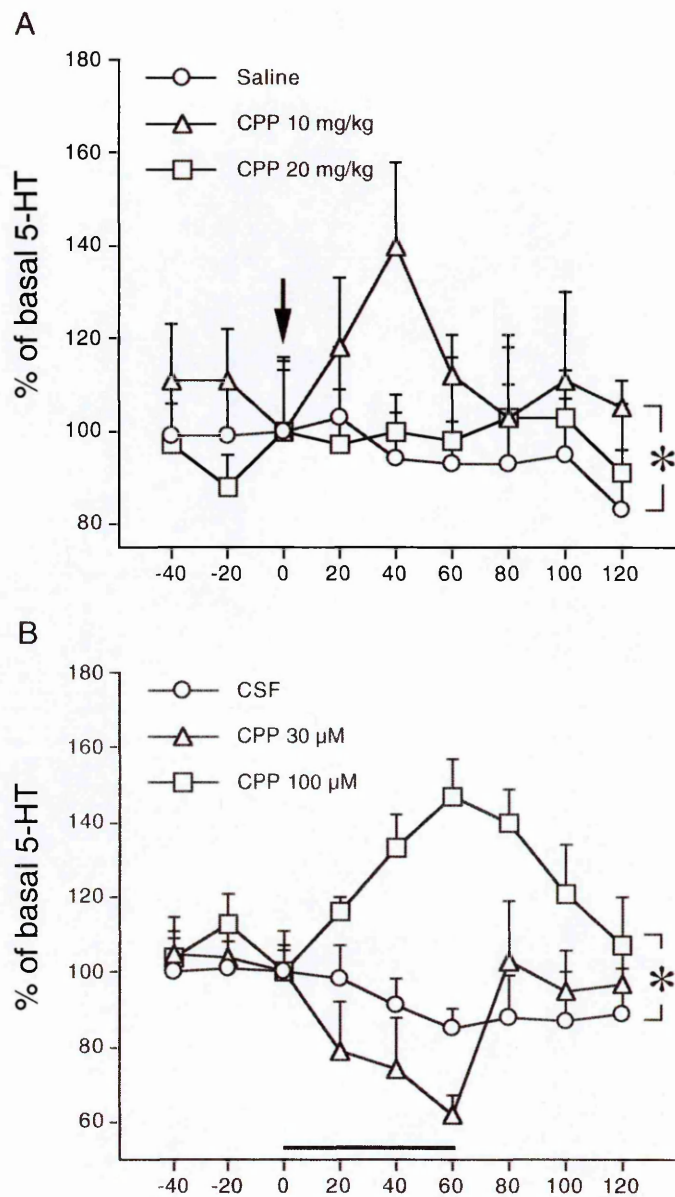
Figure 2.3
Extracellular 5-HT in the mPFC of rats given 10 and 20 mg/kg CPP or saline (arrow; panel A) or 30 and 100 μM CPP infused through the probe for 1 h (horizontal bar; panel B). Results are mean ±SEM. Basal levels of 5-HT (fmol/20 μL) were: Saline (n=4) 4.0 ± 0.6; CPP 10 mg/kg (n=5) 5.4 ± 0.6; CPP 10 mg/kg (n=5) 3.2 ± 0.5; CSF (n=4), 4.8 ± 0.4 ; CPP 30 μM (n=5), 4.9 ± 0.6; CPP 100 μM (n=5), 4.7 ± 0.3. *P<0.05 vs. saline or CSF (Tukey-Kramer's test).

DISCUSSION

Both intraperitoneal injection and intracortical infusions of the selective NMDA receptor antagonist CPP raised extracellular glutamate and 5-HT in the mPFC of awake rats. The increase of GLU reached its peak 20 min after the end of CPP infusion. This delayed effect (Figs. 2.2 and 2.3) is probably related to the use of probes made with the AN-69 membrane since it was not seen with the Cuprophan membrane (data not shown).

The ability of the microdialysis technique to reveal changes in neuronal release of glutamate is debated (Herrera-Marschitz et al., 1996; Timmerman and Westerink, 1997). Basal extracellular levels of glutamate do not reflect neuronal release but mainly depend on the activity of the cystine-glutamate exchanger (Baker et al., 2002). However, the stimulation of exocytotic release induced by increased K^+ in the perfusion medium or electrical stimulation of glutamatergic pathways raised glutamate in the dialysate (Timmerman and Westerink, 1997). Accordingly, basal levels of glutamate were largely insensitive to TTX whereas K^+-evoked release increased dialysate glutamate. In addition the infusion of nicotine through the probe increased extracellular glutamate. Nicotine at doses shown in previous studies to stimulate the neuronal release of glutamate (Gioanni et al., 1999; Lambe et al., 2003; Reno et al., 2004) increased glutamate release suggesting that under the present experimental conditions, the raised glutamate levels in the dialysate may be due to activity-dependent glutamate release. Thus, it is conceivable that the CPP-induced increase of extracellular glutamate reflects an increase of neuronal release. The finding that TTX completely abolished the increase of extracellular glutamate induced by CPP supports this interpretation. Likewise, the increase of extracellular levels of striatal glutamate induced by NMDA or stimulation of the corticostriatal

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glutamatergic pathway was prevented by locally perfused TTX (Dijk et al., 1995; Liu and Moghaddam, 1995; Lada et al., 1998).

The systemic dose and the intracortical concentration of CPP that significantly increased extracellular glutamate fit well with those used *in vitro* and *in vivo* to selectively block NMDA receptors (Chapman et al., 1987; Lehmann et al., 1987; Del Arco and Mora, 2002). In addition, intracortical infusion of the competitive NMDA receptors antagonist AP7 a structural analogue of CPP (Perkins et al., 1982) also raised extracellular glutamate. These results indicate that selective blockade of NMDA receptors in the mPFC is sufficient to increase extracellular glutamate in this brain region. The involvement of NMDA receptors in the mechanism by which CPP increases extracellular glutamate, is further supported by the finding that systemic administration of non-competitive NMDA receptor antagonists such as PCP and ketamine and CPP (present study) increase extracellular glutamate (Moghaddam et al., 1997; Moghaddam and Adams, 1998).

The mechanism by which CPP increased extracellular glutamate is unclear. The presynaptic location of some NMDA receptors means they are presumably autoreceptors (Conti et al., 1997). However, the stimulation of NMDA autoreceptors facilitated glutamate release and synaptic currents whereas their blockade had no effect or even reduced glutamate release (Connick and Stone, 1988; Martin et al., 1991; Berretta and Jones, 1996). Thus, it is unlikely that the blockade of autoreceptors contributed to the effect of CPP on extracellular glutamate. This suggests that the increase of glutamate by CPP was probably due to indirect mechanisms involving the blockade of NMDA receptors on non-glutamatergic neurons, which in turn enhance extracellular glutamate. The antagonism of CPP's effect by TTX is consistent with this.

There is evidence that glutamate release is inhibited by GABA (Pende et al., 1993) whereas DA and 5-HT may have excitatory and inhibitory effects on glutamate release depending on the receptor subtype (Yamamoto and Davy, 1992; Srkalovic et al., 1994; Dijk et al., 1995; Maura and Raiteri, 1996; Exposito et al., 1999; Abekawa et al., 2000; Scruggs et al., 2003). NMDA receptors are located on GABAergic neurons in the rat cortex (Aoki et al., 1994; DeBiasi et al., 1996) and their blockade with PCP and MK-801 reduced extracellular GABA in the mPFC (Yonezawa et al., 1998). In addition, PCP markedly reduced bursting activity of cortical pyramidal neurons (Shi and Zhang, 2003) and it has been suggested that the response of GABAergic interneurons depends on the firing pattern of pyramidal cells (Thomson, 2000; Shi and Zhang, 2003). Thus, the effect of CPP on extracellular glutamate may be mediated by direct or indirect suppression of cortical GABAergic transmission, which in turn enhances the release of glutamate from afferents to the mPFC. Studies in cortical slices and synaptosomes showing that stimulation of presynaptic $GABA_B$ receptors inhibited cortical GLU release (Potashner, 1979; Pende et al., 1993; Perkinton and Sihra, 1998) are consistent with this interpretation. CPP and the structural analogue AP-5 infused into the mPFC raised extracellular DA locally (Del Arco and Mora, 1999; Feenstra et al., 2002) suggesting that activation of dopaminergic receptors may have contributed to the effect of CPP. However, results are contrasting regarding the control exerted by D2 receptors on extracellular glutamate (Yamamoto and Davy, 1992; Exposito et al., 1999) whereas the stimulation of D1 receptors reduced extracellular glutamate in the mPFC (Abekawa et al., 2000).

The infusion of CPP through the probe raised extracellular 5-HT in the mPFC. This effect was observed with the same concentration of CPP (100 µM) that increased

extracellular glutamate whereas 30 μM CPP had no effect on extracellular glutamate and 5-HT. These results are consistent with previous microdialysis studies in which blockade of NMDA receptors by PCP and MK-801 or genetic deletion of the receptor increased extracellular 5-HT in the mPFC and/or other regions of the rat brain (Whitton et al., 1992; Yan et al., 1997; Martin et al., 1998a; Miyamoto et al., 2001). The 5-HT$_{2A}$ receptor agonist DOI increased extracellular GLU (Scruggs et al., 2003) and the frequency of the GLU-dependent late component of evoked EPSC in slices of the mPFC (Aghajanian and Marek, 1999). Thus CPP, by releasing 5-HT, may activate 5-HT$_{2A}$ receptors in the mPFC to enhance GLU release. However, this interpretation hardly accounts for the effect of systemic CPP which at 10 mg/kg increased extracellular 5-HT but had no effect on GLU whereas at 20 mg/kg it increased GLU but had no effect on 5-HT. Thus, it appears that systemic CPP may increase extracellular GLU independently of its effect on cortical 5-HT. Differences in the effects of systemic and intracortical CPP on 5-HT are not surprising since NMDA receptors are widespread in the brain and may exert opposite effects on cortical 5-HT release depending on their localization in the raphe nuclei or cortex (Tao and Auerbach, 1996).

The data show that a competitive NMDA receptor antagonist CPP injected into the mPFC induces accuracy impairment and an increase in anticipatory and perseverative responding in rats performing a 5-CSRT task. Infused through a microdialysis probe, CPP, raised extracellular concentrations of glutamate and 5-HT as measured by microdialysis technique. Together the present findings suggest that enhanced glutamate and 5-HT release induced by blockade of NMDA receptors in the mPFC might be associated with deficits in attention and loss of executive control.

CHAPTER 4.   EFFECTS OF mGLU$_{2/3}$ RECEPTOR AGONIST, LY379268,

AND AMPA RECEPTOR ANTAGONIST, NBQX

The behavioural effects of NMDA receptor antagonists in humans and in experimental animals have been primarily attributed to *hypoactivity* of glutamatergic neurotransmission due to postsynaptic blockade of NMDA receptors. However, various findings demonstrate that the NMDA receptor antagonists may exert at least in part their effects by *hyperactivation* of glutamate neurotransmission. Indeed, disinhibition of glutamate transmission has been suggested to account for the increase in human frontal cortex metabolism and resting cerebral blood flow after ketamine (Breier et al., 1997; Vollenweider et al., 1997; Holcomb et al., 2005).

Fig. 1 Adapted from (Holcomb et al., 2005).
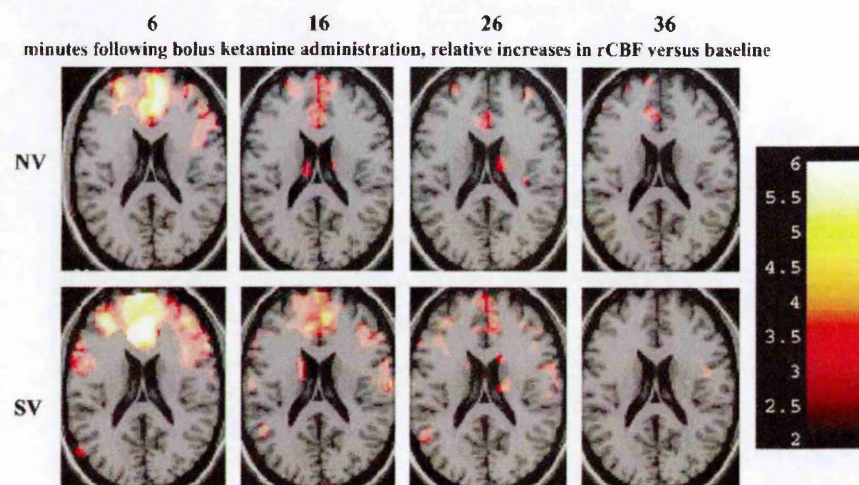
Ketamine elicited increases in ventral anterior cingulate CBF, in schizophrenic and healthy volunteers.

Studies using experimental animals show that the disruptive effects of NMDA receptor antagonists on motor activity, and working memory may be associated with their ability to raise extracellular glutamate concentrations in the PFC (Moghaddam et al., 1997; Moghaddam and Adams, 1998). Consistent with these studies are findings presented in Chapter 3 showing that the competitive NMDA receptor

antagonist CPP administered into the mPFC impaired attentional performance and enhanced glutamate release.

It is thought that glutamate released under these circumstances is more likely to stimulate non-NMDA receptors such as AMPA, kainate and metabotropic glutamate receptors and thus augment the negative impact of NMDA receptor blockade on cortical functions (Lisman et al., 1998; Krystal et al., 2003)). This hypothesis would suggest that under conditions of excessive glutamate release, activation of mechanisms capable of limiting excitatory transmission, or the blockade of AMPA receptors, might at least in part prevent the behavioural impairments induced by blockade of NMDA receptors.

One possible approach to suppressing glutamate release is through metabotropic receptors (mGluR) because many subtypes function as presynaptic autoreceptors on glutamatergic terminals (Conn and Pin, 1997). The mGluR are a heterogeneous family of G-protein coupled receptors, including $mGlu_{1-8}$ receptor sub-types, classified in 3 groups based on structural homology, signal transduction mechanisms and pharmacological properties (Group I, $mGlu_1$ and $mGlu_5$; Group II, $mGlu_2$ and $mGlu_3$; Group III $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$) (Conn and Pin, 1997; Schoepp, 2001). The group II metabotropic glutamate receptor subtypes which consists of $mGlu_2$ and $mGlu_3$ subtypes are primarily expressed in forebrain regions including PFC (Ohishi et al., 1993; Ohishi et al., 1994; Petralia et al., 1996) and function as a presynaptic negative regulatory mechanism, modulating excitatory glutamate and inhibitory GABA transmission (Anwyl, 1999; Schoepp, 2001). Using $mGlu_{2/3}$ agonists it was clearly established that stimulation of these receptors reduces postsynaptic potentials (EPSP) via a presynaptic mechanism in various brain areas (Baskys and Malenka, 1991) but may also influence postsynaptic excitability

(Tyszkiewicz et al., 2004) directly via $mGlu_2$ and $mGlu_3$ localized near active zones of postsynaptic densities in glutamatergic neurons (Petralia et al., 1996). Relevant for this study is the demonstration by Marek and Aghajanian (Marek and Aghajanian, 1998) that activation of these receptors in the mPFC suppresses glutamate neurotransmission evoked by electrical stimulation or through stimulation of $5\text{-}HT_{2A}$ receptors by iontophoretic application of 5-HT, thus suggesting that $mGlu_{2/3}$ receptor agonists may act also as "functional" $5\text{-}HT_{2A}$ antagonists.

Recently, conformationally constrained analogues of glutamate that are selective agonists or antagonists for Group II mGluR receptors at low concentrations have been developed. The systemically active $mGlu_{2/3}$ agonists include (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxilate monohydrate (LY354740) and (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268).

In pharmacological studies LY354740 and LY379268 have been shown to attenuate behavioural effects of NMDA receptor antagonists in man and experimental animals and to reduce the effects on glutamate excitability and release (Moghaddam and Adams, 1998; Cartmell et al., 1999; Krystal et al., 1999a; Anand et al., 2000; Krupitsky et al., 2001; Krystal et al., 2003; Lorrain et al., 2003a; Winter et al., 2004; Greco et al., 2005; Homayoun et al., 2005; Krystal et al., 2005).

This chapter provide evidences that the systemically active $mGlu_{2/3}$ agonist LY379268 is able to reverse some effects of CPP such as impairments in accuracy and impulsivity suggesting that *hyperactivation* of glutamate neurotransmission in the PFC may underlie dysfunctions in some aspects of attentional performance.

Findings showing that AMPA receptor antagonists reverse the NMDA receptor antagonist-induced PFC dopamine release and impairments in working memory (Moghaddam et al., 1997) and hyperlocomotion (Hauber and Andersen, 1993;

Willins et al., 1993; Bubser et al., 1995) support the hypothesis that *hyperactivity* of glutamate neurotransmission at AMPA receptors underlies the behavioural and neurochemical effects of NMDA receptor antagonists. In addition, phencyclidine markedly suppressed bursting activity of cortical pyramidal neurons but these PCP-induced changes in bursting activity were not observed when AMPA receptors were blocked (Shi and Zhang, 2003). Thus, in this chapter examining the effects of a competitive AMPA receptor antagonist, NBQX administered into the mPFC, tested whether CPP-induced attentional performance deficits may have resulted from an over-activation of AMPA receptors. The data show that blockade of AMPA receptors by NBQX had no effect on CPP-induced performance deficits but by itself caused attentional impairment and loss of executive control similar to that reported after NMDA receptor antagonists.

MATERIALS AND METHODS

Animals, food deprivation, 5-CSRT task and behavioural procedures, surgery and CPP microinjections into the mPFC, and histology were as described previously (General methods).

Drugs, treatment schedules and experimental design

Drugs   CPP (Tocris, U.K), LY379268 (gift from Eli-Lilly, USA), NBQX (Tocris, USA), were dissolved in the phosphate buffer saline (PBS composition in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.0, KH_2PO_4 1.8, pH 7.4).

Treatment schedules   In each experiment the various combinations of different doses of a particular drug (LY379268 and NBQX) with vehicle or CPP were administered according to a Latin square design. At least two days were left between test days. Rats were always tested on these "free" days to re-establish the baseline and check for lasting effects of drugs.

Systemic and intracortical injection of mGlu$_{2/3}$ agonist LY379268

A group of rats (n=9) received 1 μL vehicle (PBS) or 0.1 and 0.3 mg/kg subcutaneously 20 min before an injection of 1 μL PBS or 50 ng/μL CPP into the mPFC. Ten minutes after the rats were put into the box and the test session started. Eight rats were used to assess the effects of various combinations of vehicle and LY379268 injected into the mPFC on CPP-induced performance deficits. LY379268 (3 and 30 ng/μL) or vehicle (1 μL) injected in a volume of 1 μL bilaterally into the mPFC 5 minutes before CPP (50 ng/μL).

Intracortical injection of the AMPA receptor antagonist, NBQX

Vehicle or 12.5 and 50 ng/μL NBQX were injected bilaterally into the mPFC 5 minutes before a microinjection of 1 μL saline or 50 ng/μL CPP into the same area.

Ten min later rats were exposed to a session of a 5-CSRT task. Data from 12 rats were statistically analysed and are included in the results.

Additional 7 rats were used to assess the effects of 200 ng/µL NBQX injected bilaterally into the mPFC 10 min before the test session.

Statistical analysis

The main dependent variables selected for analysis were: (a) the percentage of correct responses; (b) percentage of omissions; (c) mean correct response latency; (d) the number of anticipatory responses and (e) the number of perseverative responses.

The data of the experiments testing the effects of CPP in combination with different doses of either systemic or intra-mPFC drugs (LY379268 and NBQX) were analyzed by separate within-subjects two-way ANOVA with factors drug (LY379268 or NBQX) and CPP. The effects of high dose of NBQX on the performance variables of the 5-CSRT task were analysed by a with-in subjects one-way ANOVA. The means of the individual treatment combinations were compared between them by Tukey's HDS test.

RESULTS

- *Systemic injection of a mGlu$_{2/3}$ receptor agonist, LY379268, reversed the CPP-induced impairments in accuracy (% correct responses) and anticipatory but not perseverative responding*

Fig. 1 and Table 1 show that 0.1 mg/kg LY379268 administered subcutaneously 20 min before bilateral injections of vehicle (1 µL) into the mPFC had no effect on any measure of rats' performance. Administered before injections of 50 ng/µL CPP into the mPFC LY379268 reversed the CPP-induced impairments in accuracy (%correct) and anticipatory responding but was without effects on CPP-induced increase in perseverative responses, omissions and correct responses latencies.

Figure 1.
Effects of LY379268 alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat was injected subcutaneously with vehicle (V) or 0.1 mg/kg LY379268 (LY) 20 min before 1 µL vehicle (+VEHICLE) or 50 ng/µL CPP (+CPP) into the mPFC. Ten min later rats started the test session. CPP and LY379268 singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 9 rats.
* P<0.05 vs. V (+VEHICLE); # P<0.05 vs. V (+CPP); (Tukey's test)

A two-way ANOVA performed on % correct response data showed a significant interaction between LY379268 and CPP ($F_{1,24}$=11.5 P=0.002) and significant effects of CPP ($F_{1,24}$=43.0 P<0.0001) and LY379268 ($F_{1,24}$=6.5 P=0.01). Post-hoc multiple comparisons of various treatments means by Tukey's test revealed that CPP significantly decreased % correct responses compared to vehicle controls (P<0.05) and that CPP-injected rats pre-treated with 0.1 mg/kg LY379268 but not vehicle made significantly more correct responses than CPP-injected rats (P<0.05) (Fig. 1A). Similarly ANOVA performed on the anticipatory response data showed a significant interaction between LY379268 and CPP ($F_{1,24}$=4.3 P=0.05) a significant effect of CPP ($F_{1,24}$=19.2 P=0.00021) but no effect of LY379268 ($F_{1,24}$=2.9 P=0.1). Again, anticipatory responding was increased by CPP (compared to vehicle control, P<0.05) and LY379268 decreased it (compared to CPP, P<0.05) (Fig. 1B). Statistical analysis of perseverative responses data by ANOVA indicated that LY379268 had no effect on CPP-induced perseverative over-responding (Fig. 1C) (LY379268 x CPP, $F_{1,24}$=0.1 P=0.8; CPP, $F_{1,24}$=13.9 P=0.001; LY379268, $F_{1,24}$=0.6 P=0.4).

- *Systemic LY379268 did not affect the CPP-induced increases in the proportion of omissions and correct response latencies*

Omissions shown in Table 1, were increased by CPP ($F_{1,24}$=116.0 P<0.0001) but not LY379268. However, the proportion of omissions made by rats receiving CPP was not further affected by LY379268 (LY379268 x CPP, $F_{1,24}$=2.2 P=0.15). Table 1 also shows that rats receiving CPP into the mPFC had longer correct response latencies ($F_{1,24}$=55.1 P<0.0001) but that pre-treatment with LY379268 had no effect (LY379268, $F_{1,24}$=1.9 P=0.18; LY379268 x CPP, $F_{1,24}$=0.13 P=0.7.

The effects of 0.3 mg/kg LY379268 on CPP-induced deficits were also tested. At this

dose LY379268 by itself greatly increased omissions and correct response latencies.

When given in combination with CPP 0.3 mg/kg LY379268 had additive effects on

omissions and latencies.   Many rats performed only few trials doing mostly

omissions. Six out of 9 animals made about 80 % omissions. In fact, the mean

proportion of omissions and correct response latencies after 0.3 mg/kg LY379268

plus CPP reached 60.3 ± 5.3 and 1.47 ± 0.11 s respectively. Due to such a high

number of omissions the data obtained with 0.3 mg/kg LY379268 were not

considered reliable and were not included in the statistical analysis.

Table 1. Effects of systemic LY379268 and CPP on omissions and correct response
latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	10.0 ± 1.4	0.54 ± 0.03
LY 0.1 + VEH	16.4 ± 2.0	0.60 ± 0.05
VEH + CPP	33.7 ± 4.2 *	0.95 ± 0.07 *
LY 0.1 + CPP	43.2 ± 3.9	1.05 ± 0.09

Each value is the mean ± SEM of 9 rats. LY379268 at a dose of 0.1 mg/kg (LY 0.1) or vehicle (VEH)
were injected subcutaneously 20 min before bilateral injections of 1 µL vehicle (VEH) or 50 ng/µL
CPP into the mPFC.   Ten min later the rats started the test sessions. The various doses were
administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH; Tukey's test

- *Injected into the mPFC LY379268 reversed the CPP-induced effects on*

  accuracy but not anticipatory and perseverative over-responding

Vehicle or 3 and 30 ng/µL LY379268 were administered bilaterally into the mPFC

before injections of 1 µL vehicle or 50 ng/µL CPP into the same cortical area. At

these doses LY379268 had no effect on % correct responses, omissions and correct

response latencies but increased anticipatory and perseverative responses. However,

LY379268 reversed the effects of CPP on accuracy (measured by % correct) whereas

the effects of CPP on anticipatory and perseverative responses as well as omissions

and correct response latencies were not affected.

Fig. 2A illustrate the effects of CPP and LY379268 on accuracy. Statistical analysis

of % correct responses by two-way repeated measure ANOVA found a significant

interaction LY379268 x CPP ($F_{2,35}$=7.0 P=0.003) effect of CPP ($F_{1,35}$=24.5 P<0.0001)

and LY379268 ($F_{1,35}$=3.5 P=0.04). Post-hoc analysis comparing various treatments

means by Tukey's test found that when injected with 30 ng/μL LY379268 plus CPP

rats made significantly higher proportion of correct responses than after CPP plus

vehicle (P<0.05). The effects of 3 ng/μL LY379268 on CPP-induced impairment in
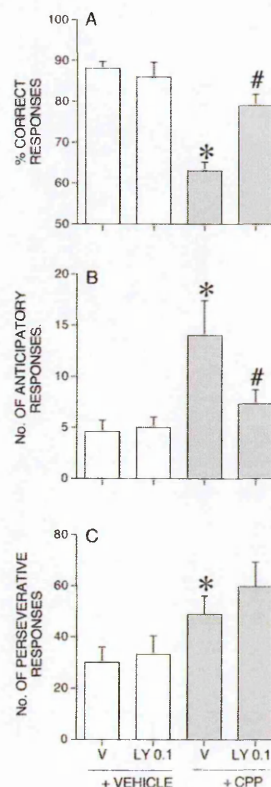
accuracy were not statistically significant (P>0.05).

Figure 2.
Effects of LY379268 alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat received vehicle (V), and 3 or 30 ng/µL LY379268 (LY) 10 min before 1 µL vehicle (+VEHICLE) or 50 ng/µL CPP (+CPP) into the mPFC. Ten min later rats started the test session. CPP and LY379268 singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 8 rats.
* P<0.05 vs. V (+VEHICLE); # P<0.05 vs. V (+CPP); (Tukey's test)

The analysis of anticipatory responses data by ANOVA showed a significant interaction LY379268 x CPP ($F_{2,35}$=3.3 P=0.04) but no effect of CPP ($F_{1,35}$=2.7 P=0.1) or LY379268 ($F_{1,35}$=0.7 P=0.5). As illustrated in Fig. 2B comparing various treatment means by Tukey's test both CPP and 30 ng/µL LY379268 increased anticipatory responses (compared to vehicle controls (P<0.05). At 3 ng/µL LY379268 the increase in anticipatory responses was not statistically significant

(P<0.05). The number of anticipatory responses induced by CPP was not affected by LY379268 (both doses, P>0.05). Analysis of perseverative responses data by ANOVA showed that these were enhanced by CPP ($F_{1,35}$=11.7 P=0.002). Either administered to vehicle or to CPP treated rats LY379268 did not affect response perseveration (LY379268, $F_{1,35}$=0.8 P=0.4; LY379268 x CPP, $F_{2,35}$=1.1 P=0.3).

- *Intra-mPFC LY379268 had no effects on the CPP-induced increases in the proportion of omissions and correct response latency*

Omissions shown in Table 2, were increased by CPP ($F_{1,35}$=19.6 P<0.0001). However, the proportion of omissions made by rats receiving CPP was not further affected by LY379268 (LY379268, $F_{1,35}$=0.2 P=0.8; LY379268 x CPP, $F_{2,35}$=0.6 P=0.5). Rats receiving CPP into the mPFC had significantly longer correct response latencies ($F_{1,35}$=27.8 P<0.0001). Either administered alone or in combination with CPP, LY379268 had no effect on correct response latencies (LY379268, $F_{1,35}$=0.5 P=0.6; LY379268 x CPP, $F_{2,35}$=1.6 P=0.2).

Table 2. Effects of systemic LY379268 and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	12.9 ± 5.5	0.53 ± 0.03
LY 3 + VEH	17.0 ± 4.8	0.58 ± 0.03
LY 30 +VEH	13.9 ± 3.8	0.56 ± 0.02
VEH + CPP	35.8 ± 6.6 *	0.83 ± 0.08 *
LY 3 + CPP	29.6 ± 6.2	0.89 ± 0.13
LY 30 +CPP	29.1 ± 6.7	0.81 ± 0.13

Each value is the mean ± SEM of 8 rats. LY379268 at doses of 3 (LY 3) and 30 (LY 30) ng/μL or 1 μL vehicle (VEH) were injected bilaterally into the mPFC 10 min before injections of 1 μL vehicle (VEH) or 50 ng/μL CPP into the same area. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH; Tukey's test

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- *The AMPA receptor antagonist, NBQX, injected into the mPFC had no effects on the CPP-induced impairments in accuracy or anticipatory and perseverative responding*

Doses of 12.5 and 50 ng/μL of an AMPA receptor antagonist, NBQX, were injected bilaterally into the mPFC of rats receiving 1 μL vehicle or 50 ng/μL CPP into the same cortical area. These doses of NBQX administered in combination with vehicle had no effects on any measure of rats' attentional performance. More importantly NBQX had no effects on CPP-induced changes in attentional performance.

Two-way ANOVA performed on % correct response data showed a significant effect of CPP ($F_{1,55}=14.2$ P=0.0004) but no effect of NBQX ($F_{1,55}=0.8$ P=0.47) or interaction NBQX and CPP ($F_{2,55}=2.4$ P=0.1). Fig. 3A shows that CPP injected rats had lower percentage of correct responses compared to vehicle controls (P<0.05, Tukey's test). CPP-injected rats administered various doses of NBQX made a similar proportion of correct responses as those injected with vehicle plus CPP (P>0.05, Tukey's test).

Overall ANOVA shows that anticipatory responses were increased by CPP ($F_{1,55}=11.4$ P=0.001). The main effect of NBQX ($F_{1,55}=0.4$ P=0.7) or its interaction with CPP were not significant ($F_{2,55}=2.1$ P=0.1). As shown in Fig. 3B CPP-injected rats made more anticipatory responses (compared to vehicle controls, P<0.05). Compared to vehicle plus CPP-injected rats no dose of NBQX affected CPP-induced increase in anticipatory responding (P>0.05, Tukey's test).

Similarly, ANOVA shows that perseverative responses were increased by CPP ($F_{1,55}=14.2$ P=0.0004) but NBQX ($F_{1,55}=1.0$ P=0.4) and interaction NBQX x CPP were not significant ($F_{2,55}=1.7$ P=0.2). Fig. 3C shows that perseverative responding was enhanced by CPP (compared to vehicle controls, P<0.05) but rats receiving

NBQX plus CPP made an equal number of perseverative responses than rats receiving vehicle plus CPP (P>0.05).

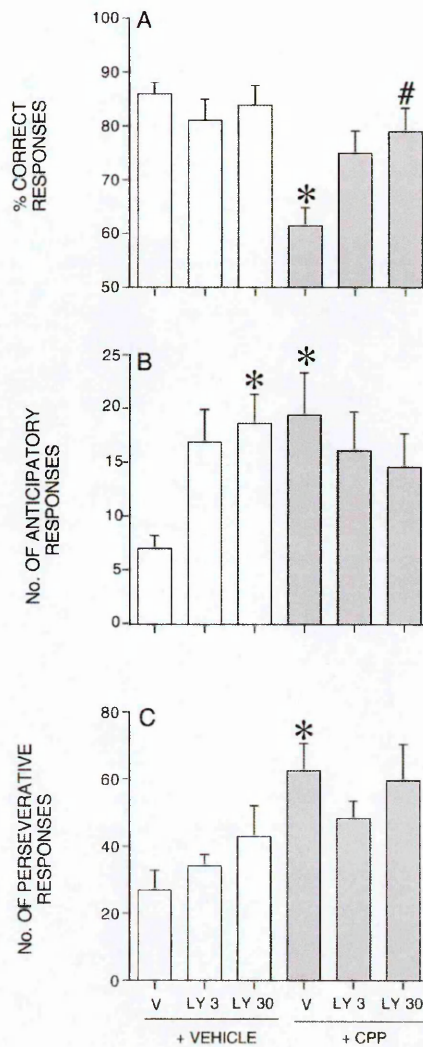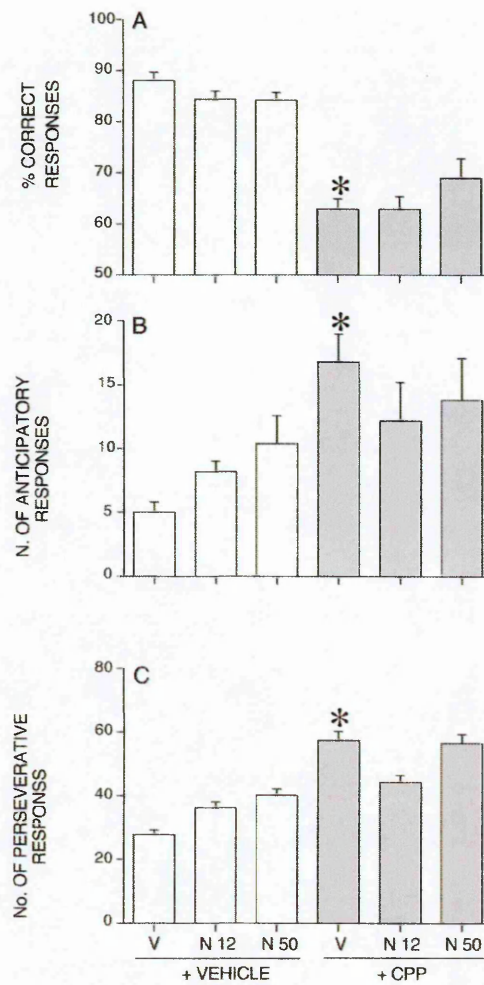
Figure 3.
Effects of NBQX alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat received vehicle (V), and 12.5 (N 12) or 50 ng/μL (N 50) NBQX 10 min before 1 μL vehicle (+VEHICLE) or 50 ng/μL CPP (+CPP) into the same area. Ten min later rats started the test session. CPP and NBQX singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 12 rats.
* P<0.05 vs. V (+VEHICLE); # P<0.05 vs. V (+CPP); (Tukey's test)

- ***Intra-mPFC NBQX had no effects on the CPP-induced omissions and correct response latency***

Omissions and mean correct response latencies were both increased by CPP (omissions, $F_{1,55}=149.0$ P<0.0001; correct latency, $F_{1,55}=53.0$ P<0.0001). Although

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NBQX had no statistically significant effects on omissions ($F_{1,55}$=0.5 P=0.6) and correct latency ($F_{1,55}$=2.0 P=0.1) and its interactions with CPP were not significant on these measures (omissions, $F_{2,55}$=0.6 P=0.5; correct latency, $F_{2,55}$=2.1 P=0.1) (Tab. 2) the proportion of omissions and correct response latencies were higher in rats injected with NBQX plus CPP.

Table 3. Effects of intra-mPFC NBQX and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	12.5 ± 2.8	0.58 ± 0.04
NBQX 12 + VEH	13.5 ± 2.0	0.57 ± 0.03
NBQX 50 +VEH	11.5 ± 1.6	0.57 ± 0.03
VEH + CPP	44.7 ± 3.8 *	0.96 ± 0.08 *
NBQX 12 + CPP	50.2 ± 4.0	1.29 ± 0.12
NBQX 50 ± CPP	51.6 ± 5.8	1.36 ± 0.20

Each value is the mean ± SEM of 12 rats. NBQX at doses of 12 (NBQX 12) and 50 (NBQX 50) ng/μL or 1 μL vehicle (VEH) were injected bilaterally into the mPFC 10 min before injections of 1 μL vehicle (VEH) or 50 ng/μL CPP into the same area. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH; Tukey's test

- *A high dose of NBQX impaired the attentional performance of rats in the 5-CSRT task*

A high dose of NBQX (200 ng/μL) impaired rats' attentional performance by itself and was not used in combination with CPP. As illustrated in Tab. 4, the effects of 200 ng/μL NBQX on attentional performance of rats performing a 5-CSRT task were similar to those reported with CPP. NBQX significantly decreased % correct responses (t_6=3.4 P=0.01) and increased anticipatory (t_6=3.3 P=0.01) and perseverative responses (t_6=3.7 P=0.01). However, the increase in omissions (t_6=1.4 P=0.2) and correct response latencies (t_6=2.2 P=0.1) was not statistically significant.

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Table 4. Effects of NBQX injected into the mPFC on various parameters of a 5-CSRT task

TREAT	CORR (%)	ANTICIP	PERSEV	OMISS (%)	CLAT (s)
VEH	89.0 ± 0.9	6.4 ± 2.0	31.6 ± 9.3	11.9 ± 3.8	0.58 ± 0.03
NBQX 200	79.1 ± 2.4*	13.7 ± 2.9*	53.0 ± 9.2*	19.7 ± 4.3	0.67 ± 0.04

Each value is the mean ± SEM of 7 rats. Bilateral injections of 1 µL vehicle (VEH) or NBQX 200 ng/µL (NBQX 200) were injected into the mPFC 10 min before the test sessions. The vehicle or NBQX were administered in a counterbalanced order 48 h apart. Abbreviations: %correct (CORR); Number of anticipatory responses (ANTICIP); Number of perseverative responses (PERSEV); omissions (OMISS); mean correct response latency (CLAT).
* $P < 0.05$ vs. VEH; (Student's t-test for paired values).

SUMMARY OF THE RESULTS

	Correct (%)	Anticipatory responses	Perseverative responses	Omissions (%)	Latency Correct
CPP	⇓	⇑	⇑	⇑	⇑
plus LY379268 systemic	0	0	=	=	=
plus LY379268 mPFC	0	=	=	=	=
plus NBQX mPFC	=	=	=	=	=

⇓ decrease; ⇑ increase; 0 reversal; = no effect.

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DISCUSSION

The data presented in this Chapter indicate that activation of mGlu2/3 receptors by a Group II selective agonist LY379268 (Monn et al., 1999) ameliorated some aspects of attentional performance disrupted by blockade of NMDA receptors in the mPFC. LY379268 either administered subcutaneously or into the mPFC reversed the CPP-induced accuracy impairments. Anticipatory over-responding induced by intra-mPFC CPP was abolished by systemic but not intra-mPFC LY379268. The effects of CPP on perseverative responses, omissions and correct response latencies were not affected by either systemic or intra-mPFC LY379268. LY379268 by itself increased anticipatory responses but only when injected into the mPFC. Other performance measures were not affected by LY379268.

The data also show that blockade of AMPA receptors in the mPFC had no effect on CPP-induced performance deficits in a 5-CSRT task. High dose of NBQX induced impairments in attentional performance that were qualitatively and quantitatively similar to those induced by CPP.

- *Stimulation of mGlu2/3 receptors*

Stimulation of $mGlu_{2/3}$ receptors in the mPFC by LY379268 was sufficient to prevent accuracy deficit induced by CPP suggesting that $mGlu_{2/3}$ receptors in the mPFC importantly contribute to the effects observed after systemic injection. Interestingly, delivered locally in the mPFC 1μM LY379268 prevented the ketamine-induced raise in extracellular glutamate (Lorrain et al., 2003a). Although in the present study the ability of LY379268 to prevent CPP-induced increase in glutamate release was not determined it could not be excluded that LY379268 reversed the CPP-induced accuracy deficits by limiting the excitatory transmission.

In the present study LY379268 was active at a dose of 0.1 mg/kg which is at least 10 times lower than those reported to block similar effects of phencyclidine in mice performing a 5-CSRT task (Greco et al., 2005), phencyclidine, ketamine and amphetamine-induced ambulations (Cartmell et al., 1999, 2000a; Swanson and Schoepp, 2002; Lorrain et al., 2003b) and ketamine-evoked glutamate release (Lorrain et al., 2003a). However, in one study LY379268 at 0.125 mg/kg significantly reversed the head twitches induced by a mixed 5-HT$_{2A/2C}$ receptor agonist DOI (Klodzinska et al., 2002).

It is important to note that a dose of 3 mg/kg LY379268 significantly reduced basal levels of locomotor activity (Swanson and Schoepp, 2002). In a preliminary experiment a few rats were administered 1 mg/kg LY379268 but they stopped responding during the task and any further testing with this dose was discontinued. A dose of 0.3 mg/kg LY379268 was tested in control and in CPP-injected rats. Although accuracy, correct response latencies and anticipatory and perseverative responses of rats were not significantly affected by 0.3 mg/kg LY379268 given alone, omissions were significantly increased. The overall performance of animals receiving 0.3 mg/kg LY379268 plus CPP was disrupted and rats completed only about 20% of trials making mostly omissions. In these conditions measures of accuracy, correct response latencies and anticipatory and perseverative responses were unreliable due to sampling error and data of 0.3 mg/kg LY379268 were not included in the statistical analysis. The optimal performance in this task requires a complex and tightly timed behavioural sequence. Active but not behaviourally disruptive doses of the majority of drugs tested in rats performing this task are generally low compared to those found effective in other tasks or on spontaneous motor behaviour. For example, amphetamine increases anticipatory responses at 0.4

mg/kg but increases spontaneous motor activity at doses > 2 mg/kg that disrupt 5-CSRT task performance (personal observations; for a review of drugs effects in this task, (Robbins, 2002)).

In a recent study Greco et al. (Greco et al., 2005) reported that in mice performing a 5-CSRT task systemic phencyclidine induced attentional performance deficits similar to those reported in the present study. At variance with the present results, in the study of Greco et al. (Greco et al., 2005) LY379268 had no effect on PCP-induced accuracy deficit but completely abolished the increase in anticipatory and perseverative responses. The reasons for these differences are not clear. In mice, behavioural and neurochemical differences in response to various drugs have often been shown to depend on the underlying genotype (Crawley et al., 1997). Differences in binding characteristics, the competitive or non-competitive nature of NMDA receptor antagonists and intracortical versus systemic route of administration may also have contributed.

In contrast to the systemic effects, intra-mPFC LY379268 by itself increased anticipatory responses. In CPP-injected rats LY379268 did not cause any further increase in anticipatory responses. It seems unlikely that the inability of intra-mPFC LY379268 to increase anticipatory responding in CPP-injected rats resulted from a ceiling effect since much higher numbers of anticipatory responses were reported in other pharmacological studies (Baunez and Robbins, 1999) thus showing that there was sufficient room for further increases in anticipatory responses. In fact, in a study by Higgins et al. (Higgins et al., 2003a) a $5-HT_{2C}$ receptor antagonist, SB242084 and a non-competitive NMDA receptor antagonist, dizocilpine increased anticipatory responses to a similar extent but when given in combination their effects were

additive. Why LY379268 was not able to increase anticipatory responses in CPP-injected rats is not obvious.

The mGlu$_{2/3}$ agonists cause dose-dependent decreases in firing rate and it has been reported to impair working memory (Aultman and Moghaddam, 2001; Higgins et al., 2004). Thus, a general depression of neuronal activity in PFC (Homayoun et al., 2005) might explain some of its effects on performance.

The number of anticipatory responses in this task was associated with high 5-HT and DA turnover and 5-HT release in the mPFC (Puumala and Sirvio, 1998; Dalley et al., 2002). Because LY379268 has been shown to increase DA and 5-HT turnover and release in the PFC it is conceivable that the increase in anticipatory responses observed in this study might be due to activation of DA and 5-HT neurotransmission (Cartmell et al., 2000b; Cartmell and Schoepp, 2000; Cartmell et al., 2001). However, this seems unlikely since infused into the mPFC LY379268 had no effect on DA or 5-HT release (Cartmell et al., 2001).

Despite differences in behavioural processes under study, route of drugs administrations and competitive or non-competitive nature of NMDA receptors antagonists used, the present findings are grossly compatible with the behavioural studies showing that mGlu$_{2/3}$ agonists such as LY354740 and LY379268 reduced hyperlocomotion, stereotypy and deficits in working memory, induced by non-competitive NMDA receptor antagonists given systemically (Moghaddam and Adams, 1998; Swanson and Schoepp, 2002).

- *Blockade of AMPA receptors*

The disinhibition of glutamate release by NMDA receptor antagonists and the consequent activation of AMPA receptors have been suggested to account for their

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effects on behaviour and monoaminergic neuotransmission in various brain areas (Moghaddam et al., 1997; Martin-Ruiz et al., 2001). Supporting this view are findings that the AMPA/kainate receptor antagonist CNQX injected into the mPFC blocked the raise in dopamine release in mPFC induced by systemic ketamine and the AMPA/kainate antagonist LY293558 administered intraperitoneally blocked the effect of ketamine on spatial delayed alternation (Moghaddam et al., 1997).

In the present study the AMPA/kainate receptor antagonist, NBQX injected into the mPFC at doses of 12.5 and 50 ng/μL was unable to affect CPP-induced performance deficits. At these doses NBQX by itself was without effect on rats' performance in the 5-CSRT task. Thus blockade of AMPA/kainate receptors in the mPFC was not sufficient to prevent the disruptive effects of CPP on attentional functioning and executive response control.

Various factors may at least in part account for the observed differences between the present and the previous study by Moghaddam et al. (1997). For example, the route of administration, chemical class and binding characteristics of the AMPA/kainate receptor antagonists employed all differed. The competitive or non-competitive nature of NMDA receptor antagonists may also be a critical factor since a study has reported that synergistic or no effects of an AMPA/kainate antagonist and NMDA receptor antagonists in a task of spatial memory may depend on whether the NMDA receptor antagonists were respectively non-competitive or competitive (Li et al., 1998).

However, the ability of AMPA receptor antagonists to antagonize the effects of NMDA receptor antagonists is not clearly established. In fact, the AMPA/kainate antagonist, GYKI 52466 reversed the effects of dizocilpine on locomotor activity (Bubser et al., 1995) and the anti-cataleptic effects of a competitive NMDA receptor

antagonist, CGP 37849 (Hauber and Waldenmeier, 1994) but other studies found no effects of AMPA antagonists and in some studies AMPA receptor antagonists potentiated NMDA receptor antagonists-induced effects. For example, systemic LY293558 had no effect on PCP-induced hyperlocomotion (Swanson and Schoepp, 2002) but injected into the mPFC impaired set-shifting ability (Stefani et al., 2003) whereas the AMPA antagonist YM90K and dizocilpine have been reported to synergistically impair working memory (Li et al., 1998).

When a high dose of NBQX was tested its effects were similar to those found after the NMDA receptor antagonist, CPP; it impaired accuracy and increased anticipatory and perseverative responses. At variance to the effects of CPP, the AMPA receptor antagonist, NBQX, did not increase the proportion of omissions or correct response latency. Accordingly, other studies have reported that NMDA and AMPA receptor antagonists both disrupted attentional set shifting (Stefani and Moghaddam, 2002; Stefani et al., 2003; Stefani and Moghaddam, 2003) and working memory (Aura and Riekkinen, 1999; Romanides et al., 1999) thus suggesting that glutamatergic transmission through NMDA and AMPA receptors in the mPFC importantly controls the various cognitive processes associated with PFC functions. However, various studies have reported that NMDA and AMPA receptors may be differently involved in distinct stages of cognitive functions (Day et al., 2003; Winters and Bussey, 2005). In conclusion, activation of mechanisms in the mPFC that provide a negative regulatory mechanism on glutamate release is sufficient to reduce accuracy deficit induced by blockade of NMDA receptors in the same area. This suggests that attentional dysfunctions may be caused by excessive cortical glutamate release. However, in the conditions of blocked NMDA receptors, activation of the AMPA

receptor does not appear to play a relevant role in the attentional performance deficits.

- ***Functional analogy of mGlu_{2/3} receptor agonists and 5-HT_{2A} receptor antagonists***

The mGlu$_{2/3}$ agonists have been shown to block 5-HT-evoked glutamate release. In the *ex-vivo* slice recordings from rat PFC, Marek et al. (Marek et al., 2000) demonstrated that 5-HT-evoked EPSCs were enhanced by the mGlu$_{2/3}$ antagonist LY311495 and inhibited by agonists LY379268 and LY354740. In this system the 5-HT-evoked EPSC appears to involve presynaptic impulse-flow-independent release of glutamate mediated by 5-HT$_{2A}$ receptors since it was antagonised by M100907 (Aghajanian and Marek, 2000). The likely neuronal location for this induction are the apical dendrites of neocortical layer V pyramidal cells (Aghajanian and Marek, 1999) and correspond to the laminae that are rich in 5-HT terminals and 5-HT$_{2A}$ receptors (Blue et al., 1988; Aghajanian and Marek, 1997). Additionally, activation of 5-HT$_{2A}$ receptors by DOI increases excitatory postsynaptic currents (EPSC) and potentials (EPSP), glutamate release (Scruggs et al., 2003), activates c-*fos* in PFC and induces a behavioural syndrome characterized by head twitches. These effects were blocked by LY354740 and LY379268 (Gewirtz and Marek, 2000; Marek et al., 2000; Klodzinska et al., 2002; Zhai et al., 2003) suggesting that mGlu$_{2/3}$ receptor agonists may act as "functional" 5-HT$_{2A}$ antagonists (Marek and Aghajanian, 1998). An underlying neuroanatomical basis for this interaction between 5-HT$_{2A}$ and mGlu$_{2/3}$ receptors might reside in the co-localization of mGlu$_{2/3}$ and 5-HT$_{2A}$ binding to the same lamina, Va, in the mPFC as opposed to that found in other cortical regions such as fronto-parietal where mGlu$_{2/3}$ and 5-HT$_{2A}$ binding

appears to localize to different laminas (II-IV and Va, respectively) (Marek et al., 2000). Thus, $mGlu_{2/3}$ and $5\text{-}HT_{2A}$ receptors may play an important role in the integration of synaptic activity by apical dendrites of the layer V pyramidal neurons. Despite the "functional" analogy between $mGlu_{2/3}$ agonists and $5\text{-}HT_{2A}$ antagonists it is unlikely that they share a common underlying mechanism with respect to their effects on glutamate neurotransmission. In fact, systemic CPP increased extracellular glutamate independently of its effect on cortical 5-HT (present study, Chapter 3). Additionally in a preliminary experiment it was found that CPP was still able to increase extracellular glutamate but not 5-HT in the mPFC of animals administered PCPA to decrease 5-HT synthesis (Dr. RW Invernizzi and Mirjana Carli, Unpublished results).

Interestingly the behavioural effects of NMDA antagonists were still present in 5-HT depleted animals. The $mGlu_{2/3}$ agonist LY379268 but not the $5\text{-}HT_{2A}$ receptor antagonists, ketanserine or M100907 reversed the NMDA antagonist-induced behaviours in 5-HT depleted rats (Martin et al., 1998b; Swanson and Schoepp, 2002).

Despite the differences in the underlying mechanisms by which $mGlu_{2/3}$ agonists and $5\text{-}HT_{2A}$ receptor antagonists may exert their effects the "functional" analogy between them provide the rational for examining the effects of $5\text{-}HT_{2A}$ receptor antagonists on attentional performance deficit induced by blockade of NMDA transmission in the mPFC.

CHAPTER 5: EFFECTS OF 5-HT$_{1A}$, 5-HT$_{2A}$ AND 5-HT$_{2C}$ RECEPTOR AGONISTS AND ANTAGONISTS

Serotonin exerts both inhibitory and excitatory functions in neural networks through the coupling of different 5-HT receptor types to distinct ion channels. One of the main target structures of the 5-HT systems originating in the mesencephalic dorsal (DR) and median (MR) raphé nuclei is the frontal cortex (Azmitia and Segal, 1978; Steinbusch, 1981). The 5-HT projections target both the glutamatergic pyramidal principal neurons and GABAergic interneurons (Smiley and Goldman-Rakic, 1996). However, the exact role of serotonergic transmission in the mPFC in cognitive functions is poorly understood.

As reviewed in the Introduction the 5-HT_{1A} and 5-HT_{2A} receptor mechanisms have been implicated in cognitive functions (Carli and Samanin, 1992; Williams et al., 2002; Balducci et al., 2003; Meltzer et al., 2003). The 5-HT_{1A} and 5-HT_{2A} receptor types are particularly dense in the PFC (Pazos and Palacios, 1985; Pompeiano et al., 1992, 1994)) where these receptors are highly co-localized (80%) in nearly half of the glutamatergic pyramidal neurons (Santana et al., 2004). Through 5-HT_{1A} and 5-HT_{2A} receptors serotonin hyperpolarises and depolarises glutamatergic pyramidal neurons and has opposite effects on glutamate release (Araneda and Andrade, 1991; Dijk et al., 1995; Aghajanian and Marek, 1997, 2000)).

Immunohistochemical studies have revealed the presence of 5-HT_{2C} receptors in many forebrain areas including the prefrontal cortex (Abramowski et al., 1995; Pasqualetti et al., 1999; Lopez-Gimenez et al., 2001). Although 5-HT_{2C} receptors have received little attention in studies of cognitive functions, evidence has accumulated showing that 5-HT_{2C} and 5-HT_{2A} receptors exert opposing effects on behaviour and similarly to global 5-HT depletion blockade of the 5-HT_{2C} receptors increases impulsivity in the 5-CSRT task (Fletcher et al., 2002; Winstanley et al., 2004b).

Therefore the experiments reported in this chapter examined the effects of the 5-HT_{2A} receptor antagonist M100907, the 5-HT_{2C} receptor agonist, Ro60-0175 and the 5-HT_{1A} receptor agonist 8-OH-DPAT on the impairments in attentional performance induced by CPP injected into the mPFC. The effects of systemic M100907 and Ro60-0175 on CPP-induced deficits are reported in Section 1 and Section 2, respectively. In section 3, are reported the data of experiments that compared the contribution of PFC 5-HT_{1A} and 5-HT_{2A} receptor activity to various aspects of attentional performance. Table A, reports the binding profile of compounds used in these studies.

TABLE A. Ki (nM) Values of 8-OH-DPAT, WAY 100635, M100907 and Ro 60-0175 at selected binding sites

Receptor	8-OH-DPAT	WAY100635	M100907	Ro 60-0175
Serotonin receptors				
$5\text{-}HT_{1A}$	0.89	0.24	>10,000[h]	1,584
$5\text{-}HT_{1B}$	33,165	8,310[p]	>10,000	>1,000
$5\text{-}HT_{1D}$	820	2,2450[p]		>1,000
$5\text{-}HT_{1E}$	826[h]		>10,000[h]	
$5\text{-}HT_{1F}$	1,772[h]			
$5\text{-}HT_{2A}$	7,143	1,100	1.92	398[h]
$5\text{-}HT_{2B}$	4,120		261[h]	3[h]
$5\text{-}HT_{2C}$	11,000	282[p]	88	79[h]
$5\text{-}HT_{3}$	5,500	>10,000[p]	>10,000[h]	
$5\text{-}HT_{4}$	>10,000			
$5\text{-}HT_{5a}$	1,000		>10,000[h]	
$5\text{-}HT_{5b}$	398.1[m]			
$5\text{-}HT_{6}$	>10,000[c]		3,694[h]	
$5\text{-}HT_{7}$	41.29[c]			
Adrenergic				
$\alpha\text{-}1$	2,691	45		>1,000
$\alpha\text{-}1A$		18.6	128	
$\alpha\text{-}1B$		660.7	424.7	
$\alpha\text{-}2$	301	1,258		
$\alpha\text{-}2A$	186[c]		1,900[h]	1,995
$\alpha\text{-}2C$	117[c]		1,442[h]	
$\beta\text{-}$		>1,000	>10,000	
Dopamine				
D1	>10,000[h]	>1,000	2,964[h]	
D2	2,187	346[h]	2,250[h]	3,162
D3	361[h]	67[h]	6,700[h]	3,162
D4	6,770		540[h]	
D5			10,000[h]	

Unlabeled, determination made in rat cortical tissue.
h, determination made using human cloned receptors;
m, determination made using mouse cortical tissue;
p, determination made using pigeons brain tissue;
c, determination made using HT29 cells;
The values are taken from PDSP Ki Data base. http://pdsp.cwru.edu/pdsp.php.
Ro 60-0175 binding data were adapted from Gobert et al. 2000.

MATERIALS AND METHODS

Animals, food deprivation, 5-CSRT task apparatus and training, procedures for measures of motor activity, surgery, microinjection and histology were as described previously (General Methods).

Drugs, treatment schedules and experimental design

Drugs   CPP (Tocris, U.K), 8-OH-DPAT (Tocris, USA), WAY100635 (gift from Pharmacia, Nerviano, Italy) and Ro60-0175 (gift from Hofman-LaRoche, Switzerland) were dissolved in the phosphate buffer saline (PBS composition in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.0, KH_2PO_4 1.8, pH 7.4). M100907 (gift from Aventis, USA) was dissolved in vehicle (PBS containing 2-3 drops of 90% lactic acid; pH of the solution was adjusted to 7 with 1M NaOH).

Treatment schedules   In each experiment the various combinations of different doses of a particular drug (8-OH-DPAT, M100907 and Ro60-0175) with vehicle or CPP were administered according to a Latin square design.   At least two days were left between test days. Rats were always tested on these "free" days to re-establish the baseline and check for lasting effects of drugs.

Intracortical injection of 5-HT$_{1A}$ agonist 8-OH-DPAT and antagonist WAY100635

A group of rats (n=11) received 1 µL vehicle (PBS) or 30 and 100 ng/µL 8-OH-DPAT into the mPFC 5 min before an injection of 1 µL PBS or 50 ng/µL CPP into the same cortical area. Ten minutes after the last intra-cortical injection rats were put into the box and the test session started.

Eight rats were used to assess the effects of various combinations of vehicle, WAY100635 and 8-OH-DPAT on CPP-induced performance deficits. WAY100635 (30 ng/0.5 µL) or vehicle (0.5 µL) were mixed with the solution of 8-OH-DPAT

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(100 ng/0.5 μL) or vehicle (0.5 μL) and injected in a volume of 1 μL into the mPFC

5 minutes before CPP (50 ng/μL). Using thin-layer chromatography (TLC) it was

shown that WAY100635 and 8-OH-DPAT do not form stable complex when mixed

in a solution (Carli et al. 1998).

Systemic and intracortical injection of the 5-HT$_{2A}$ receptor antagonist, M100907

Vehicle or 10 and 40 μg/kg M100907 were given subcutaneously 20 minutes before

a microinjection of 1 μL saline or 50 ng/μL CPP into the mPFC. Ten min later rats

were exposed to a session of a 5-CSRT task. Only data from 9 rats were statistically

analysed and are included in the results.

Bilateral injection of vehicle (1 μL) or 100 and 300 ng/mL M100907 were made into

the mPFC 5 min before a microinjection of 1 μL saline or 50 ng/μL CPP into the

same cortical region. Again, 10 min later they were given a test session on the 5-

CSRT task. Data from 14 rats were statistically analysed and are included in the

results.

Effects of systemic M100907 on CPP-induced motor hyperactivity

After habituation to the activity cages the rats were injected subcutaneously with

vehicle (2 mL/kg) or M100907 (10 and 40 μg/kg) and 20 min later received bilateral

injections of CPP (50 ng/μL) or vehicle (1 μL) into the mPFC. Ten minutes later

they were transferred to the activity cages and their motor activity was recorded over

a 2 h period in 5-min bins. The data collected during the first 30 min of testing were

statistically analyzed and are presented in the Results section.

Systemic injection of the 5-HT$_{2C}$ receptor agonist, Ro60-0175

On each test day rats were injected subcutaneously with 2 mL/kg vehicle (PBS) or

0.03 and 0.1 mg/kg Ro60-0175 and 15 min later received a bilateral injection of

Vehicle (1 μL) or CPP (50 ng/μL) into the mPFC. Data of 8 rats were statistically analysed and are included in the results.

A different group of 11 rats were injected subcutaneously with 0.3 mg/kg and 5 min later tested on a variable intertrial interval (VAR-ITI) version of the 5-CSRT task. In this version of the task rats had 50 trials at each ITI duration. Due to random intermixing of ITIs of different durations (3, 5, 7 and 9 sec) the presentation of the stimuli is rendered unpredictable. The session lasted 60 min or 200 trials whichever was completed sooner.

Statistical analysis

The main dependent variables selected for analysis were: (a) the percentage of correct responses; (b) percentage of omissions; (c) mean correct response latency; (d) the number of anticipatory responses and (e) the number of perseverative responses.

The data of the experiments testing the effects of CPP in combination with different doses of either systemic or intra-mPFC drugs (8-OH-DPAT, M100907 and Ro60-0175) were analyzed by separate within-subjects two-way ANOVA with factors drug (8-OH-DPAT, M100907 or Ro60-0175) and CPP. The effects of Ro60-0175 and VAR-ITI were also analysed by a with-in subject two-way ANOVA with factors Ro60-0175 and ITI. The effects of WAY100635 plus 8-OH-DPAT on the performance of CPP injected rats were analysed by a with-in subjects two-way ANOVA with factors WAY100635 and 8-OH-DPAT. The means of the individual treatment combinations were compared between them by Tukey's HDS test.

The motor activity data were analysed by ANOVA and the means of individual treatments compared between them by Tukey's HDS test.

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Section 1.   Effects of systemic M100907 on CPP-induced attentional performance deficit and motor activity

Several studies show functional interactions between 5-HT$_{2A}$ receptors and glutamate neurotransmission in the mPFC and had been reviewed in the Introduction. Notably, in rats performing a 5-CSRT task capable of indexing attentional functioning and executive control, agonists at 5-HT$_{2A}$ receptors have been shown to impair both attentional functioning and inhibitory response control (Carli and Samanin, 1992; Koskinen et al., 2000). Conversely the 5-HT$_{2A}$ receptor antagonists such as M100907 and ketanserin improved rats' attentional performance and decreased anticipatory "impulsive" responding in the same task (Winstanley et al., 2003b; Passetti et al., 2003a).

The 5-HT$_{2A}$ antagonists have been shown to reduce almost all behavioural deficits induced by NMDA antagonists. However, in the 5-CSRT task the interaction between 5-HT$_{2A}$ and NMDA receptor mechanisms appears to be involved in impulsivity but not perseveration. Systemic NMDA receptor antagonists enhance anticipatory and perseverative responding in the 5-CSRT task but have inconsistent effects on accuracy (Higgins et al., 2003b; Le Pen et al., 2003). M100907 reversed impulsivity but not perseverative over-responding induced by systemic administration of the NMDA receptor antagonists, dizocilpine and Ro 63-1908 (Higgins et al., 2003a).

The results presented in Chapter 3 show that the attentional performance of rats performing the 5-CSRT task is profoundly affected by blockade of NMDA receptors in the mPFC; accuracy of attention is impaired and executive control lost as shown by "impulsivity" and "compulsive" perseveration. This shows that the effects of

central injection of a competitive NMDA receptor antagonist CPP into the mPFC are qualitatively similar to those observed after excitotoxic lesions of the mPFC.

The present experiments investigated the contribution of 5-HT$_{2A}$ receptors to CPP-induced impairment in attentional performance using a selective antagonist at these receptors such as M100907, which has 300 times more affinity for 5-HT$_{2A}$ receptors than other receptor subtypes including 5-HT$_{2C}$ and α-1 adrenergic-receptors (Kehne et al., 1996). Several doses of M100907 were administered systemically in rats, which were given microinjections of vehicle or CPP into the mPFC.

RESULTS

- *Systemic injection of a 5-HT_{2A} receptor antagonist, M100907, reduced the CPP-induced impairments in accuracy and anticipatory but not perseverative responding*

Fig. 1.1A shows how the effects of 50 ng/µL CPP in the mPFC on accuracy were modified by the selective 5-HT_{2A} receptor antagonist, M100907 administered subcutaneously. Doses of 10 and 40 µg/kg M100907 by themselves had no effect on accuracy but dose-dependently prevented the effects of CPP on the percentage of correct responses (M100907 x CPP, $F_{(2,40)}=6.1$, P=0.005; M100907, $F_{(2,40)}=2.9$, P=0.07; CPP, $F_{(1,40)}=13.5$, P=0.0007). M100907 10 µg/kg significantly reduced (P<0.05; Tukey's test) and 40 µg/kg completely abolished the accuracy impairment induced by CPP (P<0.05 Tukey's test).

The CPP-induced increase in anticipatory responses (Fig. 1.1B) was completely abolished by 10 and 40 µg/kg of M100907 (M100907 x CPP, $F_{(2,40)}$ =6.8, P=0.003; M100907, $F_{(2,40)}$ =11.4, P=0.0001; CPP, $F_{(1,40)}$ =27.8, P=0.0001). The number of perseverative responses shown in Fig. 1.1C was reduced by M100907 (both doses, P<0.05, Tukey's test), however, M100907 did not affect CPP-induced perseverative over-responding (M100907 x CPP, $F_{(2,40)}=0.06$, P>0.05; M100907, $F_{(2,40)}=0.8$, P=0.47; CPP, $F_{(1,40)}=17.3$, P=0.0002).

Although M100907 tended to decrease anticipatory responses when the rats received vehicle in the mPFC this effect was not statistically significant due to an already low number of anticipatory responses at baseline task conditions. On one occasion the effects of M100907 were determined in task condition of increased ITI. Increasing the ITI from 5 s to 7 s resulted in an overall increase in anticipatory responses (ITI 5 s, vehicle = 3.3 ± 1.4; ITI 7 s, vehicle = 31.5 ± 5.2; P<0.05 Student's t-test).

Anticipatory responses significantly decreased with M100907 ($F_{(2,10)}=15.1$, $P<0.001$)(Table 1.1).
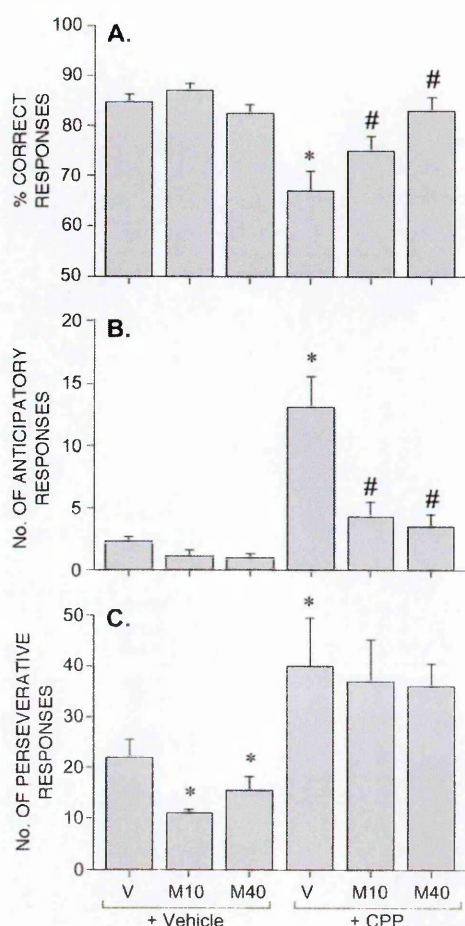
Figure 1.1
Effects of M100907 alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat was injected subcutaneously with vehicle (V), 10 or 40 μg/kg M100907 (M) 20 min before 1 μL vehicle (V) or 50 ng/μL CPP into the mPFC, 10 min before the test session. CPP and M100907 singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 9 rats. * $P<0.05$ vs. V+V; # $P<0.05$ vs. V+CPP; (Tukey's test)

Table 1.1. Effect of M100907 on anticipatory responses in condition of increased ITI (7 sec)

TREATMENT	ANTICIPATORY RESPONSES
VEH	31.5 ± 5.2
M 10	11.8 ± 2.6 *
M 40	5.8 ± 1.2 *

Each value is the mean ± SEM of 9 rats. M100907, 10 (M 10) and 40 μg/kg (M 40), or vehicle (2 mL/kg) was injected subcutaneously 30 min before the test. * $P < 0.05$ vs. VEH (Dunnett's t test)

- *M100907 had additive effects on the CPP-induced omissions and correct response latency*

As shown in Table 1.2, the combination of CPP plus 40 μg/kg M100907 had additive effects on omissions (M100907 x CPP, $F_{(2,40)}=0.87$, P>0.05; M100907, $F_{(2,40)}=6.4$, P=0.004; CPP, $F_{(1,40)}=43.0$, P=0.0001) and correct response latency (M100907 x CPP, $F_{(2,40)}=0.5$, P>0.05; M100907, $F_{(2,40)}=9.8$, P=0.0003; CPP, $F_{(1,40)}=25.7$ P=0.0001).

Table 1.2. Effects of systemic M100907 and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	7.9 ± 1.6	0.51 ± 0.03
M 10 + VEH	15.2 ± 3.5 *	0.67 ± 0.06 *
M 40 + VEH	15.6 ± 3.4 *	0.62 ± 0.04 *
VEH + CPP	22.8 ± 3.9 *	0.67 ± 0.03 *
M 10 + CPP	25.3 ± 4.2 °	0.84 ± 0.08
M 40 + CPP	35.8 ± 3.5 +	0.85 ± 0.08 $

Each value is the mean ± SEM of 9 rats. M100907 at doses of 10 (M 10) and 40 μg/kg (M 40) were injected subcutaneously 20 min before bilateral injections of 1 μL vehicle (VEH) or 50 ng/μL CPP into the mPFC. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH; ° P < 0.05 vs. M 10+VEH; + P < 0.05 vs. VEH+CPP;
$ P < 0.05 vs. M 40+VEH; (Tukey's test).

- *M100907 reduced the motor hyperactivity induced by CPP*

Table 1.3 presents the effects of M100907 on CPP-induced motor hyperactivity. A two-way ANOVA on activity counts showed that 50 ng/μL of CPP injected into the mPFC significantly increased motor activity, as indicated by the significant main effect of CPP ($F_{(1,34)}=17.0$ P=0.0002). The main effect of M100907 was also significant ($F_{(2,34)}=11.9$ P=0.0001). However, the interaction between M100907 pretreatment and CPP was not significant ($F_{(2,34)}=2.4$ P=0.10). Tukey's HDS tests comparing the means of various individual treatments indicated both doses of

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M100907 reduced the motor activity of control rats (P<0.05) but that only 40 µg/kg

of M100907 significantly reduced the CPP-induced hyperactivity (P<0.05).

Table 1.3. Effect of M100907 on CPP-induced motor hyperactivity

TREATMENT	ACTIVITY COUNTS
VEH + VEH	159.6 ± 13.1
M 10 + VEH	89.3 ± 11.9*
M 40 + VEH	97.5 ± 23.0*
VEH + CPP	246.0 ± 33.6*
M 10 + CPP	191.0 ± 13.7
M 40 + CPP	114.4 ± 15.5#

Each value is the mean ± SEM of 6-7 rats. Motor activity is expressed as the total number of activity counts measured in the first 30 min of testing. CPP 50 ng/µL or vehicle (VEH) (1 µL) was injected bilaterally into the mPFC 10 min before the test session. M100907, 10 (M 10) and 40 µg/kg (M 40), or vehicle (2 mL/kg) was injected subcutaneously 20 min before CPP.
* $P < 0.05$ vs. VEH + VEH ; # $P < 0.05$ vs. VEH + CPP (Tukey's test)

Section 1.   SUMMARY OF THE RESULTS

	Correct (%)	Anticipatory responses	Perseverative responses	Omissions (%)	Latency Correct	Motor activity
CPP	⇓	⇑	⇑	⇑	⇑	⇑
plus **M100907** systemic	0	0	=	+	+	0

⇓ decrease; ⇑ increase; + additive effects; 0 reversal; = no effect.

DISCUSSION

These experiments show that M100907, a selective 5-HT$_{2A}$ receptor antagonist

(Kehne et al., 1996), injected subcutaneously at 10 and 40 µg/kg, had no effect on

accuracy (% correct responses) but dose-dependently prevented the impairment

induced by intra-mPFC 50 ng/µL CPP. The dose of 10 µg/kg M100907 already

completely abolished CPP-induced anticipatory responding but perseverative over-

responding was not affected by any dose. Both doses of M100907 decreased motor

activity whereas 40 but not 10 µg/kg M100907 reversed CPP-induced motor

hyperactivity.

Despite the profound impairment in attentional performance induced by blockade of

NMDA receptors in the mPFC, the selective and potent 5-HT$_{2A}$ antagonist M100907

administered systemically dose-dependently reversed the accuracy impairment

induced by CPP. Systemic M100907 alone had no effect on accuracy in a 5-CSRT

task (present result; (Le Pen et al., 2003; Winstanley et al., 2003b) whereas when

injected into the mPFC it boasted accuracy (Winstanley et al., 2003b). This may be

due to opposite effects of prefronto-cortical and sub-cortical 5-HT$_{2A}$ receptor

blockade when the drug is administered by the systemic route. Previous work has

shown that 5-HT lesion of the DR nucleus improves attentional functioning

(Harrison et al., 1997b). This 5-HT depletion, restricted to certain forebrain areas

such as the cortex and striatum, presumably lowers 5-HT neurotransmission at all 5-

HT receptor subtypes within the fronto-cortico-striatal circuitry but, as shown by

Winstanley et al. (Winstanley et al., 2003b) blockade of 5-HT$_{2A}$ receptors in the

mPFC has effects similar to 5-HT lesions of the DR nucleus. On the other hand,

stimulation of 5-HT$_{2A}$ receptors by DOI had no effect on accuracy (Koskinen et al.,

2000). These findings suggest that serotonin, through 5-HT$_{2A}$ receptors, exerts a

tonic control on attentional functioning, so reducing serotonergic function at 5-HT$_{2A}$ receptors might help preserve attentional selectivity.

M100907 added its effects on the rate of omissions and correct response latencies to those of CPP, suggestive of some effects on motivation or motor activity. However, combined treatment did not cause a general disruption of performance and the majority of rats completed 100 trials within the allotted time (30 min). Although M100907 by itself reduced motor activity, thus supporting the interpretation that the increase in omissions and response latency might reflect motor factors, it completely abolished CPP-induced hyperactivity. As a whole, these data again suggest that the effects of M100907 and CPP on omissions and correct response latency cannot be explained in terms of a simple change in motor activity.

That the effects of CPP on attentional functioning may be dissociated from its effects on inhibitory response control is further suggested by the fact that 10 µg/kg M100907, a dose that only partially counteracted CPP's effects on accuracy, completely abolished the CPP-induced increase in anticipatory responses. Although in our study M100907 tended to reduce the anticipatory responses of rats performing under control conditions, the effect was not statistically significant, probably because of the small number of anticipatory responses by controls. However, we found that 10 and 40 µg/kg M100907 significantly reduced anticipatory responding when the ITI was increased from 5 s to 7 s, thus allowing more anticipatory responses.

The effects of M100907 on CPP-induced anticipatory over-responding are similar to those reported recently by Higgins et al. showing that M100907, although at doses ten times those used in the present study, reversed the effects of the non-competitive NMDA receptor antagonist dizocilpine and an NR2B-selective NMDA receptor antagonist Ro 63-1908 on anticipatory responding in the 5-CSRT task (Higgins et al.,

2003b). It is interesting that the NMDA antagonists increased the release of 5-HT in the mPFC (Martin et al., 1998a) and that poor inhibitory response control in a 5-CSRT task, measured by anticipatory responses, was associated with high 5-HT turnover (Puumala and Sirvio, 1998) or release in the mPFC (Dalley et al., 2002). Consistent with these findings is that stimulation of 5-HT$_{2A}$ receptors by a variety of non-selective 5-HT$_{2A}$ agonists increased while 5-HT$_{2A}$ receptor antagonists reduced anticipatory responses (Carli and Samanin, 1992; Koskinen et al., 2000; Koskinen and Sirvio, 2001; Winstanley et al., 2003b; Passetti et al., 2003a).

Therefore, over-activation of 5-HT$_{2A}$ receptors in the mPFC as a consequence of elevated 5-HT release in this cortical area may be an important mechanism that increases active responding in anticipation of reward. However, these findings challenge the view that loss of response control is necessarily mediated by diminished 5-HT function, since global forebrain 5-HT depletion consistently results in enhanced impulsivity in the rat (Harrison et al., 1997a). This apparent discrepancy may be explained by 5-HT exerting tonic inhibition on impulsivity through 5-HT$_{2C}$ receptors since blocking them greatly increased anticipatory responding in a 5-CSRT task (Higgins et al., 2003a; Winstanley et al., 2004b). Thus 5-HT, probably through opposite action on 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, contributes to the mechanisms responsible for preventing the disruptive consequences of loss of inhibitory response control on attentional performance.

It is interesting that M100907 did not prevent the compulsive perseveration induced by CPP. This implies that the mechanisms of executive control impaired in perseveration may be different from those involved in anticipatory responding. Hyperactivity elicited by CPP injected into the mPFC was reduced by M100907 at the dose of 40 µg/kg. This dose is similar to the ED$_{50}$ of 30 µg/kg reported to block

dizocilpine-induced hyperactivity in rats (Higgins et al., 2003a). However, in contrast to published results we found that M100907 reduced spontaneous motor activity at doses 300 times lower than those previously reported in mice (Martin et al., 1997).

The present experiments provide evidence that some aspects of inhibitory response control such as anticipatory and perseverative responding may be differentiated at the level of 5-HT$_{2A}$ receptor function. Therefore, it could be concluded that 5-HT$_{2A}$ receptor function is relevant to processes that permit appropriate response selection and attentional selectivity in the face of interference induced by dysfunctional glutamate transmission in the prefrontal-cortex.

These findings present an interesting analogy with those reported after the mGlu$_{2/3}$ receptor agonist, LY379268 (Chapter 4). Both systemic LY379268 and M100907 abolished the CPP-induced deficit in attentional accuracy and impulsivity. M100907 at doses active in behavioural experiments prevented CPP-induced increase in extracellular glutamate (Chapter 6) and LY379268 was reported to reduce the increase in glutamate efflux induced by NMDA antagonists. It is interesting however that neither M100907 nor LY379268 prevented the compulsive perseveration induced by CPP. This may suggest that the *hyperactive* glutamatergic neurotransmission, which to some extent may underlie deficits in accuracy and in aspects of inhibitory response control such as those measured by anticipatory responses are not sufficient to account for compulsive perseveration.

Section 2. **Effects of stimulation of 5-HT$_{2C}$ receptors on CPP-induced attentional performance deficits**

There are several behavioural responses that have been associated with activation of central 5-HT$_{2C}$ receptors. These include hypolocomotion, hypophagia, anxiety, penile erection, sleep and hyperthermia (Koek et al., 1992; Barnes and Sharp, 1999; Frank et al., 2002). Recent studies using relatively selective agonists and antagonists have reported that activation of 5-HT$_{2C}$ receptor by Ro60-0175 inhibit the rewarding and locomotor effects of cocaine (Grottick et al., 2000), nicotine (Grottick et al., 2001) and stress-induced DA release in the mPFC (Pozzi et al., 2002) while their blockade by SB242084 enhance the behavioural effects of cocaine (Fletcher et al., 2002), hyperactivity induced by NMDA receptor antagonists (Hutson et al., 2000), increase DA release (Millan et al., 1998) and potentiate the effects of NMDA receptor antagonists on DA release (Hutson et al., 2000). Interestingly, blockade of 5-HT$_{2A}$ receptors attenuated the effects of cocaine (Fletcher et al., 2002), hyperlocomotion induced by NMDA receptor antagonists (Martin et al., 1997) and stress-induced DA release (Pehek et al., 2005) while activation of 5-HT$_{2A}$ receptors increased DA release (Gobert and Millan, 1999). In rats performing the 5-CSRT task, activation of 5-HT$_{2A}$ receptors by DOI increased and their blockade reduced "impulsive" (anticipatory) responding (Carli and Samanin, 1992; Koskinen et al., 2000; Koskinen and Sirvio, 2001; Higgins et al., 2003a; Winstanley et al., 2003b; Passetti et al., 2003a; Winstanley et al., 2004b). By contrast blockade of 5-HT$_{2C}$ receptors greatly increased anticipatory responding in this task (Winstanley et al., 2004b) and had additive effects on dizocilpine-induced anticipatory over-responding

(Higgins et al., 2003a). Together, these findings suggest that the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors exert functionally opposing roles.

The 5-HT$_{2C}$ receptor agonists such as metachlorophenylpiperazine (mCPP) had no effect on accuracy but increased omissions and decreased the speed to respond correctly and to collect the earned food reward in a 5-CSRT task (Carli and Samanin, 1992). These effects were suggested to result from changes in motivation and general arousal, as they were similar to pre-feeding (Carli and Samanin, 1992). Correspondingly, blockade of 5-HT$_{2C}$ receptors by SB242084 did not affect accuracy but speeded up correct responding (Higgins et al., 2003a; Winstanley et al., 2004b).

In addition, *in-vitro* studies have implicated the 5-HT$_{2C}$ receptors in the modulation of glutamate NMDA and GABA$_A$ receptor transmission (Huidobro-Toro et al., 1996; Maura et al., 2000).

Therefore, it was of interest to examine whether stimulation of 5-HT$_{2C}$ receptors could have effects on CPP-induced deficits similar to those reported after blockade of the 5-HT$_{2A}$ receptor. Ro60-0175 is a potent 5-HT$_{2C}$ receptor agonist, with approximately 10-fold higher affinity at the human 5-HT$_{2C}$ over the 5-HT$_{2A}$ receptors *in-vitro* (Porter et al., 1999). This selectivity was confirmed *in-vivo* for doses in a range between 0.3 and 3 mg/kg (Millan et al., 1997; Clifton et al., 2000; Kennett et al., 2000). The present experiments investigated first, the ability of Ro60-0175 (0.3 mg/kg) to decrease impulsivity under task conditions capable to increase anticipatory responding (VAT-ITI). Second, the effects of Ro60-0175 (0.03 mg/kg) on CPP-induced deficits in the 5-CSRT task were examined.

RESULTS

- *Systemic administration of a 5-HT$_{2C}$ receptor agonist, Ro60-0175, decreased anticipatory responding of rats performing the 5-CSRT task with variable inter-trial-interval (VAR-ITI)*

Vehicle (2 mL/kg) or a dose of 0.3 mg/kg Ro60-0175 was administered to surgically naive rats (n=10). These rats were not used in subsequent experiments examining the effects of systemic Ro60-0175 on CPP-induced performance deficit. Fig. 2.1 illustrates the effects of VAR-ITI and 0.3 mg/kg Ro 60-0175 on accuracy (A) and anticipatory (B) and perseverative (C) responses.
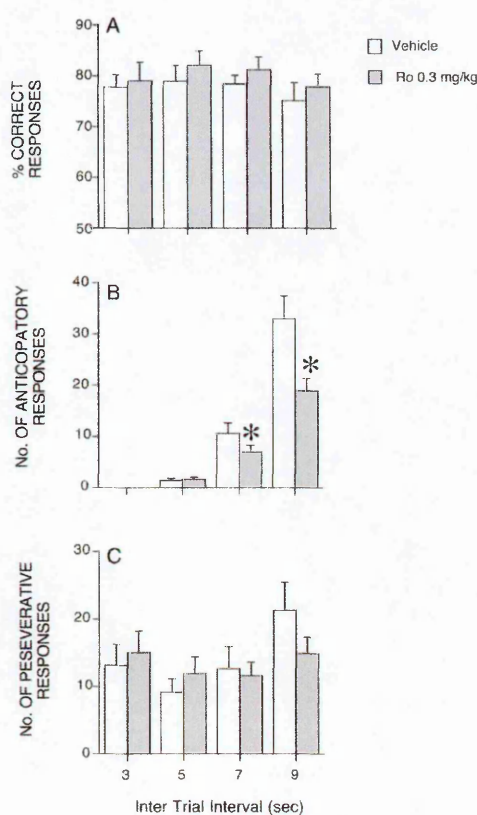
Figure 2.1
Effects of systemic injections of Ro60-0175 on %correct responses (A), anticipatory responses (B) and perseverative responses (C) of rats performing a variable inter-trial interval (VAR-ITI) version of a 5-CSRT task. Vehicle (2 ml/kg) or 0.3 mg/kg Ro60-0175 were injected subcutaneously 10 min before the test sessions. In this version the 5-CSRT task had 200 trials and the stimulus presentations were made unpredictable by intermixing the ITIs of different durations (3, 5, 7 and 9 sec). The Ro60-0175 or vehicle were administered at least 48h apart, according to a Latin-square design. Each value is the mean ± S.E.M. of 11 rats.
*P<0.05 vs. Vehicle (Tukey's test)

Intermixing the ITI of varying durations had no effect on accuracy (ITI, $F_{3,30}$=0.4 P=0.4). The effects of 0.3 mg/kg Ro60-0175 ($F_{1,9}$=2.46 P=0.15) and its interaction with ITI ($F_{3,54}$=0.5 P=0.5) were not significant (Fig. 2.1 A). With VAR-ITI anticipatory ($F_{3,30}$=79.2 P<0.001) and perseverative responses ($F_{3,30}$=6.6 P=0.002) increased. Anticipatory over-responding was decreased by Ro60-0175 (Ro60-0175 $F_{1,9}$=16.5 P=0.003; Ro60-0175xITI, $F_{3,54}$=6.4 P=0.009) at 7 and 9 sec ITIs (both P<0.05)(Fig. 2.1 B) whereas perseverative responding was not affected (Ro60-0175, $F_{1,9}$=0.3 P=0.6; Ro60-0175xITI, $F_{3,54}$=1.8 P=0.15) (Fig. 2.1 C).

- *Ro60-175 had no effect on omissions or correct response latency*

As shown in Table 2.1, the percentage of omissions ($F_{3,30}$=9.4 P=0.002) and correct response latency ($F_{3,30}$=10.8 P=0.0001) decreased with increasing duration of the ITI but were not affected by Ro60-0175 (omissions $F_{1,9}$=2.9 P=0.1; correct response latencies $F_{1,9}$=4.4 P=0.07).

Table 2.1. Effects of systemic Ro60-0175 on omissions and correct response latency of a 5-CSRT task with variable inter-trial-interval (VAR-ITI)

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
ITI=3 sec		
VEH	27.9 ± 5.0	0.89 ± 0.06
Ro 0.3	27.5 ± 6.4	0.95 ± 0.07
ITI=5 sec		
VEH	14.8 ± 2.6*	0.74 ± 0.03
Ro 0.3	15.2 ± 3.0#	0.74 ± 0.05
ITI=7 sec		
VEH	11.4 ± 2.3*	0.63 ± 0.04
Ro 0.3	12.8 ± 2.5#	0.78 ± 0.05
ITI=9 sec		
VEH	18.5 ± 4.4	0.72 ± 0.03
Ro 0.3	15.3 ± 2.3	0.68 ± 0.05

Each value is the mean ± SEM of 11 rats. Vehicle (2mL/kg) or 0.3 mg/kg RO60-0175 were injected subcutaneously 10 min before the test sessions. The vehicle or Ro60-0175 were administered at least 48 h apart, according to a Latin-square design.
* P<0.05 vs. VEH (ITI=3 sec); # P<0.05 vs. Ro 0.3 (ITI=3 sec); Tukey's test

- *Systemic Ro60-0175 reduced the CPP-induced impairments in accuracy and anticipatory but not perseverative over-responding*

As shown in Figure 2.2 A, the reduced percentage of correct responses of rats injected with CPP was completely prevented by 0.03 mg/kg Ro60-0175. In fact, two-way repeated measure ANOVA showed a significant interaction between Ro60-0175 and CPP ($F_{1,21}=30.5$ P<0.0001) and a significant effect of CPP, ($F_{1,21}=64.3$ P<0.0001) and Ro60-0175 ($F_{1,21}=36.6$ P<0.0001). Post-hoc Tukey's HDS test showed that compared to vehicle controls CPP significantly reduced the percentage of correct responses (P<0.05) and that CPP-injected rats pre-treated with Ro60-0175 had a significantly higher percentage of correct responses (P<0.05) compared to CPP plus vehicle.

Statistical analysis of anticipatory responses reported that CPP enhanced, whereas Ro60-0175 decreased the number of anticipatory responses (Ro60-0175 x CPP, $F_{1,21}=2.3$ P=0.1; Ro60-0175, $F_{1,21}=4.5$ P=0.04; CPP, $F_{1,21}=4.6$ P=0.03). Fig. 2.2 B shows multiple comparisons of treatments group means by Tukey's test. CPP increased the number of anticipatory (P<0.05) responses and Ro60-0175 decreased anticipatory responses of CPP injected rats (P<0.05). The increased number of perseverative responses induced by CPP (Fig. 2.2C) was not affected by Ro60-0175 (Ro60-0175 x CPP, $F_{1,21}=0.2$ P=0.6; Ro60-0175, $F_{1,21}=0.3$ P=0.6; CPP, $F_{1,21}=24.0$ P<0.0001).

The dose of 0.03 mg/kg of Ro60-0175 employed in these experiments is one tenth that used in the experiments with VAR-ITI schedule. However, in this group of rats 0.3 mg/kg Ro60-0175 had sedative effects and some rats made many omissions while others stopped responding. In fact, in some experimental conditions 0.3 mg/kg was reported to cause sedation (Kennett et al. 2000). Thus the dose of Ro60-0175 had to

be drastically reduced. I have tested the effects of 0.1 mg/kg Ro60-0175 in combination with CPP but these data are unreliable and are not presented. These animals made a great number of omissions and completed only few trials.
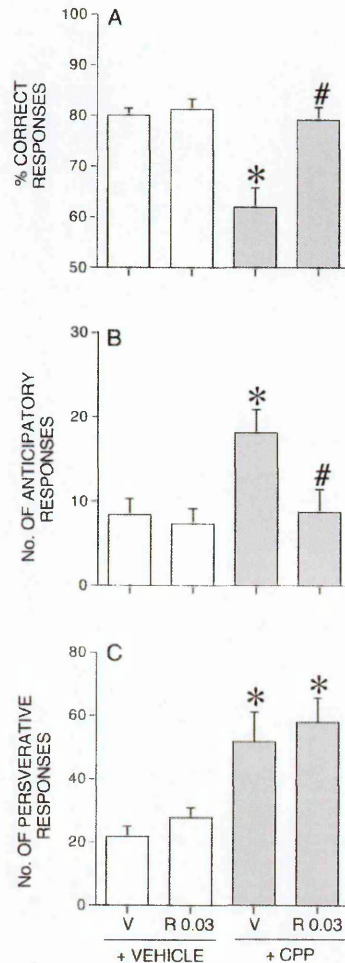
Figure 2.2
Effects of Ro60-0175 alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat was injected subcutaneously with vehicle (V) or 0.03 mg/kg Ro60-0175 (R) 20 min before 1 µL vehicle (+VEHICLE) or 50 ng/µL CPP (+CPP) into the mPFC. Ten min later the rats started the test session. CPP and Ro60-0175 singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 8 rats. * P<0.05 vs. V(+VEHICLE); # P<0.05 vs. V(+CPP); (Tukey's test)

- ***Ro60-0175 had no effects on the CPP-induced increase in omissions and correct response latency***

The effects of Ro60-0175 and CPP alone and of their combination on omissions and correct response latencies are shown in Table 2.2. CPP increased both omissions and

correct response latencies whereas Ro 60-0175 had no effect by itself and did not change the effects of CPP (omissions: Ro 60-0175 x CPP, $F_{1,21}=0.2$ P=0.6; Ro 60-0175, $F_{1,21}=0.4$ P=0.5; CPP, $F_{1,21}=68.3$ P<0.0001; correct response latency: Ro 60-0175 x CPP, $F_{1,21}=0.1$ P=0.7; Ro 60-0175, $F_{1,21}=0.02$ P=0.9; CPP, $F_{1,21}=33.2$ P<0.0001).

Table 2.2. Effects of systemic RO60-0175 and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	5.8 ± 0.7	0.50 ± 0.03
Ro 0.03 + VEH	6.6 ± 1.5	0.52 ± 0.02
VEH + CPP	$43.0 \pm 6.1*$	$1.10 \pm 0.13 *$
Ro 0.03 + CPP	48.1 ± 7.7	1.05 ± 0.14

Each value is the mean ± SEM of 8 rats. Vehicle (2 mL/kg) or 0.03 mg/kg (Ro 0.03) were injected subcutaneously 20 min before bilateral injections of 1 µL vehicle (VEH) or 50 ng/µL CPP into the mPFC.   Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH (Tukey's test).

Section 2.   SUMMARY OF RESULTS

	Correct (%)	Anticipatory responses	Perseverative responses	Omissions (%)	Latency Correct
VAR-ITI	=	⇑	=	=	=
plus Ro60-175 systemic	=	⇓	=	=	=
CPP	⇓	⇑	⇑	⇑	⇑
plus Ro60-175 systemic	O	O	=	=	=

⇓ decrease; ⇑ increase; = no effect; O reversal.

DISCUSSION

Despite the intensity of changes induced by CPP, the 5-HT$_{2C}$ agonist Ro60-0175 was able to normalise impulsivity (by reducing anticipatory responding). However, Ro60-0175 did not normalise all aspects of response control since, perseverative responding was not affected by Ro60-0175. Evidence of improved attentional functioning was demonstrated by the fact that Ro60-0175 increased correct responses of CPP-injected rats. When tested alone in the baseline version of a 5-CSRT task, Ro60-0175 had no effects on any measure of task performance. However, in conditions of increased demands on response inhibition such as in a 5-CSRT task with ITIs of variable durations, Ro 60-0175 improved performance by reducing impulsivity. The data show that stimulation of 5-HT$_{2C}$ receptors by Ro60-0175 resembles that of blockade of 5-HT$_{2A}$ receptors by M100907.

In the VAR-ITI schedule, the stimuli are made temporally unpredictable. The less predictable VAR-ITI schedule means that the rat cannot rely on automatic processing to control orientation to the location of stimuli at a particular time and they have to monitor their readiness to respond on a continuous basis. This manipulation may increase alertness or arousal (Robbins, 2002) and increase attentional demands to the timing of the stimulus presentation. Under these condition the accuracy of animals was not affected but the anticipatory and perseverative responses were increased when the animals had to wait longer than the usual 5 s for the stimulus presentation. In this condition 0.3 mg/kg Ro60-0175 had no effect on accuracy but selectively decreased anticipatory responses. The fact that Ro60-0175 by itself had no effect on accuracy confirms previous findings with the less selective 5-HT$_{2C}$ agonist mCPP (Carli and Samanin, 1992). However, it is unlikely that the effects of Ro60-0175 on anticipatory responding may be accounted for by Ro60-0175 blunting motivation or

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having sedative effects since omissions, correct response latencies and perseverative responses were not affected.

Previous studies have strongly implicated the 5-HT system in impulsivity (General Introduction). Global reduction of 5-HT neurotransmission increases impulsivity (Harrison et al., 1997a). In contrast, decreasing 5-HT function at 5-HT_{1A}, 5-HT_{2A} and 5-HT_3 receptors reduces impulsivity induced by long ITI, lesions or drugs (Muir et al., 1995; Balducci et al., 2003; Higgins et al., 2003a) (previous section showing the effects of M100907). The present findings together with observations that blockade of 5-HT_{2C} receptors by SB242084 increases anticipatory responding (Higgins et al., 2003a; Winstanley et al., 2004b), suggest that under normal circumstances some aspects of inhibitory response control such as impulsivity might be maintained by signalling through 5-HT_{2C} receptors.

Similarly to long ITI, increases in anticipatory responses are reported after systemic d-amphetamine (Cole and Robbins, 1987; Baunez and Robbins, 1999). The d-amphetamine-induced increase in impulsive responding in this task appears to be dependent on the ability of d-amphetamine to enhance DA transmission in the NAcc (Cole and Robbins, 1987, 1989). Evidence suggests that 5-HT_{2C} receptors are involved in the modulation of DA neurotransmission (Di Matteo et al., 2002). Activation of 5-HT_{2C} receptors inhibits the firing rate of DA neurons in the VTA through activation of GABA interneurons (Di Matteo et al., 2001; Di Matteo et al., 2002) and decreases DA release in the NAcc (Di Matteo et al., 2002) whereas blockade of 5-HT_{2C} receptors by SB242084 increases DA release in the PFC and NAcc (Millan et al., 1998; Gobert et al., 2000; Pozzi et al., 2002). In microdialysis studies Ro60-0175 had no effect on basal DA release but inhibited DA release in the PFC induced by immobilization stress (Pozzi et al., 2002). Thus, the interaction of

5-HT$_{2C}$ receptors with DA transmission may be relevant to how Ro60-0175 reversed the increase in anticipatory responding.

DA mechanisms have been implicated in the control of perseverative responses. In fact, perseverative responses in the 5-CSRT task are increased after systemic d-amphetamine and 6-OHDA lesions of the caudate nucleus (Baunez and Robbins, 1999). However, despite of its modulation of DA neurotransmission Ro60-0175 had no effect on perseverative responding.

This study shows that Ro60-0175 at a very low dose (0.03 mg/kg s.c.) improved accuracy and reduced anticipatory responses in CPP injected rats. Again perseverative over-responding induced by CPP was not affected. There is evidence that, under some circumstances, the constitutive activity of 5-HT$_{2C}$ receptor (Berg et al., 1999) may be enhanced (Gurevich et al., 2002) or reduced (Englander et al., 2005) thus increasing or reducing agonist affinity and potency. It could not be excluded that increased constitutive activity of the 5-HT$_{2C}$ receptor in CPP-injected rats might explain why such low doses had to be used.

The behavioural and neurochemical effects of NMDA receptor antagonists are further enhanced by 5-HT$_{2C}$ receptor antagonists (Hutson et al., 2000; Higgins et al., 2003a) and Higgins et al. (Higgins et al., 2003a) showed that SB242084 had additive effects while M100907 reduced dizocilpine-induced increase in anticipatory responding. This suggests an opposing role of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors on anticipatory responding. However, in the study by Higgins et al. (Higgins et al., 2003a) systemic administration of NMDA receptor antagonist, M100907 and SB242084 alone or in combination had no effect on accuracy. The experiments reported in this thesis show that CPP administered into the mPFC consistently impairs accuracy. The present results showing that blockade of 5-HT$_{2A}$ and

activation of 5-HT$_{2C}$ receptors abolished CPP-induced decrements in accuracy extend

the suggested opposing roles of these 5-HT receptors to attentional functioning.

Section 3. Effects of 8-OH-DPAT and M100907 injected into the mPFC on CPP-induced deficits

The anatomical localisation and functions of the $5-HT_{1A}$ and $5-HT_{2A}$ receptors have been reviewed in the Introduction and only the most important features will be presented here, briefly. The $5-HT_{1A}$ receptor subtype can be considered functionally antagonistic to the $5-HT_{2A}$ receptors as shown by electrophysiological and behavioural studies (Backus et al., 1990; Sharp et al., 1990; Ashby et al., 1994). Interestingly, the opposing action of $5-HT_{1A}$ and $5-HT_{2A}$ receptors in the mPFC has been extended to attentional functioning (accuracy) (Winstanley et al., 2003b) but not to processes controlling response inhibition. In fact, the $5-HT_{2A}$ but not $5-HT_{1A}$ receptors in the mPFC appear particularly involved in impulsivity (Koskinen et al., 2000; Winstanley et al., 2003b).

In addition, as shown in the previous section the $5-HT_{2A}$ receptor antagonist, M100907 administered systemically reduced the effects of intra-mPFC NMDA antagonists on attentional functioning and anticipatory but not perseverative responding, confirming the results obtained by Higgins et al. (Higgins et al., 2003a). However, the contribution of PFC $5-HT_{2A}$ receptors to the effects of systemic M100907 is not clear. The $5-HT_{1A}$ receptor partial agonists and full antagonists attenuated spatial learning and working memory deficits as well as psychotomimetic effects induced by NMDA receptor antagonists (Carli et al., 2000; Harder and Ridley, 2000; Wedzony et al., 2000; Carli et al., 2001). Whether $5-HT_{1A}$ agonists may reverse the NMDA receptor antagonists induced deficits in attentional performance is not known.

This investigation compared the contribution of mPFC 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor activity to various aspects of performance in the 5-CSRT task. Various doses of a 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (Peroutka, 1986; Hoyer et al., 1994) or M100907 (Kehne et al., 1996) were microinjected into the mPFC and their effects examined on attentional performance deficits induced by CPP (Lehmann et al., 1987) injected into the same area. As 8-OH-DPAT acts on both 5-HT$_{1A}$ and 5-HT$_7$ receptors (Hedlund et al., 2004) the involvement of 5-HT$_{1A}$ receptors in the effects of 8-OH-DPAT was examined by testing the ability of the selective 5-HT$_{1A}$ antagonist WAY100635 (Forster et al., 1995) to antagonize any effect of 8-OH-DPAT on performance.

RESULTS

- *The $5\text{-}HT_{1A}$ receptor agonist, 8-OH-DPAT, injected into the mPFC reduced the CPP-induced impairment in accuracy and perseverative but not anticipatory responding*

Fig. 3.1A shows that 8-OH-DPAT prevented the CPP-induced decrease in the percentage of correct responses (8-OH-DPAT x CPP, $F_{2,50}=3.6$, P=0.03; 8-OH-DPAT, $F_{2,50}=11.0$, P=0.0001; CPP, $F_{1,50}=47.8$, P<0.0001). Multiple comparison of the various treatment group means by Tukey's HDS test indicated that 30 and 100 ng/µL 8-OH-DPAT (both P<0.05) prevented the decrease in correct responses induced by CPP (P<0.05). By itself 8-OH-DPAT had no such effect (P>0.05).

The CPP-induced increase in the number of anticipatory responses (Fig. 3.1B) was not affected by 8-OH-DPAT (8-OH-DPAT x CPP, $F_{2,50}=1.5$, P=0.2; 8-OH-DPAT, $F_{2,50}=0.3$, P=0.7; CPP, $F_{1,50}=5.6$, P=0.02). By itself 8-OH-DPAT tended to increase anticipatory responses, but not significantly (P>0.05).

The CPP-induced perseverative over-responding (Fig. 3.1C) was significantly reduced by 8-OH-DPAT (8-OH-DPAT x CPP, $F_{2,50}=3.9$, P=0.02; 8-OH-DPAT, $F_{2,50}=1.0$, P=0.4; CPP, $F_{1,50}=16.5$, P=0.0002). Doses of 30 and 100 ng/µL 8-OH-DPAT by themselves had no effect on perseverative responses but dose-dependently lowered the effects of CPP (P<0.05).
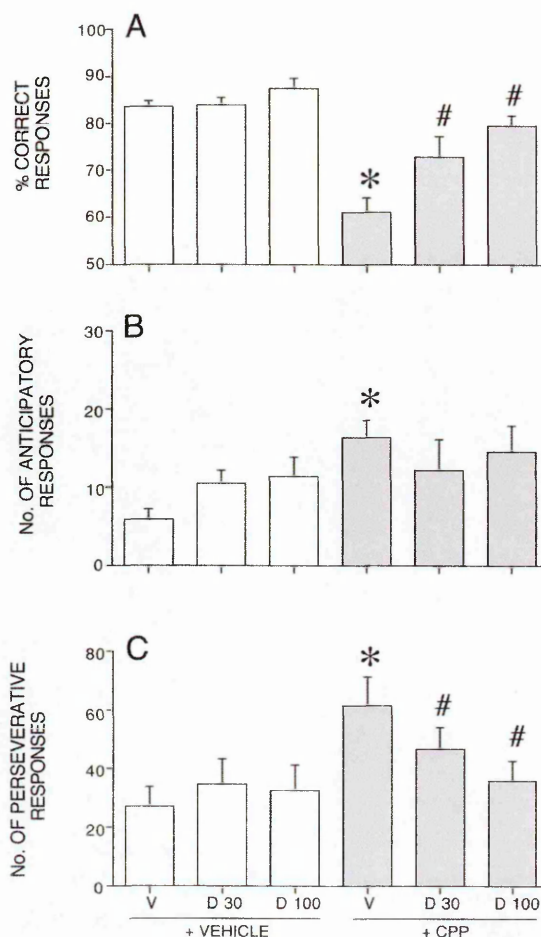
Figure 3.1.

The effects of 8-OH-DPAT and CPP alone and in combination on the percentage of correct responses (A), the number of anticipatory (B) and the number of perseverative (C) responses. Vehicle 1 μL (V) or 8-OH-DPAT at doses of 30 (D 30) and 100 ng/μL (D 100) were injected into the mPFC 5 min before bilateral injections of 1 μL vehicle (VEHICLE) or 50 ng/μL CPP (CPP) into the same area. Ten min later the rats started the test sessions. The various treatment combinations were administered at least 48 h apart, according to a Latin-square design. The histograms represent the mean ± SEM of 11 rats. *P < 0.05 vs. V (+ VEHICLE); # P < 0.05 vs. V (+ CPP) (Tukey's test).

- *8-OH-DPAT reduced the CPP-induced increases in omissions and correct*

  response latency

Table 3.1 shows the effects of 8-OH-DPAT on the CPP-induced increase in the proportion of omissions (8-OH-DPAT x CPP, $F_{2,50}=2.6$, P=0.08; 8-OH-DPAT, $F_{2,50}=3.5$, P=0.03; CPP, $F_{1,50}=30.5$, P=0.0001) and the mean latency to make a correct response (8-OH-DPAT x CPP, $F_{2,50}=1.9$, P=0.15; 8-OH-DPAT, $F_{2,50}=5.3$, P=0.008; CPP, $F_{1,50}=22.3$, P=0.0001). Further analysis by comparing individual treatment

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means indicated that by itself 8-OH-DPAT had no effect on omissions but reduced the effects of CPP on omissions, although only at 30 ng/µL (P<0.05). In the control condition 8-OH-DPAT speeded up correct responding, although only at 30 ng/µL (p<0.05). Similarly, the CPP-induced increases in correct response latency were reduced by 30 ng/µL (P<0.05) but not 100 ng/µL 8-OH-DPAT.

Table 3.1. Effects of 8-OH-DPAT, CPP and their combination on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	10.1 ± 2.2	0.61 ± 0.02
DPAT 30 + VEH	9.1 ± 1.5	0.54 ± 0.02 *
DPAT 100 + VEH	11.4 ± 2.5	0.56 ± 0.03
VEH + CPP	29.0 ± 4.9 *	0.93 ± 0.11 *
DPAT 30 + CPP	15.9 ± 3.9 #	0.67 ± 0.04 #
DPAT 100 + CPP	22.0 ± 4.5	0.72 ± 0.06

Each value is the mean ± SEM of 11 rats. 8-OH-DPAT at doses of 30 (DPAT 30) and 100 ng/µL (DPAT 100) were injected into the mPFC 5 min before bilateral injections of 1 µL vehicle (VEH) or 50 ng/µL CPP into the same area. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH; # P < 0.05 vs. VEH+CPP (Tukey's test).

- *Co-infused with 8-OH-DPAT into the mPFC the 5-HT$_{1A}$ receptor antagonist, WAY100635, abolished the effects of 8-OH-DPAT on accuracy and perseverative responding in CPP-injected rats*

I examined the effects of 30 and 100 ng/µL WAY100635 on performance of rats injected with vehicle (1 µL) or CPP (50 ng/µL) into the mPFC. WAY100635 had no effects on any measure of performance in rats receiving vehicle (1 µL) into the mPFC (Data not shown). However, 100 ng/µL WAY100635 interfered with the performance of rats given CPP (50 ng/µL); they stopped performing and made a large proportion of omissions (data not shown). Thus, 30 ng/µL WAY100635 was

selected to examine the selectivity of 8-OH-DPAT's effects on CPP-induced performance deficit.

All animals received bilateral injections of 50 ng/μL CPP into the mPFC. This experiment showed that 8-OH-DPAT increased accuracy ($F_{1,21}$=27.7, P<0.0001) and that this effect was blocked by WAY100635 (WAY100635 x 8-OH-DPAT; $F_{1,21}$= 4.6, P=0.04; WAY100635, $F_{1,21}$=12.6, P=0.002). Multiple comparison of the treatment group means by Tukey's HDS test is illustrated in Table 3.2; after 100 ng/μL 8-OH-DPAT in the mPFC rats made a higher proportion of correct responses than after vehicle (P<0.05) thus replicating in a new group of rats the results of the experiment presented in Fig. 3.1A. WAY100635 30 ng/μL abolished the effect of 100 ng/μL 8-OH-DPAT on the percentage of correct responses since rats receiving both drugs made fewer correct responses than animals receiving vehicle with 8-OH-DPAT (P<0.05). Similarly WAY100635 blocked the effects of 8-OH-DPAT on the number of perseverative responses (WAY100635 x 8-OH-DPAT, $F_{1,21}$=7.6, P=0.05).

Table 3.2. Effects of intra-cortical co-infusion of WAY100635 plus 8-OH-DPAT in rats receiving CPP in the mPFC

TREATMENT	%CORRECT	PERSEVERATIVE
VEH + VEH	62.1 ± 4.6	62.1 ± 4.3
WAY 30 + VEH	58.1 ± 1.8	53.7 ± 6.8
VEH + DPAT 100	83.1 ± 3.1*	41.0 ± 7.3*
WAY 30 + DPAT 100	67.0 ± 3.9#	57.5 ± 4.9#

Each value is the mean ± SEM of 8 rats. All animals received 50 ng/μL CPP into the mPFC. A solution containing WAY100635 30 ng/μL (WAY 30) plus 8-OH-DPAT 100 ng/μL (DPAT 100) was co-injected into the mPFC 5 min before CPP. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design. Abbreviations: The percentage of correct responses (%CORRECT); Number of perseverative responses (PERSEVERATIVE) * P < 0.05 vs. VEH + VEH; # P < 0.05 vs. VEH + DPAT 100 (Tukey's test).

Table 3.2 shows that after 8-OH-DPAT rats made fewer perseverative responses than after vehicle (P<0.05) whereas when WAY100635 was added to 8-OH-DPAT, rats made more perseverative responses than those receiving only 8-OH-DPAT (P<0.05). Other measures of rats' performance such as anticipatory responses, proportion of omissions and correct response latencies were not affected by 8-OH-DPAT, WAY100635 or the combination (data not shown) and are not commented further.

- *Injected into the mPFC the 5-HT$_{2A}$ receptor antagonist, M100907, reduced the CPP-induced impairments in accuracy and anticipatory but not perseverative responding*

Figure 3.2A shows that doses of 100 and 300 ng/μL M100907 by themselves had no effect on accuracy (% correct responses) but prevented the CPP-induced impairment in accuracy (M100907 x CPP, $F_{2,65}$=5.8, P=0.004; M100907, $F_{2,65}$=5.4, P=0.006; CPP, $F_{1,65}$=31.7, P<0.0001). Both doses of M100907 were equally potent in preventing the accuracy impairment induced by CPP (both P<0.05).
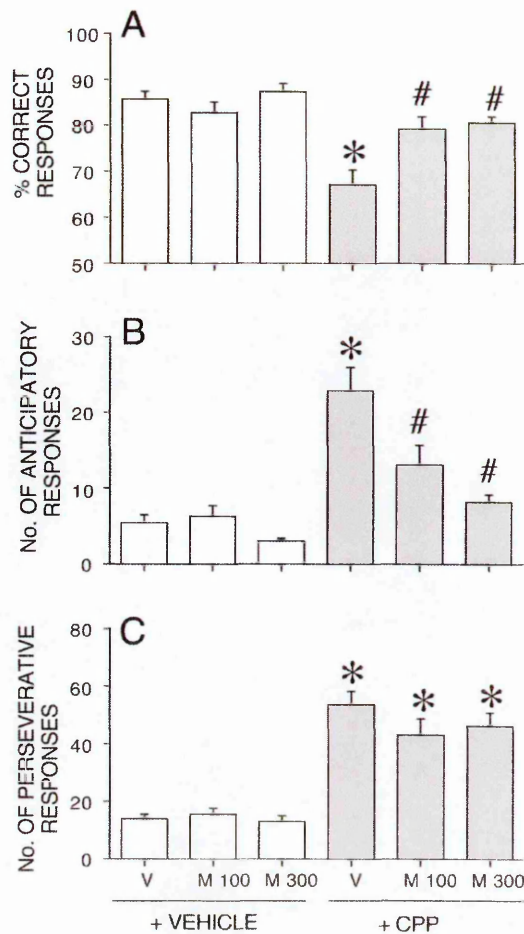
Figure 3.2.
The effects of M100907 and CPP alone and in combination on the percentage of correct responses (A), the number of anticipatory (B) and the number of perseverative (C) responses. Vehicle 1 μL (V) or M100907 at doses of 100 (M 100) and 300 ng/μL (M 300) were injected into the mPFC 5 min before bilateral injections of 1 μL vehicle (VEHICLE) or 50 ng/μL CPP (CPP) into the same area. Ten min later the rats started the test sessions. The various treatment combinations were administered at least 48 h apart, according to a Latin-square design. The histograms represent the mean ± SEM of 14 rats. * P < 0.05 vs. V (+ VEHICLE); # P < 0.05 vs. V (+ CPP) (Tukey's test).

The CPP-induced increase in anticipatory responses (Fig. 3.2B) was dose-dependently reduced by 100 and 300 ng/μL M100907 (M100907 x CPP, $F_{2,65}=7.9$, P=0.0009; M100907, $F_{2,65}=13.2$, P<0.0001; CPP, $F_{1,65}=52.2$, P<0.0001). In the control condition, 300 ng/μL M100907 tended to reduce anticipatory responses, but not significantly, possibly because the number of anticipatory responses was already low. Fig. 3.2C shows that M100907 had no effect by itself on perseverative

responses nor did it affect CPP-induced perseverative over-responding (M100907 x

CPP, $F_{2,65}=1.2$, P=0.3; M100907, $F_{2,65}=0.9$, P=0.4; CPP, $F_{1,65}=116.1$, P<0.0001).

- *M100907 had no effects on the CPP-induced increases in omissions and correct response latency*

As shown in Table 3.3, in the control condition M100907 did not affect omissions or

the speed of correct responding. The CPP-induced increases in omission (M100907 x

CPP, $F_{2,65}=0.6$, P=0.6; M100907, $F_{2,65}=1.4$, P=0.2; CPP, $F_{1,65}=32.9$, P<0.0001) and

mean latency to a correct response were unaffected by M100907 (M100907 x CPP,

$F_{2,65}=1.6$, P=0.2; M100907, $F_{2,65}=2.5$, P=0.08; CPP, $F_{1,65}=33.6$, P<0.0001).

Table 3.3. Effects of M100907, CPP and their combination on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	15.3 ± 2.4	0.58 ± 0.02
M 100 + VEH	11.6 ± 1.4	0.56 ± 0.02
M 300 + VEH	16.0 ± 2.4	0.63 ± 0.04
VEH + CPP	24.2 ± 3.7 *	0.74 ± 0.06 *
M 100 + CPP	25.1 ± 3.5	0.99 ± 0.11
M 300 + CPP	29.6 ± 4.1	0.98 ± 0.12

Each value is the mean ± SEM of 14 rats. M100907 at doses of 100 (M 100) and 300 ng/μL (M 300) were injected into the mPFC 5 min before bilateral injections of 1 μL vehicle (VEH) or 50 ng/μL CPP into the same area. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH (Tukey's test).

Section 3.   SUMMARY OF THE RESULTS

	Correct (%)	Anticipatory responses	Perseverative responses	Omissions (%)	Latency Correct
CPP	⇓	⇑	⇑	⇑	⇑
plus **8-OH-DPAT** mPFC	0	=	0	↓	↓
plus **M100907** mPFC	0	0	=	=	=

⇓ decrease; ⇑ increase; 0 reversal; = no effect.

↓ reversal only at certain doses

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DISCUSSION

This is the first study to demonstrate a dissociable contribution of serotonin 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in the mPFC to aspects of executive control such as impulsivity and compulsive perseveration. It also shows that these receptors exert opposite action on attentional functioning.

Like systemic M100907, intra-mPFC injections of 100 and 300 ng/μL M100907 abolished the deficit in attentional accuracy and anticipatory but not perseverative responding induced by 50 ng/μL CPP. Various behavioural deficits induced by NMDA antagonists have been associated with enhanced glutamate release in the mPFC (Moghaddam et al., 1997; Moghaddam and Adams, 1998) and present findings indicate that CPP in the mPFC increases glutamate efflux locally (Chapter 3) and that this was prevented by systemic or intra-mPFC M100907 (Chapter 6). Thus, 5-HT$_{2A}$ receptors in the mPFC play a major role in controlling CPP-induced glutamate release and aspects of attentional performance such as accuracy and impulsivity in a 5-CSRT task.

Impairments in attentional accuracy induced by CPP were completely abolished by 30 and 100 ng/μL 8-OH-DPAT injected into the mPFC. The 5-HT$_{1A}$ receptor subtype can be considered functionally antagonistic to the 5-HT$_{2A}$ receptors. They are highly co-localised (80%) in pyramidal neurons of the PFC (Santana et al., 2004) and electrophysiological studies have shown that M100907 potentiates 8-OH-DPAT suppression on firing rate (Ashby et al., 1994). In addition, 5-HT$_{2A}$ receptor antagonists ICI 180,809 and ritanserin potentiate the 5-HT syndrome produced by 8-OH-DPAT (Backus et al., 1990; Sharp et al., 1990) whereas 8-OH-DPAT inhibits head twitching behaviour induced by systemic DOI (Berendsen and Broekkamp, 1990; Darmani et al., 1990; Dursun and Handley, 1993) or microinjection into the

mPFC of (-) DOB, a congener of DOI and a $5\text{-HT}_{2A/2C}$ receptors agonist (Granhoff et al., 1992). Clearly, the opposition between the two 5-HT receptor subtypes suggests that the improvement produced by M100907 and 8-OH-DPAT on CPP-induced accuracy deficit may result from a functionally antagonistic activity of these receptors on a common intracellular mechanism.

Impairments in accuracy in the 5-CSRT task were reported after systemic 8-OH-DPAT, most likely due to activation of pre-synaptic 5-HT_{1A} receptors in the DR nucleus since they were blocked by selective 5-HT lesion and WAY100635 injected into the DR nucleus (Carli and Samanin, 2000). Other studies with systemic 8-OH-DPAT found improvements in accuracy but no effect on anticipatory responses (Winstanley et al., 2003b) while the selective 5-HT lesions of DR nucleus improved accuracy and increased anticipatory responding (Harrison et al., 1997b). Interestingly, opposite behavioural effects of 8-OH-DPAT were often reported depending on whether the drug was administered directly into the DR or into its projecting areas (Carli et al., 1995b; Warburton et al., 1997; Carli et al., 1998).

8-OH-DPAT but not M100907 had some additional effects on rats' attentional functioning but only at the low dose of 30 ng/μL; it speeded up correct response latencies and reduced CPP-induced omissions. Substantial evidence implicates the dopaminergic (DA) system in decision processes in this task (Robbins, 2002). The speeding up of correct responses and a decrease in omissions in a 5-CSRT task had been observed after systemic amphetamine and the dopamine D1 receptor agonist SKF 38393 in the mPFC (Granon et al., 2000; Robbins, 2002). Thus, the fact that intra-mPFC 8-OH-DPAT increases DA efflux in this cortical region (Sakaue et al., 2000) may have contributed to its effect on speed and omissions.

In contrast to the effects of M100907, injections of 8-OH-DPAT into the mPFC did

not have any effect on CPP-induced anticipatory responding. However, CPP-induced perseverative over-responding was significantly reduced by pre-treatment with 8-OH-DPAT. These results clearly demonstrate the selectivity of executive control processes and indicate that impulsivity and perseveration may be dissociated by 5-HT_{1A} and 5-HT_{2A} receptor mechanisms in the PFC. Evidently response inhibition operates independently for preparing responses and for monitoring performance, thus providing behavioural flexibility. This conclusion is generally consistent with emerging evidence of distinct neural systems in the control of "impulsive" behaviour, as, for example after lesions of the infralimbic (IL) prefrontocortical region and in "compulsive" behaviours associated with lesions of the prelimbic and orbitofrontal regions of the rat PFC (Chudasama and Muir, 2001; Passetti et al., 2002; Chudasama et al., 2003).

A selective 5-HT_{1A} receptor antagonist, WAY100635, blocked the effects of 8-OH-DPAT on CPP-induced accuracy deficits and perseverative over-responding suggesting that activation of 5-HT_{1A} receptors in the mPFC results in pharmacologically and behaviourally specific improvements in attentional performance.

It is interesting that the NMDA receptor antagonists either infused into the mPFC or injected systemically increase the release of 5-HT in the mPFC (Martin et al., 1998a). Impulsivity in a 5-CSRT task is positively associated with high 5-HT turnover (Puumala and Sirvio, 1998) or release in the mPFC (Dalley et al., 2002). Stimulation of 5-HT_{2A} receptors by a variety of 5-HT_{2A} agonists increased whereas blockade of 5-HT_{2A} receptors (by antagonists) reduced anticipatory responses (Koskinen et al., 2000; Higgins et al., 2003a; Winstanley et al., 2003b; Passetti et al., 2003b). Systemic 8-OH-DPAT also increased impulsivity in a 5-CSRT task but

through stimulation of pre-synaptic 5-HT_{1A} autoreceptors in the DR nucleus (Carli and Samanin, 2000); stimulation of post-synaptic 5-HT_{1A} receptors in the mPFC had no effect (Winstanley et al., 2003b). Thus over-activation of 5-HT_{2A} but not 5-HT_{1A} receptors in the mPFC as a consequence of elevated 5-HT release may be an important mechanism that increases active responding in anticipation of reward. However, this view is challenged by findings presented in Chapter 6 showing that decreasing 5-HT release by 8-OH-DPAT had no effect on anticipatory responding and that global 5-HT depletion consistently enhanced anticipatory responding in the 5-CSRT task in the rat (Harrison et al., 1997a, 1997b).

That enhanced glutamate and 5-HT release may not be involved in CPP's effects on perseverative responses is indicated respectively by studies showing that lowering CPP-induced glutamate release by M100907 does not abolish perseverative responding. In addition reducing but not increasing 5-HT function in the PFC leads to response perseveration in tasks such as reversal learning (Clarke et al., 2004; Clarke et al., 2005), decision-making (Rogers et al., 1999a; Rogers et al., 1999b) and in some instances in a 5-CSRT (Winstanley et al., 2004b). Both 5-HT_{1A} and 5-HT_{2A} receptors are involved in regulating a feedback projection from the mPFC to dorsal raphe (Casanovas et al., 1999; Hajos et al., 1999) through a glutamate-dependent mechanism (Celada et al., 2001; Martin-Ruiz et al., 2001). Activation of 5-HT_{1A} or antagonism of 5-HT_{2A} receptors may reduce the activity of 5-HT neurons in the DR nucleus through this projection. However, as shown by the present results, blockade of 5-HT_{2A} receptors has no effect while stimulation of 5-HT_{1A} receptors reduced perseverative responding, suggesting that stimulation of 5-HT_{1A} receptors in the mPFC by 8-OH-DPAT exerts its effects on perseverative responding through indirect action in other, possibly non-5-HT mechanisms.

It is interesting that the NMDA receptor antagonists including CPP increased DA release in the mPFC (Moghaddam et al., 1997; Del Arco and Mora, 1999; Feenstra et al., 2002) and that perseverative responses in the 5-CSRT task increased after systemic amphetamine (Baunez and Robbins, 1999). The exact mechanism by which 8-OH-DPAT might reduce CPP-induced perseverative responding is not clear. Both systemic and intra-mPFC 8-OH-DPAT increased mPFC DA efflux (Arborelius et al., 1993; Sakaue et al., 2000). However, 8-OH-DPAT actually reduced the rise in DA release in the mPFC induced by amphetamine, stress and isolation rearing (Rasmusson et al., 1994; Kuroki et al., 1996; Ago et al., 2002) and attenuated the locomotor effects of amphetamine (Przegalinski and Filip, 1997). It is of particular interest that mPFC neurons expressing 5-HT$_{1A}$ receptors simultaneously project to the 5-HT cells of DR nucleus and DA cells of the ventral tegmental areas (VTA) and influence their activity (Thierry et al., 1983; Sesack and Bunney, 1989; Carr and Sesack, 2000; Celada et al., 2001; Hajos et al., 2003). Cognitive functions of the prefrontal cortex are influenced by the 5-HT system (Robbins, 2000a) and by an optimal level of mesocortical dopamine (DA) function (Roberts et al., 1994; Arnsten, 1997; Zahrt et al., 1997; Granon et al., 2000). Therefore, the decrease in perseverative responding after intra-mPFC 8-OH-DPAT might be due to some interaction with DA neurotransmission.

The data indicate that 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in the mPFC exert opposing actions on the attentional impairment induced by blockade of NMDA receptors. Furthermore, this is the first demonstration that multiple executive mechanisms that co-operate to preserve accurate response selection can be dissociated at the levels of 5-HT$_{2A}$ and 5-HT$_{1A}$ receptor mechanisms.

CHAPTER 6.   EFFECT OF M100907 AND 8-OH-DPAT ON CPP-INDUCED

GLUTAMATE AND 5-HT RELEASE IN THE mPFC

The results presented in Chapter 3 show that CPP impaired accuracy and executive control and raised extracellular glutamate levels in the mPFC. It is thought that the increased availability of glutamate presumably on non-NMDA receptors contributes to the behavioural effects of NMDA receptor antagonists (Moghaddam et al., 1997; Moghaddam and Adams, 1998). This hypothesis would suggest that, under conditions of excessive glutamate release, activation of mechanisms capable of limiting excitatory transmission might at least in part prevent the behavioural impairments induced by blockade of NMDA receptors. In fact, the data presented in Chapter 4 shows that activating $mGlu_{2/3}$ receptors that function as negative regulatory mechanisms on excitatory glutamate transmission abolished the effects of CPP on accuracy and impulsivity, but not compulsive perseveration. These data suggest that some aspects of attentional performance deficits might be associated with enhanced glutamate release.

As reviewed in the General Introduction, 5-HT through 5-HT_{1A} and 5-HT_{2A} receptor mechanisms in the PFC importantly contribute to the modulation of glutamate NMDA receptor neurotransmission. However, no data were available on the effects of 5-HT_{1A} and 5-HT_{2A} receptor ligands on glutamate efflux in awake animals induced by blockade of NMDA receptors in the mPFC. Several studies demonstrating a functional interaction between 5-HT_{1A} and 5-HT_{2A} receptor mechanisms and behaviours related to NMDA receptor blockade have also been reviewed (see General Introduction). The data presented in Chapter 5 show that activation of 5-HT_{1A} or blockade of 5-HT_{2A} receptors in the mPFC remediate the accuracy impairment by blockade of NMDA receptors in this area. Furthermore the data demonstrate that inhibitory response control can be dissociated at the levels of 5-HT_{2A} and 5-HT_{1A} receptor mechanisms. M100907 abolished the CPP-induced

anticipatory responding but had no effects on perseverative over-responding, while 8-OH-DPAT reduced the perseverative over-responding but had no effects on anticipatory responding induced by CPP. *In-vivo* neurochemistry has attempted to provide some insight into neurochemical mechanisms associated with behavioural changes induced by 5-HT_{1A} and 5-HT_{2A} receptors.

The microdialysis experiments in freely moving rats examined the ability of the $5\text{-}HT_{1A}$ receptor agonist, 8-OH-DPAT (Middlemiss and Fozard, 1983), and 5-HT_{2A} receptor antagonist, M100907, to modify the rise in extracellular glutamate and 5-HT efflux induced by blockade of NMDA receptors in the mPFC. The selectivity of 8-OH-DPAT s effects on 5-HT_{1A} receptors was evaluated by studying the ability of the 5-HT_{1A} receptor antagonist, WAY 100635 (Forster et al., 1995) infused through the probe, to antagonize the effects of 8-OH-DPAT on the raise of extracellular GLU induced by the infusion of CPP into mPFC. As both 5-HT_{1A} and NMDA receptors control the release of 5-HT *in-vivo* (Sharp and Hjorth, 1992; Martin et al., 1998a; Casanovas et al., 1999), we also measured the effect of 5-HT_{1A} agents on CPP-evoked changes of cortical 5-HT release. The contribution of 5-HT_{2A} receptors to CPP-induced raise in extracellular glutamate and 5-HT was investigated using M100907 injected subcutaneously or co-infused with CPP through the microdialysis probe.

MATERIALS AND METHODS

Animals, surgical implantation of microdialysis probes, drugs and reagents, chemical determination of GLU concentrations in dialysate samples were as described previously (General methods).

Drugs and experimental design

All drugs or vehicle were infused during the phase of stable glutamate and serotonin output defined as three consecutive baseline samples not differing by more than 20%.

Effects of M100907 on CPP-induced glutamate and 5-HT release

M100907 was dissolved in CSF and administered subcutaneously (10 and 40 µg/kg) or through the probe (0.1 µM) 20 min before 100 µM CPP. The effects of M100907 on CPP-induced 5-HT release were examined using 40 µg/kg. The systemic doses used were based on their ability to block the CPP-induced attentional performance deficit and DOI-mediated rise in extracellular DA (Gobert and Millan, 1999). In these experiments concentric dialysis probes for measurements of exctracellular glutamate were constructed using AN69 membrane. Dialysis probes used for the measurements of 5-HT extracellular concentrations were constructed with Cuprophan membranes.

The *in-vitro* relative recovery of M100907 measured at room temperature through the AN69 probe was 22–3% (n=3). Based on this result, the concentration of M100907 in the extracellular fluid surrounding the probe should be about 20 nM.

Effects of 8-OH-DPAT on CPP-induced glutamate and 5-HT release

These experiments involved perfusion of the mPFC with 8-OH-DPAT (Research Biochemical International, MA, USA) and WAY100635 (Pharmacia and Upjohn,

Nerviano, Italy) and CPP. All drugs were dissolved in aCSF and infused through the probe into the mPFC at the concentrations indicated. Control rats were perfused with normal aCSF. Perfusion of 8-OH-DPAT, WAY100635 and their combination started 20 min before perfusion of CPP. In these experiments concentric dialysis probes were constructed with Cuprophan membrane (216 m outer diameter, 3000 Da cutoff, Sorin Biomedica, Italy). The in-vitro recovery of 8-OH-DPAT and WAY 100,635 was $18.9\% \pm 0.2$ and $7.7\% \pm 0.4$ respectively (mean $\pm$ SEM).

Correct probe placement was checked by visual inspection of the probe tracks on 30-m coronal sections from the mPFC of each rat. Only rats with correct probe placement were considered in the results.

Chemical determination of glutamate and 5-HT in the perfusion liquid was as described in Chapter 2 (General Methods)

Statistical analysis

Extracellular levels of GLU or 5-HT, not corrected for in vitro recovery, are expressed as percentages of basal values. Basal values of glutamate or 5-HT in different experiments were compared by one-way ANOVA. All time-course data were analyzed by ANOVA for repeated measures with treatments (8-OH-DPAT or M100907 x CPP) as between factors and time as within factor. Post-hoc comparisons between pre- and post-injection values were made with Dunnett's test whereas Tukey-Kramer's test was used for comparisons between treatments. Values missing because of occasional problems in sample collection or analysis were replaced by the mean of the samples immediately before and after. Statistical analyses were done on raw data using the StatView 5.0 for Apple Macintosh computer (SAS Institute Inc., Cary, NC, USA).

RESULTS

- *Activation of 5-HT_{1A} receptors in the mPFC by 8-OH-DPAT prevents CPP-induced increase of extracellular glutamate levels*

Fig. 1A, shows that extracellular levels of GLU in the mPFC of rats infused with 100 µM CPP were significant higher than in rats infused with aCSF. The effect was significant at 20 and 80 min (about +100%). Intracortical infusion of 8-OH-DPAT prevented the raise of extracellular GLU induced by CPP. ANOVA indicated a significant effect of 8-OH-DPAT ($F_{3,19}$=7.3, P=0.002), time ($F_{3,57}$=5.9, P=0.001) and their interaction ($F_{9,57}$=2.9, P=0.006). Post-hoc analysis showed that 3 µM 8-OH-DPAT completely abolished the increase of extracellular GLU induced by CPP whereas 0.3 µM 8-OH-DPAT had no effect. The infusion of 0.3 and 3 µM 8-OH-DPAT by itself had no effect on extracellular GLU (8-OH-DPAT, $F_{2,14}$=0.5, P=0.6; time, $F_{3,42}$=0.96, P=0.4; 8-OH-DPAT x time, $F_{6,42}$=1.2. P=0.3)(Fig. 1B).

Figure 1

A) Effect of 0.3 and 3 μM 8-OH-DPAT (DPAT) on CPP-induced increase of extracellular GLU. Mean basal levels of GLU in pmol/20μL (± SEM) were: aCSF, 10.2 ± 2.0 (n = 6); CPP 100 μM, 10.1 ± 1.2 (n = 6); DPAT 0.3 μM + CPP, 9.9 ± 2.2 (n = 6); DPAT 3 μM + CPP, 9.7 ± 2.8 (n = 6). DPAT perfusion started 20 min before CPP and continued for 1 h and 20 min. The duration of drugs application is indicated by horizontal bars. * $P < 0.05$ (Tukey-Kramer's test). Solid symbols indicate $P < 0.05$ vs. basal values (Tukey-Kramer's test).

B) Effect of 0.3 and 3 μM DPAT on extracellular levels of GLU in rats given vehicle. Horizontal bar indicates the duration of DPAT infusion. Mean basal levels of GLU (± SEM) in pmol/20μL were: DPAT 0.3 μM, 6.4 ± 0.9 (n = 5); DPAT 3 μM, 9.4 ± 2.4 (n = 6). Rats infused with normal aCSF are the same as in panel A.

- *Activation of 5-HT$_{1A}$ receptors in the mPFC by 8-OH-DPAT prevents CPP-induced increase of extracellular 5-HT levels*
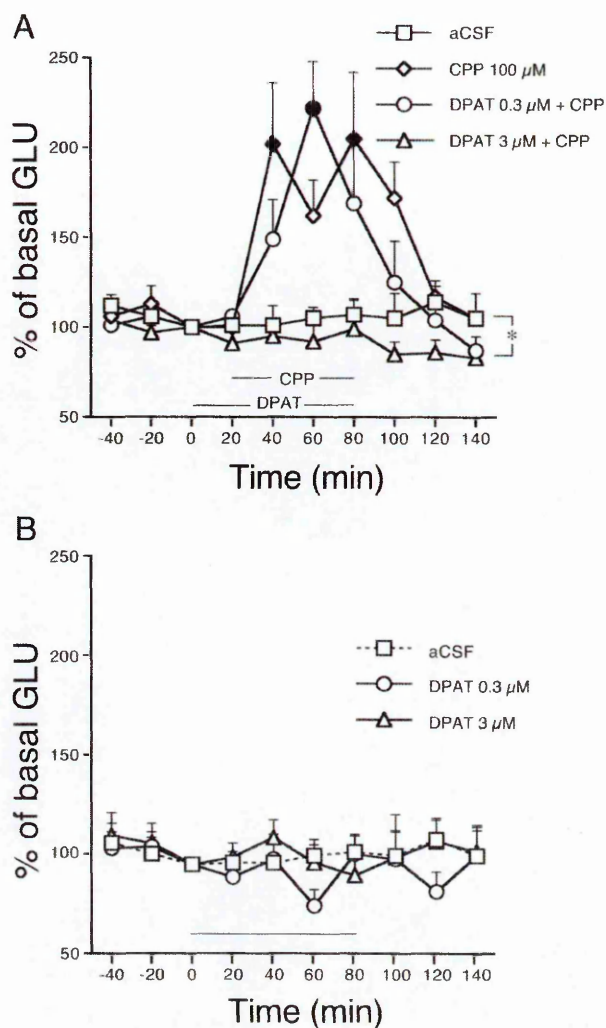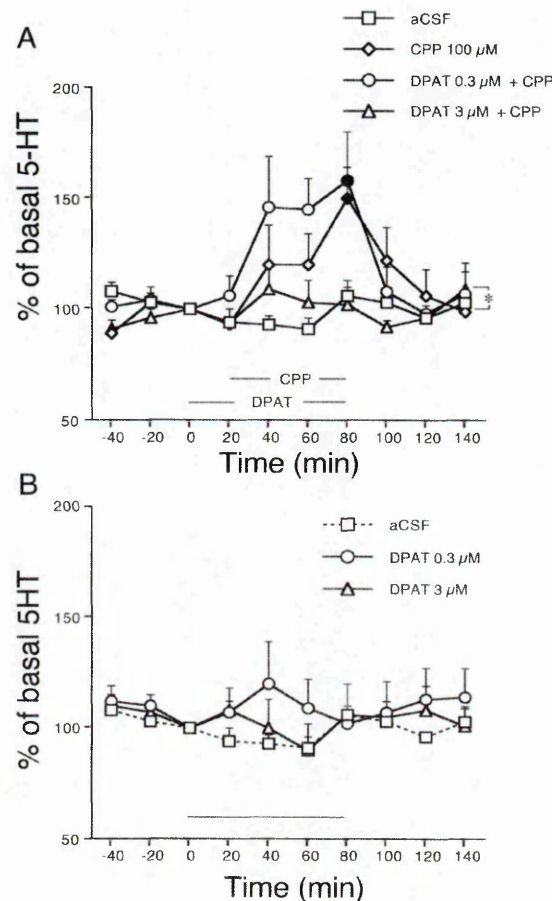

Figure 2

A) Effect of 0.3 and 3 µM 8-OH-DPAT (DPAT) on CPP-induced increase of extracellular 5-HT. Mean basal levels of 5-HT (± SEM) in fmol/20µL were: aCSF, 3.7 ± 0.3 (n = 6); CPP 100 µM, 3.6 ± 0.3 (n = 6); DPAT 0.3 µM + CPP, 4.3 ± 1.4 (n = 6); DPAT 3 µM + CPP, 3.1 ± 0.6 (n = 6). DPAT perfusion started 20 min before the perfusion with CPP and continued for 1 h and 20 min. Horizontal bars indicate the duration of drugs perfusion. *P< 0.05 (Tukey-Kramer's test). Solid symbols indicate P< 0.05 vs. basal values (Tukey-Kramer's test).

B) Effect of 0.3 and 3 µM DPAT on extracellular 5-HT. Horizontal bar indicates the duration of DPAT infusion. Mean basal levels of 5-HT (± SEM) in fmol/20µL were: DPAT 0.3 µM, 3.7 ± 0.9 (n = 5); DPAT 3 µM, 2.9 ± 0.4 (n = 6). Rats infused with normal aCSF are the same as in panel A.

Fig. 2A shows that infusion of CPP through the probe significantly raised extracellular 5-HT. The maximal increase (about +50%) was reached between 20 and 80 min after the start of the infusion. However, this increase was statistically

significant only at 80 min. Intracortical infusion of 8-OH-DPAT antagonized CPP-induced effects on extracellular 5-HT (8-OH-DPAT, $F_{3,20}=3.0$ P=0.05; time, $F_{3,60}=8.3$ P=0.001; 8-OH-DPAT x time, $F_{9,60}=1.9$ P=0.06). Post-hoc analysis showed that extracellular 5-HT was significantly reduced in rats given 3 μM 8-OH-DPAT + CPP whereas in those receiving 0.3 μM 8-OH-DPAT the effect of CPP was unchanged. Fig. 2B shows that the infusion of 0.3 and 3 μM 8-OH-DPAT by itself had no effect extracellular 5-HT (8-OH-DPAT, $F_{2,14}=0.1$, P=0.9; time, $F_{3,42}=0.5$, P=0.7; 8-OH-DPAT x time, $F_{6,42}=1.2$, P=0.3).

- ***The 5-HT$_{1A}$ receptor antagonist, WAY100635, reduces the effect of 8-OH-DPAT on CPP-induced raise of extracellular glutamate levels***
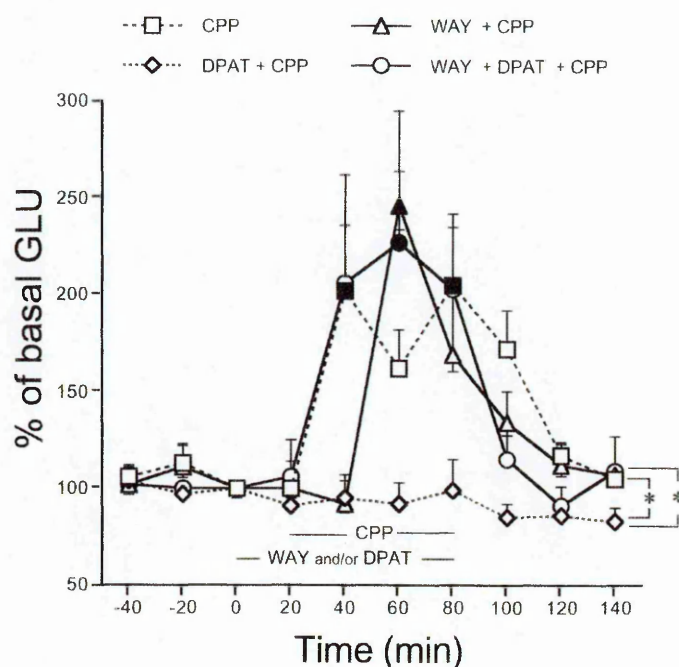
Figure 3

The 5-HT$_{1A}$ antagonist WAY 100635 (WAY) at 100 μM prevented the effect of 3 μM 8-OH-DPAT (DPAT) on the increase of extracellular GLU induced by 100 μM CPP. WAY and DPAT were co-perfused for 80 min starting 20 min before CPP as indicated by the horizontal bars. Mean basal levels of GLU (± SEM) in pmol/20μL were: WAY + CPP, 15.7 ± 2.1 (n = 6); WAY + DPAT + CPP, 13.7 ± 2.9 (n = 7). Rats infused with CPP and DPAT + CPP are the same as in Fig. 1. *P< 0.05 (Tukey-Kramer's test). Solid symbols indicate P< 0.05 vs. basal values (Tukey-Kramer's test).

As shown in Fig. 3, WAY100635 had no significant effects by itself (data not shown) but completely antagonized the effect of 8-OH-DPAT on CPP-induced raise of extracellular GLU. ANOVA indicated no significant effect of WAY100635 ($F=3.9$, $P=0.06$) or 8-OH-DPAT ($F_{1,21}=1.0$, $P=0.3$) but a significant interaction between WAY100635 and 8-OH-DPAT ($F_{1,21}=7.8$ $P=0.01$). In addition, factor time was significant ($F_{3,63}=7.3$, $P=0.0003$) as well as its interaction with 8-OH-DPAT x WAY100635 ($F_{3,63}=2.9$, $P=0.04$).

- ***WAY100635 reduces the effect of 8-OH-DPAT on CPP-induced raise of extracellular 5-HT levels***
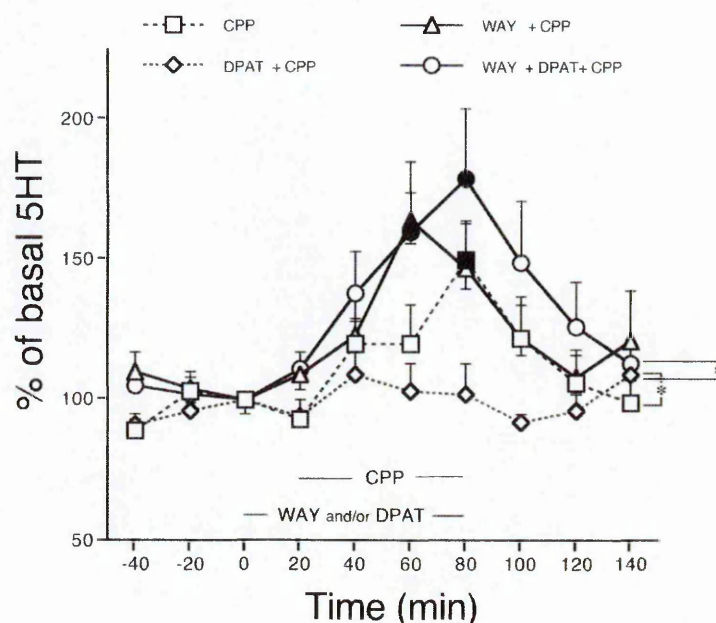
Figure 4
Effects of 100 μM WAY 100,635 (WAY) plus 3 μM 8-OH-DPAT (DPAT) on CPP-induced raise of extracellular 5-HT. WAY and DPAT were co-perfused for 80 min starting 20 min before the infusion of CPP as indicated by the horizontal bars. Mean basal levels of 5-HT ($\pm$ SEM) in fmol/20 μL were: WAY + CPP, 3.6 ± 0.8 (n = 6); WAY + DPAT + CPP, 3.0 ± 0.6 (n = 6). Rats infused with CPP and DPAT + CPP are the same as in Fig. 2. *$P < 0.05$ (Tukey-Kramer's test). Solid symbols indicate $P < 0.05$ vs. basal values (Tukey-Kramer's test).

Similarly, 100 μM WAY100635 reversed the effects of 3 μM 8-OH-DPAT on CPP-induced raise of extracellular 5-HT (WAY100635, $F_{1,21}=12.3$, P=0.0002; 8-OH-DPAT, $F_{1,21}=0.2$, P=0.7; 8-OH-DPAT x WAY100635, $F_{1,21}=4.3$, P=0.05). The factor time was significant ($F_{3,63}=11.4$, P<0.0001) whereas interaction 8-OH-DPAT, WAY100635 and time was not significant ($F_{3,63}=2.5$, P=0.07) (Fig. 4).

Lower concentrations of WAY100635 (10 μM) were unable to block the effects of 8-OH-DPAT on glutamate or 5-HT release induced by CPP (data not shown).

WAY100635 had no effects by itself on extracellular levels of GLU (WAY100635, $F_{2,15}=0.1$, P=0.9; time, $F_{3,45}=0.1$, P=0.95; WAY100635 x time, $F_{6,45}=0.4$, P=0.9; Fig. 3) and 5-HT (WAY100635, $F_{2,15}=1.1$, P=0.3; time, $F_{3,45}=0.7$, P=0.5; WAY100635 x time, $F_{6,45}=0.8$, P=0.6; Fig. 4).

Basal extracellular concentrations of GLU and 5-HT in different experimental groups were not significantly different and were pooled (GLU, $F_{7,40}=1.7$, P=0.1; 5-HT, $F_{7,39}=0.4$, P=0.9). Basal extracellular concentrations of prefrontocortical GLU and 5-HT were respectively 10.8±0.8 pmol/20 μL (n=48) and 3.5±0.3 fmol/20 μL (n=47).

- **_Blockade of 5-HT$_{2A}$ receptors by M100907 prevents the CPP-induced rise in extracellular glutamate levels in the mPFC_**

As shown in Fig. 5A, extracellular levels of GLU in the mPFC of rats infused with 100 μM CPP were significantly higher than after artificial CSF (P<0.05; Tukey-Kramer's test). Subcutaneous and intracortical injection of M100907 prevented the rise of extracellular GLU in response to CPP. ANOVA indicated a significant effect of treatment ($F_{3,18}=7.8$, P<0.01), time ($F_{5,90}=2.4$, P<0.05) but not treatment by time interaction ($F_{3,18}=0.7$, P>0.05). Post-hoc analysis showed that 40 μg/kg M100907 completely prevented the increase of extracellular GLU induced by CPP (P<0.05 vs.

CPP alone; Tukey-Kramer's test). Likewise, the co-perfusion of 0.1 µM M100907 abolished the effect of 100 µM CPP on extracellular GLU (P<0.05 vs. CPP alone; Tukey-Kramer's test). M100907 10 µg/kg also attenuated the increase of extracellular GLU induced by CPP but the effect was not significant (P>0.05 vs. CPP alone; Tukey-Kramer's test).

Fig. 5B shows that by itself M100907 (40 µg/kg s.c.) or 0.1 µM through the probe had no effect on extracellular GLU (M100907, $F_{1,10}$=0.01, P>0.05; time, $F_{7,70}$=1.8, P>0.05; M100907 x time $F_{7,70}$=1.7, P>0.05). Basal levels of glutamate in pmol/20 µL were: VEH+CPP, 14.8 ± 3.5 (n=5); 10 µg/kg M100907+CPP, 13.9 ± 2.1 (n=6); 40 µg/kg M100907+CPP, 18.6 ± 4.9 (n=6); 0.1 µM M100907+CPP, 13.5 ± 2.7 (n=6); 40 µg/kg M100907+CSF, 12.9 ± 2.1 (n=6); 0.1 µM M100907+CSF, 12.3 ± 1.0 (n=6).
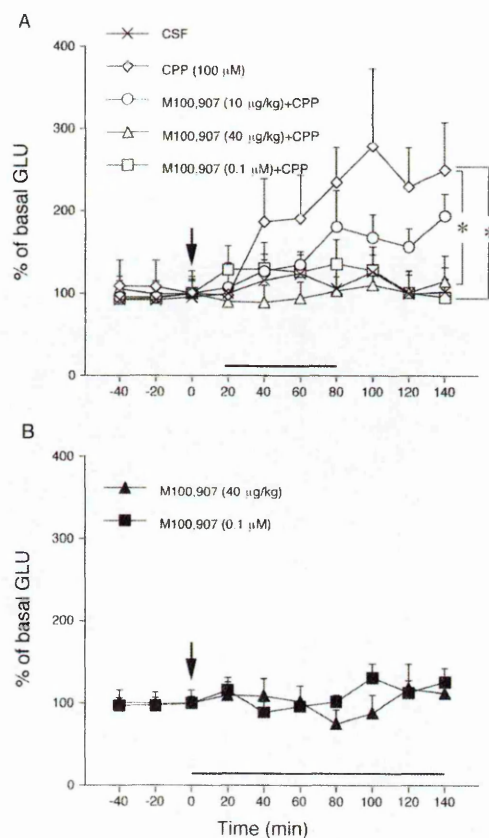
Figure 5

The 5-HT$_{2A}$ receptors antagonist M100907 prevents CPP increasing extracellular glutamate (GLU) in the mPFC. (A) Rats were injected subcutaneously with vehicle (VEH), 10 or 40 µg/kg M100907, 20 min before the perfusion of 100 µM CPP (horizontal bar) through the probe. One group of rats was co-perfused with 0.1 µM M100,907 and 100 µM CPP through the probe. M100907 started 20 min before CPP and continued for the rest of the experiment. Panel B shows the effect of 40 µg/kg s.c.M100,907 (arrow) or 0.1 µM M100,907 perfused through the probe (horizontal bar) on extracellular glutamate. Results are mean ± SEM. *P<0.05 vs. VEH+CPP (Tukey-Kramer's test).

- **Blockade of 5-HT$_{2A}$ receptorsby M100907 prevents CPP-induced increase of extracellular 5-HT levels**

Fig. 6 shows that infusion of CPP through the probe raised extracellular 5-HT levels in the mPFC. The maximal increase (about +50%) was reached between 80 min after the start of the infusion. Subcutaneous injection of 40 µg/kg M100907 by itself had no effect on extracellular 5-HT but reduced CPP-induced increase in extracellular 5-HT. The overall ANOVA showed that interaction between M100907

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and CPP was not statistically significant (M100907 x CPP, $F_{1,17}$=3.6 P=0.07). However, comparing the treatments means showed that pretreatment with M100907 significantly reduced (P<0.05) extracellular 5-HT in CPP-injected rats.
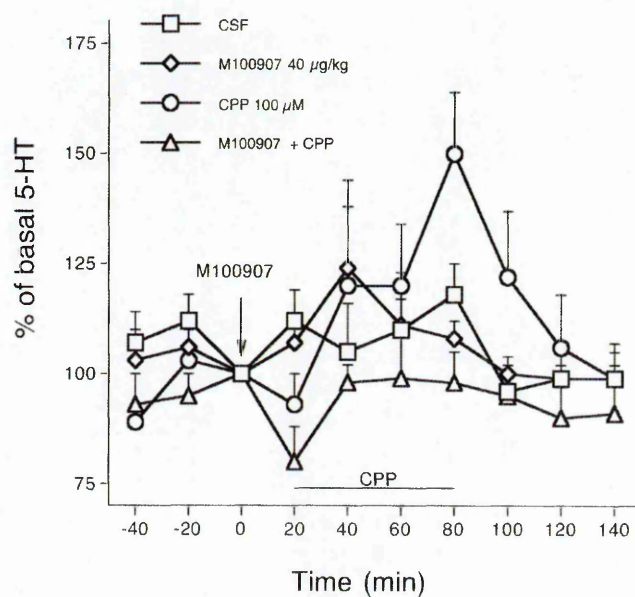
Figure 6.
Effect of M100907 on CPP-induced increase of extracellular 5-HT. M100907 40 µg/kg was injected subcutaneously 20 min before the perfusion with CPP and continued for 1 h and 20 min. Horizontal bars indicate the duration of CPP perfusion.

DISCUSSION

The intracortical infusion of CPP raised the extracellular concentrations of glutamate (GLU) in the mPFC and confirms the results obtained in the previous experiments. The major finding of the present study is that intracortical infusion of 8-OH-DPAT or M100907 suppressed the raise of extracellular glutamate and 5-HT induced by CPP.

Stimulation of 5-HT$_{1A}$ receptors in the mPFC

The 8-OH-DPAT in vivo acts on both 5-HT$_{1A}$ and 5-HT$_7$ receptors (Hedlund et al., 2004). The finding that the selective 5-HT$_{1A}$ receptor antagonist, WAY100635, antagonised the effects of 8-OH-DPAT indicates that 5-HT$_{1A}$ receptors are mainly involved. The relatively large concentrations of WAY100635 (100 μM in the probe) needed to block the effects of 8-OH-DPAT are consistent with those used in previous studies (Casanovas et al., 1999; Celada et al., 2001) and are partially justified by the low rate of diffusion of WAY100635 through the probe.

The lack of effect of WAY100635 on basal and CPP-evoked release of glutamate suggests that cortical 5-HT$_{1A}$ receptors do not exert a tonic control on glutamate release in the mPFC. However, the 5-HT$_{1A}$ receptor antagonists NAN-190 and WAY100135 increased glutamate release in slices of the guinea pig dentate gyrus (Matsuyama et al., 1996) and in the rat striatum (Dijk et al., 1995). Differences in selectivity between 5-HT$_{1A}$ antagonists (WAY100635 is the most selective among the three), partial agonist activity of WAY100135 and NAN-190 (Fletcher et al., 1993) and brain region examined may probably account for these differences.

The failure of 8-OH-DPAT to affect basal extracellular glutamate is consistent with previous findings (Dijk et al., 1995; Matsuyama et al., 1996). This is not surprising

since the inhibition of neurotransmission with tetrodotoxin does not affect basal glutamate, probably reflecting the lack of relationship between basal glutamate and neuronal activity in microdialysis studies (Timmerman and Westerink, 1997; Melendez et al., 2005). Interestingly, *in-vivo* studies showed that 8-OH-DPAT potently inhibited the K^+-evoked release of glutamate in cerebellar synaptosomes (Maura et al., 1988) but did not affect veratridine-evoked release of glutamate in the rat mPFC (Golembiowska and Dziubina, 2002). K^+-evoked release is Ca^{2+} dependent (Maura et al., 1988) whereas the effect of veratridine may involve both Ca^{2+} dependent and independent components (Villanueva et al., 1988; Szatkowski et al., 1990; Dickie and Davies, 1993). Thus, these findings suggest that 8-OH-DPAT's effect may depend on the way glutamate is released.

Intracortical CPP raised extracellular 5-HT in the mPFC. This effect is likely due to the blockade of NMDA receptors as other antagonists such as PCP or genetic deletion of the NMDA receptor had similar effects (Martin et al., 1998a; Adams and Moghaddam, 2001; Miyamoto et al., 2001). Additionally, CPP had no affinity for 5-HT_{1A} and 5-HT_2 receptors in the rat cortex (Lehmann et al., 1987). As the infusion of AMPA through the microdialysis probe increased extracellular 5-HT in the mPFC (Martin-Ruiz et al., 2001), it is conceivable that CPP-induced increase of extracellular 5-HT is secondary to the elevation of glutamate and stimulation of AMPA receptors. Consistently, NMDA receptor blockade increases extracellular glutamate and stimulates the firing rate of cortical pyramidal neurons (Jodo et al., 2003; Jackson et al., 2004; Homayoun et al., 2005) and it has been shown that the excitation of 5-HT neurons induced by electrical stimulation of cortical afferents is prevented by the blockade of AMPA receptors (Celada et al., 2001).

It is well known that the inhibitory effect of 8-OH-DPAT on 5-HT neurotransmission is mainly due to the stimulation of somatodendritic 5-HT$_{1A}$ autoreceptors in the raphe nuclei (Kreiss and Lucki, 1994). However, the stimulation of cortical 5-HT$_{1A}$ receptors controlling glutamatergic afferents originating in the mPFC may contribute (Ceci et al., 1994; Casanovas et al., 1999; Hajos et al., 1999; Celada et al., 2001). The present results clearly show that CPP's effect on extracellular 5-HT was prevented by the intracortical infusion of 8-OH-DPAT at a concentration that by itself had no effect. It could be argued that 3 µM 8-OH-DPAT is not sufficient to activate cortical 5-HT$_{1A}$ receptors controlling 5-HT release. The recovery of 8-OH-DPAT estimated *in-vitro*, revealed that about 19% of the drug crossed the probe membrane (Kreiss and Lucki, 1994; Assie and Koek, 1996) and it could be estimated that the concentration of 8-OH-DPAT in the tissue surrounding the probe was about 0.6 µM. Given the high affinity of 8-OH-DPAT (about 1 nM) for cortical 5-HT$_{1A}$ receptors (Middlemiss and Fozard, 1983), this concentration appears sufficient to stimulate 5-HT$_{1A}$ receptors. Accordingly, the effects of 8-OH-DPAT on CPP-induced raise of extracellular glutamate and 5-HT were completely prevented by WAY100635.

In line with the present findings, 8-OH-DPAT at concentrations similar to those used in the present study do not affect basal extracellular 5-HT (Assie and Koek, 1996; Ago et al., 2003). However, intracortical infusion of 100-300 µM 8-OH-DPAT reduced 5-HT release in the mPFC (Casanovas et al., 1999; Martin-Ruiz et al., 2001). Thus, it appears that the inhibitory effect of 8-OH-DPAT on CPP-induced raise of extracellular glutamate and 5-HT found here is independent from its ability to suppress basal 5-HT release.

Blockade of 5-HT$_{2A}$ receptors in the mPFC

Our results clearly show that subcutaneous injection of the 5-HT$_{2A}$ receptor antagonist M100907 completely prevented the increase of extracellular glutamate induced by CPP. Thus the effect of CPP on glutamate may therefore depend on the stimulation of 5-HT$_{2A}$ receptors in the mPFC as a consequence of the increased tone of endogenous 5-HT. As discussed in Chapter 3, it appears that systemic CPP may increase extracellular glutamate independently of its effect on cortical 5-HT. Interestingly, the hypermotility induced by systemic MK-801 was not affected by 5-HT depletion whereas its reversal by M100907 was prevented by the depletion of endogenous 5-HT (Martin et al., 1998b). However, it cannot be excluded that the blockade of 5-HT$_{2A}$ receptors unmasks the effect of endogenous serotonin on other receptor subtypes, such as 5-HT$_{1A}$ and 5-HT$_3$ receptors that inhibit glutamate release in the rat brain (Srkalovic et al., 1994; Dijk et al., 1995; Maura and Raiteri, 1996). 5-HT$_{1A}$ receptors are particularly interesting in this respect since their activation reduced CPP-induced glutamate release.

The infusion of 0.1 µM M100907 through the probe prevented the increase in glutamate induced by CPP. This finding indicates that blockade of 5-HT$_{2A}$ receptors in the mPFC plays a major role in controlling the effect of CPP on extracellular glutamate. It may appear surprising that the CPP-induced raise of extracellular glutamate was antagonized here using 0.1 µM M100907, as higher concentrations (100-300 µM) were used in previous studies to block cortical 5-HT$_{2A}$ receptors (Martin-Ruiz et al., 2001; Amargos-Bosch et al., 2003). However, brain extracellular concentrations of the drug in rats given 5 mg/kg M100907, a dose 125 times that used in the present study, were about 1 µM (Scott and Heath, 1998). Taking into account the *in vitro* recovery of M100907 (about 20%, present paper; (Scott and

Heath, 1998), its concentration in the extracellular fluid should be about 20 nM. This is proportional to the concentration found in rats given 5 mg/kg M100907 (Scott and Heath, 1998) and well above the Ki of the drug (about 1 nM) for 5-HT$_{2A}$ receptors (Kehne et al., 1996).

As reviewed in the General Introduction the 5-HT$_{2A}$ receptors are expressed, albeit in small amounts, by axons and terminals of the mPFC (Miner et al., 1997; Willins et al., 1997; Jakab and Goldman-Rakic, 2000). Glutamatergic afferents to the mPFC arise from different regions of the brain including thalamus, hippocampus, amygdala and other cortical regions (Groenewegen and Uylings, 2000). Thus, it is conceivable that the reversal of the CPP-induced increase of extracellular glutamate by M100907 is mediated by presynaptic 5-HT$_{2A}$ heteroreceptors on glutamatergic axon terminals. The DOI-induced increase of EPSC in the mPFC slices and c-fos expression in the somatosensory cortex depends on the integrity of thalamo-cortical afferents (Aghajanian and Marek, 1997, 1999; Scruggs et al., 2000; Scruggs et al., 2003). However, this interpretation is challenged by *in vivo* findings that DOI-induced activation of pyramidal neurons in the mPFC of anesthetized rats did not depend on thalamo-cortical afferents (Puig et al., 2003). The effect of M100907 on the increase of glutamate induced by CPP could be mediated by 5-HT$_{2A}$ receptors on glutamate afferents arising from other brain regions or, most probably, from those present in large amounts on cortical pyramidal neurons (Miner et al., 1997; Jakab and Goldman-Rakic, 2000). Electrophysiological studies in slices of the mPFC have shown that the major effect of 5-HT is on pyramidal cells and M100907 prevented PCP-induced blockade of NMDA responses in pyramidal neurons (Wang and Liang, 1998). 5-HT$_{2A}$ receptors are also present on GABAergic interneurons (Willins et al., 1997; Jakab and Goldman-Rakic, 2000). However, the local infusion of DOI raised

extracellular GABA levels and c-fos expression while M100907 inhibited K$^+$-evoked [^{3}H]GABA release in rat cortical slices (Abi-Saab et al., 1999). Thus, a direct effect of M100907 on GABAergic interneurons is unlikely to be involved in the reversal of CPP's effect on extracellular glutamate.

In contrast with the present findings, Adams and Moghaddam (Adams and Moghaddam, 2001) found that M100907 did not antagonize the increase of cortical extracellular glutamate induced by PCP. This discrepancy may be due to the fact that these experiments used different NMDA receptor antagonists (CPP vs. PCP) and route of administration (intracortical vs. intraperitoneal). PCP has considerable affinity for 5-HT$_2$ and sigma receptors, ion channels other than NMDA receptors, and DA and 5-HT transporters (Nabeshima et al., 1988; Wong et al., 1988; Javitt and Zukin, 1991; Kapur and Seeman, 2002) (Maurice et al., 1991) whereas CPP is far more selective (Lehmann et al., 1987). Thus, besides NMDA receptor blockade, the interaction with these mechanisms may contribute to the effect of PCP on cortical glutamate. It cannot be excluded that different routes of administration of PCP and CPP might account for the discrepancy between the present results and those by Adams and Moghaddam (2001).

In conclusion, NMDA receptor antagonists-induced behavioral deficits have been associated to increased extracellular glutamate in the mPFC (Moghaddam et al., 1997; Moghaddam and Adams, 1998). Both 8-OH-DPAT and M100907 completely prevented the increase of extracellular glutamate induced by CPP. However, as shown in Chapter 5 although both drugs counteracted the accuracy deficit induced by CPP their effects on different aspects of inhibitory response control were dissociable suggesting that the suppression of cortical glutamate release does not account for all the effects of these drugs in the 5-CSRT task.

CHAPTER 7. EFFECTS OF DA D$_2$ ANTAGONISTS

Numerous lines of evidence suggest that the 5-HT and DA systems, although both implicated in functions associated with PFC, mediate different forms of neuromodulation, which is shown by their distinct contribution to various aspects of PFC functions such as working memory, vigilance, decision-making, reversal learning, attentional set shifting, sustained and selective attention and inhibitory response control (reviewed in the General Introduction). As reviewed in the General Introduction, distinct contributions of these chemically defined systems to attention and inhibitory response control have been revealed when the behavioural effects of their manipulation by drugs or selective lesions were examined systematically on a common behavioural task such as the 5-CSRT task. Notably, either decreasing 5-HT or increasing DA function increase anticipatory responding (Harrison et al., 1997a) (Cole and Robbins, 1987). The attentional functioning is ameliorated by activation of 5-HT$_{1A}$ or blockade of 5-HT$_{2A}$ receptors in the mPFC (Winstanley et al., 2003b) as well as by the partial DA D$_1$ agonist SKF 38393 (Granon et al., 2000). The DA D$_1$ receptor antagonist SCH23390 impaired accuracy only in rats with high baseline performance; the DA D$_2$ receptor antagonist l-sulpiride had no effect (Granon et al., 2000). The 5-HT$_{2A}$ receptors are involved in "impulsivity" (Winstanley et al., 2003b; present results Chapter 5) whilst the role of DA D$_1$ and D$_2$ receptor subtypes in inhibitory response control is less clearly defined. In contrast, the attentional impairments of rats with mPFC lesions were alleviated by a DA D$_2$ antagonist l-sulpiride but not by a DA D$_1$ antagonist SCH 23390 (Passetti et al., 2003b).

As shown in this thesis, 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor mechanisms in the mPFC all play distinct and to some extent dissociable roles in various aspects of attentional performance (Chapter 6). There is considerable evidence that these 5-HT

receptors modulate DA functions in the PFC and subcortical regions along the frontocortico-striatal-thalamic circuitry (reviewed in the General Introduction). Microdialysis studies show that concomitant blockade of 5-HT$_{2A}$ and DA D$_2$ receptors may stimulate the frontocortical DA release but inhibit that in the NAcc (Liegeois et al., 2002). The 5-HT$_{1A}$ receptor agonists stimulate the release of DA in the PFC as well as potentiate the effects of DA D$_2$ antagonists on DA release (Ichikawa and Meltzer, 1999a). Administration of 5-HT$_{2C}$ receptor antagonists can directly increase DA release in the PFC and in NAcc (Di Matteo et al., 1998) and agonists such as Ro60-0175 inhibit dialysate levels of DA in the PFC (Millan et al., 1998). Thus, the 5-HT/DA interaction may be critical for attentional and executive functioning. Therefore, it was of interest to compare the pattern of effects of the some DA D$_2$ antagonists with those obtained with 5-HT$_{1A}$ and 5-HT$_{2C}$ agonist and 5-HT$_{2A}$ antagonists on CPP-induced deficits.

The highest density of DA D$_2$ receptor (measured by selective radioligand binding and immunocytochemistry) has been found in the striatum (caudate-putamen), the olfactory tubercle, in the core of NAc where it is expressed by GABAergic neurons co-expressing enkephalins and in the septal pole of the shell of the NAc. Medium-high densities were found in the ventral pallidum, zona incerta, globus pallidum, central amygdala, hippocampus laterodorsal septal area, subiculum, subthalamic nucleus, lateral mammillary bodies and in various cortical fields: prefrontal, anterior cingulate, entorhinal and perirhinal cortices (Bentivoglio and Morelli, 2005).

In the mPFC, D$_2$ receptor binding revealed the presence of D$_2$ receptor in cell bodies of layer II-VI, with the highest density in deep layers V and VI. The laminar distribution of receptors is similar to that of mesocortical DA afferents, suggesting that D$_2$ receptors are functionally related to these inputs. D$_2$ receptors are mainly

located in cell populations different from those expressing D_1 receptors (Vincent et al., 1993). In the cortex the greater expression of D_2 mRNA was found in the mPFC, cingulate and insular cortices, restricted to layer V and to corticostriatal and cortico-cortical neurons (Gaspar et al., 1995). About 50% of D_2 mRNA was found on GABA interneurons expressing parvalbumin (Le Moine and Gaspar, 1998). The DA D_2 receptor is coupled to a G-protein and has been mainly characterised as an inhibitor of adenylyl cyclase. It also activates K^+ channels, stimulate phospholipase A_2 and affects Ca^{2+} channels (Missale et al., 1998).

Table A. Pharmacological profiles of DA receptors

	D1-like		D2-like		
	D1	D5	D2	D3	D4
Haloperidol	+	+	++++	++	+++
Clozapine	+	+	+	+	+
L-sulpiride	-	-	++	++	++

++++, Inhibition constant (Ki) <0.5nM; +++, 0.5nM <Ki< 5nM; ++, 5nM <Ki< 50 nM; +, 50 nM <Ki< 500nM; -, Ki> 5 μM; Adapted from Missale et al. (Missale et al., 1998).

Although blockade of DA D_2 receptors by both haloperidol and clozapine is sufficient to produce antipsychotic action and to improve cognitive functions in schizophrenic patients (Lee et al., 1999; Green et al., 2002), the lack of side effects such as tardive diskinesia and the greater efficacy on negative symptoms and on cognitive deficits of atypical antipsychotics such as clozapine have been proposed to relate to their high 5-HT_{2A}/D_2 affinity ratio (Meltzer et al., 2003). Additionaly, partial and inverse agonist effects of the atypical antipsychotics on 5-HT_{1A} and 5-HT_{2C} receptors, respectively, have been implicated in their greater efficacy (Meltzer et al., 2003). For example, activation of 5-HT_{1A} receptors decreases the cataleptogenic effects of haloperidol (Invernizzi et al., 1988) while blockade of the 5-HT_{2A} receptors potentiate the conditioned avoidance response and the cataleptogenic

effects of typical antipsychotics (Wadenberg et al., 2001); the effects on locomotor

activity by atypical antipsychotics appear to involve the antagonist activity at 5-HT$_{2C}$

receptor (Prinssen et al., 2000). The binding profiles of haloperidol, clozapine and l-

sulpiride on DA D$_1$-like and D$_2$-like receptors are presented in Table A. Table B

shows the binding profile of haloperidol and clozapine on non-DA receptors.

Table B. In vitro binding affinities (Ki values, nmol/L)

	5-HT$_{1A}$	5-HT$_{2A}$	5-HT$_{2C}$	α1	α2	H1	M	5-HT$_{2A}$/D$_2$
Haloperidol	2600	28	1500	7.3	1600	>730	570	0.06
Clozapine	710	4	5	3.7	51	17	0.9	46

α1 (α1-adrenergic receptor); α2 (α2-adrenergic receptor); H1 (histaminergic receptor); M (muscarinic receptors) Adapted from Arnt and Skarsfeldt (Arnt and Skarsfeldt, 1998). The 5-HT$_{2A}$/D$_2$ ratio is taken from Blin, (Blin, 1999).

Acute intoxication with the glutamate NMDA receptor antagonists either

administered systemically or into the mPFC has become a pharmacological model of

positive and negative symptoms and cognitive impairment of schizophrenia (Javitt

and Zukin, 1991).

In *in-vitro* studies haloperidol and clozapine have a facilitatory effect on NMDA-

evoked responses in the rat pyramidal cells of the PFC (Arvanov et al., 1997). Both

drugs inhibit the evoked glutamate release from rat nerve terminals isolated from

PFC (Yang and Wang, 2005). In microdialysis studies clozapine but not haloperidol

increased extracellular glutamate in the PFC and striatum (Daly and Moghaddam,

1993; Yamamoto et al., 1994) but neither drug had any effect on PCP-induced

glutamate release (Adams and Moghaddam, 2001). Haloperidol attenuates

hyperlocomotion and some of the cognitive effects of ketamine and PCP in rats

(Ogren and Goldstein, 1994; Verma and Moghaddam, 1996) and humans (Krystal et

al., 1999b). Clozapine, but not, haloperidol consistently ameliorate PCP-induced,

deficits in sensory motor gating of startle response and impairments in working memory (Hoffman et al., 1993; Bakshi et al., 1994; Svensson et al., 1995; O'Neill et al., 1998).

In this chapter, the effects of systemic haloperidol and clozapine were compared to reveal the relative contribution of DA D_2 and "non-D_2" receptors to their action on CPP-induced deficits in the 5-CSRT task. Furthermore the contribution of DA D_2 receptors in the mPFC was studied by injecting l-sulpiride into the mPFC. L-sulpiride does not block "D_1-like" receptors (Table A) as well as adrenergic, cholinergic, GABAergic, histaminergic or 5-HT receptors to an appreciable extent (Caley and Weber, 1995).

MATERIALS AND METHODS

Animals, food deprivation, 5-CSRT task apparatus and training, procedures for measures of motor activity, surgery, microinjection and histology were as described previously (General Methods).

Drugs, treatment schedules and experimental design

CPP (Tocris, U.K) dissolved in phosphate buffered saline; clozapine (Sandoz, CH), haloperidol (Lusofarmaco, Italy) and l-sulpiride (Ravizza, Italy) were dissolved in vehicle (PBS containing 2-3 drops of 90% lactic acid; pH of the solution was adjusted to 7 with 1M NaOH).

Treatment schedules In each experiment the various combinations of different doses of a particular drug (clozapine, haloperidol and l-sulpiride) with vehicle or CPP were administered according to a Latin square design. At least two days were left between test days. Rats were always tested on these "free" days to re-establish the baseline and check the lasting effects of drugs.

Systemic haloperidol and clozapine On each test day rats were injected intraperitonealy with 2 mL/kg vehicle (PBS) or 0.03 mg/kg haloperidol and 50 min later received a bilateral injection of vehicle (1 μL) or CPP (50 ng/μL) into the mPFC. Data of 8 rats were statistically analysed and are included in the results. A different group of rats was employed to examine the effects of clozapine. On each test day rats were injected intraperitonealy with 2 mL/kg vehicle (PBS) or 2.5 mg/kg clozapine and 50 min later received a bilateral injection of vehicle (1 μL) or CPP (50 ng/μL) into the mPFC. Data of 9 rats were statistically analysed and are included in the results.

Intracortical injection of l-sulpiride Bilateral injection of vehicle (1 μL) or 200

ng/μL l-sulpiride were made into the mPFC 5 min before a microinjection of 1 μL saline or 50 ng/μL CPP into the same cortical region. Ten min later rats were given a test session on the 5-CSRT task. Data from 8 rats were statistically analysed and are included in the results.

Statistical analysis

The main dependent variables selected for analysis were: (a) the percentage of correct responses; (b) percentage of omissions; (c) mean correct response latency; (d) the number of anticipatory responses and (e) the number of perseverative responses.

The data of the experiments testing the effects of CPP in combination with different doses of either systemic or intra-mPFC drugs (clozapine, haloperidol and l-sulpiride) were analyzed by separate within-subjects two-way ANOVA with factors drug (clozapine, haloperidol or l-sulpiride) x CPP. The means of the individual treatment combinations were compared between them by Tukey's HDS test.

RESULTS

- *Systemic haloperidol reversed the CPP-induced increase in anticipatory and perseverative responding but not the accuracy deficit*

Figure 1.
Effects of haloperidol alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat was injected intraperitonealy with vehicle (V) or 0.03 mg/kg haloperidol (H) 50 min before 1 μL vehicle (+VEHICLE) or 50 ng/μL CPP (+CPP) into the mPFC. Ten min later rats started the test session. CPP and haloperidol singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 8 rats.
* P<0.05 vs. V (+VEHICLE); # P<0.05 vs. V (+CPP); (Tukey's test)

As illustrated in Fig. 1A the reduction in the percentage of correct responses induced by CPP was not prevented by administration of 0.03 mg/kg haloperidol (haloperidol x CPP, $F_{1,21}$=2.3, P>0.05; CPP, $F_{1,21}$=21.6, P=0.0001; haloperidol, $F_{1,21}$=0.81, P>0.05).

This dose of haloperidol completely reversed the CPP-induced increase in the number of anticipatory (Fig. 1B) (haloperidol x CPP, $F_{1,21}$=8.7 P=0.007; haloperidol, $F_{1,21}$=7.1

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P=0.01; CPP, $F_{1,21}$=13.9 P=0.001) and perseverative responses (Fig. 1C) (haloperidol x CPP, $F_{1,21}$=4.2 P=0.05; haloperidol, $F_{1,21}$=5.9 P=0.02; CPP, $F_{1,21}$=23.2 P=0.0001) (Fig. 1C).

- *Haloperidol had no effects on the CPP-induced increase in omissions*

As shown in Table 1 the CPP-induced increases in the percentage of omissions (haloperidol x CPP, $F_{1,21}$=0.08 P>0.05; haloperidol, $F_{1,21}$=1.1 P>0.05; CPP, $F_{1,21}$=14.7 P=0.001) and correct response latencies (haloperidol x CPP, $F_{1,21}$=0.08 P>0.05; haloperidol, $F_{1,21}$=0.05 P>0.05; CPP, $F_{1,21}$=1.92 P>0.05) were not affected by haloperidol.

Table 1. Effects of systemic haloperidol and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	9.3 ± 2.0	0.58 ± 0.02
H 0.03 + VEH	6.9 ± 1.3	0.61 ± 0.05
VEH + CPP	16.5 ± 2.7 *	0.65 ± 0.06
H 0.03 + CPP	14.6 ± 2.2	0.64 ± 0.04

Each value is the mean ± SEM of 8 rats. Haloperidol at a dose of 0.03 mg/kg (H 0.03) was injected intraperitonealy 50 min before bilateral injections of 1 µL vehicle (VEH) or 50 ng/µL CPP into the mPFC. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design. * P < 0.05 vs. VEH+VEH; (Tukey's test).

A higher dose of haloperidol (0.1 mg(kg) was also tested. However, data obtained with 0.1mg/kg haloperidol alone and in combination with 50 ng/µL CPP were excluded from the analysis and are not shown since the rats completed less than 20 trials doing mostly omissions.

- *Systemic administration of clozapine reversed the CPP-induced impairments in accuracy and anticipatory but not perseverative responding*
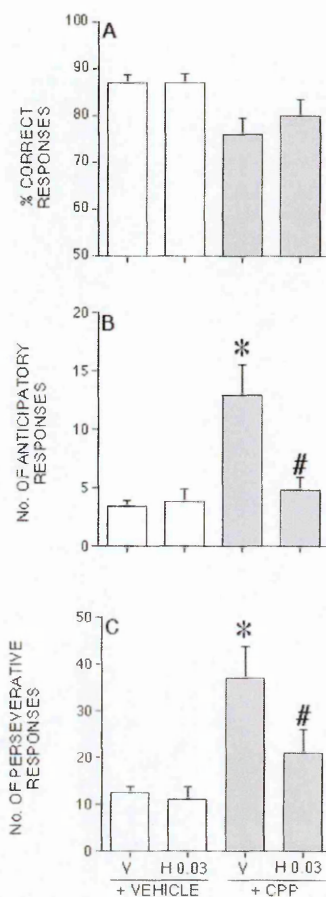

Figure 2.
Effects of clozapine alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat was injected intraperitonealy with vehicle (V) or 2.5 mg/kg clozapine (C) 50 min before 1 μL vehicle (+VEHICLE) or 50 ng/μL CPP (+CPP) into the mPFC. Ten min later rats started the test session. CPP and clozapine singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 9 rats.
* P<0.05 vs. V (+VEHICLE); # P<0.05 vs. V (+CPP); (Tukey's test)

Clozapine at 2.5 mg/kg prevented the CPP-induced reduction in the percentage of correct responses (Fig. 2A). The two-way repeated measure ANOVA showed a significant interaction between clozapine plus CPP ($F_{1,24}$=7.6 P=0.01) and significant effects of CPP, ($F_{1,24}$=10.5 P=0.003) and clozapine, ($F_{1,24}$=4.7 P=0.04). The CPP-induced increase in anticipatory responding (Fig. 2B) was abolished by 2.5 mg/kg clozapine (clozapine x CPP, $F_{1,24}$=5.8 P=0.02; clozapine, $F_{1,24}$=12.9 P=0.002; CPP,

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$F_{1,24}$=29.6 P=0.0001) whereas perseverative responding was not affected (clozapine x

CPP, $F_{1,24}$=1.3 P>0.05; clozapine, $F_{1,24}$=0.3 P>0.05; CPP, $F_{1,24}$=16.5 P=0.0005) (Fig.

2C).

- **Clozapine had no effect on CPP-induced omissions but had additive effects on correct response latency**

The increase in the percentage of omissions (Table 2) induced by CPP was not

affected by clozapine (clozapine x CPP, $F_{1,24}$ =3.6 P=0.07; clozapine, $F_{1,24}$=1.2,

P>0.05; CPP, $F_{1,24}$=4.9 P=0.04). Correct response latencies were increased by both

CPP and clozapine (clozapine, $F_{1,24}$=4.7 P=0.03; CPP, $F_{1,24}$=17.2 P=0.0004). When

administered in combination CPP and clozapine had additive effects on correct

response latency (clozapine x CPP, $F_{1,24}$=0.1) (Table 2).

Table 2. Effects of systemic clozapine and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	12.9 ± 2.0	0.52 ± 0.04
C 2.5 + VEH	26.9 ± 4.8	0.60 ± 0.05
VEH + CPP	33.3 ± 7.9 *	0.67 ± 0.05*
C 2.5 + CPP	27.9 ± 4.9	0.74 ± 0.05#

Each value is the mean ± SEM of 9 rats. Clozapine at a dose of 2.5 mg/kg (C 2.5) was injected intraperitonealy 50 min before bilateral injections of 1 µL vehicle (VEH) or 50 ng/µL CPP into the mPFC. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH; # P<0.05 vs. VEH+CPP; (Tukey's test).

- *Blockade of DA D₂ receptors in the mPFC by l-sulpiride reduced the CPP-induced impairments in accuracy and anticipatory and perseverative responding*
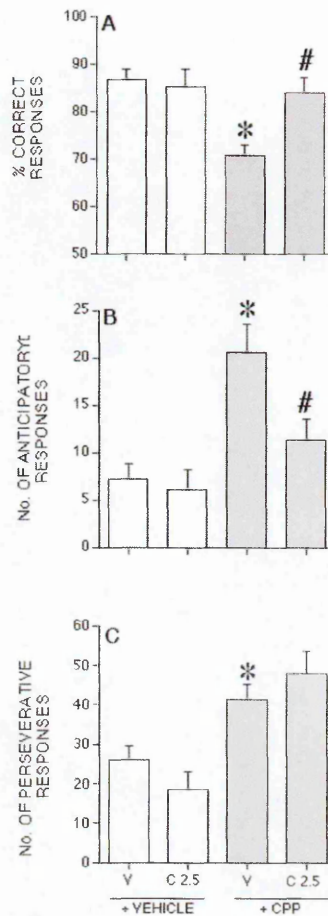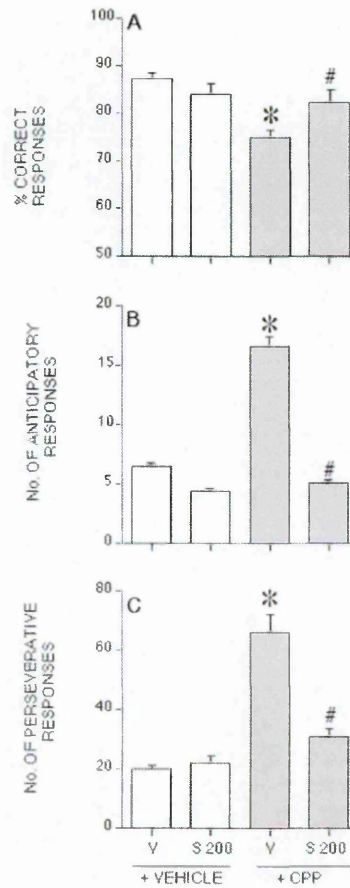

Figure 3.
Effects of l-sulpiride alone or with CPP on correct responses (A), anticipatory responses (B) and perseverative responses (C). Each rat was injected into the mPFC with vehicle (V) or 200 ng/μL l-sulpiride (S) 10 min before 1 μL vehicle (+VEHICLE) or 50 ng/μL CPP (+CPP) into the mPFC. Ten min later rats started the test session. CPP and l-sulpiride singly or combined were administered at least 48 h apart, according to a Latin-square design. The histograms show mean ± S.E.M. of 8 rats.
* P<0.05 vs. V (+VEHICLE); # P<0.05 vs. V (+CPP); (Tukey's test)

As shown in Fig. 3A the CPP-induced reduction in the percentage of correct responses was prevented by 200 ng/μL l-sulpiride. The two-way repeated measure ANOVA showed a significant interaction between l-sulpiride and CPP ($F_{1,21}$=5.4 P=0.03) and significant effects of CPP, ($F_{1,21}$=11.0 P=0.003) and but not l-sulpiride ($F_{1,21}$=0.6 P=0.4).

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L-sulpiride completely reversed CPP-induced increase in the number of anticipatory

(fig. 3B)(l-sulpiride x CPP, $F_{1,21}$=4.9 P=0.03; l-sulpiride, $F_{1,21}$=10.4 P=0.004; CPP,

$F_{1,21}$=6.6 P=0.01) and perseverative responses (Fig. 3C)(l-sulpiride x CPP, $F_{1,21}$=22.0

P<0.0001; l-sulpiride, $F_{1,21}$=17.7 P<0.0004; CPP, $F_{1,21}$=48.5 P<0.0001).

- *L-sulpiride injected into the mPFC had no effect on the CPP-induced omissions but reduced the effects on correct response latency*

The increase in the percentage of omissions (Table 3) induced by CPP was not

affected by l-sulpiride (l-sulpiride x CPP, $F_{1,21}$ =0.9 P=0.4; l-sulpiride, $F_{1,21}$=3.0,

P=0.09; CPP, $F_{1,21}$=11.5 P=0.002). As shown in Table 3, after CPP the rats were

slower to make a correct response and l-sulpiride made them faster (l-sulpiride x CPP,

$F_{1,21}$=2.1 P=0.2; l-sulpiride, $F_{1,21}$=12.4 P=0.002; CPP, $F_{1,21}$=23.5 P<0.0001).

Table 3. Effects of intra-mPFC l-sulpiride and CPP on omissions and correct response latency

TREATMENT	OMISSIONS (%)	CORRECT RESPONSE LATENCY (s)
VEH + VEH	12.9 ± 1.5	0.59 ± 0.03
S 200 + VEH	10.4 ± 1.9	0.53 ± 0.02
VEH + CPP	26.5 ± 3.8 *	0.76 ± 0.03*
S 200 + CPP	18.1 ± 3.9	0.62 ± 0.02#

Each value is the mean ± SEM of 8 rats. L-sulpiride 200 ng/µL (S 200) was injected into the mPFC 10 min before bilateral injections of 1 µL vehicle (VEH) or 50 ng/µL CPP into the same area. Ten min later the rats started the test sessions. The various doses were administered at least 48 h apart, according to a Latin-square design.
* P < 0.05 vs. VEH+VEH; # P<0.05 vs. VEH+CPP; (Tukey's test).

SUMMARY OF THE RESULTS

	Correct (%)	Anticipatory responses	Perseverative responses	Omissions (%)	Latency Correct
CPP	⇓	⇑	⇑	⇑	⇑
plus **Haloperidol** systemic	=	0	0	=	=
plus **Clozapine** systemic	0	0	=	=	+
plus **I-Sulpiride** mPFC	0	0	0	=	0

⇓ decrease; ⇑ increase; 0 reversal; = no effect; + additive effect.

DISCUSSION

The effects of CPP on performance of the 5-CSRT task are consistent with those presented in previous chapters. The main findings of the present study concern the effects of pharmacological challenges and are: first, blockade of DA D_2 receptors in the mPFC by l-sulpiride (200 ng/ L) reduced the CPP-induced accuracy deficit. Additionally an intraperitoneal injection of clozapine (2.5 mg/kg) improved accuracy of CPP-injected rats whereas intraperitoneal injection of 0.03 mg/kg haloperidol had no effect. Second, haloperidol and intra-mPFC l-sulpiride reduced CPP-induced anticipatory and perseverative over-responding while clozapine reduced anticipatory but had no effect on perseverative responding. At doses used in this study these drugs had no effect on any measure of performance in the control condition (intra-mPFC vehicle). These data implicate DA D_2 mechanisms in the PFC in the control of attention and inhibitory response control i.e. executive function. Moreover, they suggest that non-D_2 receptor mechanisms may be importantly implicated in the effects of clozapine on attention and impulsivity.

The finding that intracortical injection of l-sulpiride, but not systemic haloperidol (0.03 mg/kg) ameliorated accuracy could suggest that the dose of haloperidol used in the present study was insufficient to block DA D_2 receptors in the PFC. By contrast this dose of haloperidol reduced anticipatory and perseverative responding and the effects were similar to those of intra-mPFC l-sulpiride. A higher dose of haloperidol 0.1 mg/kg was tested but the animals injected with combined treatment (haloperidol plus CPP) stopped responding and the data collected were not reliable. Haloperidol at 0.1 mg/kg has been reported to have sedative effects; it greatly increased omissions and latency to respond correctly (Carli and Samanin, 1992). Differential occupancy of striatal versus cortical D_2 receptors after systemic haloperidol could not

explain its lack of effect on accuracy since *in-vivo* evaluation of D_2 receptor occupancy in primate brain has shown that haloperidol do not discriminate between striatal (caudate-putament) and extrastriatal (thalamus and cortical regions) DA D_2 receptors (Mukherjee et al., 2001). Haloperidol at 0.05 mg/kg occupied 85% of D_2 receptors in the caudate-putament and 74% in the frontal cortex. Similarly no difference between striatal and extrastriatal occupancy of D_2/D_3 receptors was reported in schizophrenic subjects given haloperidol (Kessler et al., 2005. Moreover, the protein structure of the D2 receptors throughout the brain is similar and so is their in-vitro affinity (Seeman et al., 1983). Haloperidol shows 10 to 20-fold higher affinity at the D_2 than D_3 receptor while l-sulpiride do not discriminate between these receptor subtypes (Sokoloff et al., 1990). It is unlikely that blockade of D_3 receptors could explain the efficacy of l-sulpiride as D_3 receptors in the mPFC are scarce (Sokoloff et al., 1990). L-sulpiride does not show appreciable binding to non-D2 receptors such as DA D_1-like (D_1 and D_5 subtypes), 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, which could have explained at least in part its superior efficacy on accuracy compared to haloperidol (Blin, 1999). In fact the D_1 antagonist SCH 23390 tended to ameliorate the accuracy of mPFC lesioned rats although the effect did not reach statistical significance (Passetti et al., 2003b). The 5-HT_{1A} agonist 8-OH-DPAT and 5-HT_{2A} antagonist M100907 reversed attentional impairment induced by CPP (present study).

In contrast to the lack of effect of systemic haloperidol, clozapine completely abolished the CPP-induced accuracy deficit. The efficacy of clozapine in abolishing the effects of CPP is unlikely to be due to a greater occupancy of D_2 receptors since it has been repeatedly shown that occupancy of these receptors is lower after clozapine than haloperidol (Mukherjee et al., 2001). Notably, after 5 mg/kg IP

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clozapine about 20% of D_2 receptors were occupied in the striatum of rats while it could be estimated a 40% occupancy after 0.03 mg/kg haloperidol. In monkeys given 9.7 mg/kg clozapine the occupancy in the striatum was about 64% and in the frontal cortex 39% (Mukherjee et al., 2001). Compared with haloperidol clozapine occupies less D_2 receptors but has greater efficacy on accuracy. Clozapine binds with relatively high affinity to other receptors and in particular to 5-HT_{2A}, 5-HT_{2C} and α-1 adrenoceptors (Table B). As shown in Chapter 5, blockade of the 5-HT_{2A} by M100907 or stimulation of 5-HT_{2C} receptors by Ro60-0175 completely reversed the CPP-induced deficit in accuracy. However, it is unlikely that the effects of clozapine are due to interaction with 5-HT_{2C} receptor as clozapine has inverse agonist effects on 5-HT_{2C} receptors; reduces the constitutive activity of this receptor (Herrick-Davis et al., 2000) and prevents the effects of Ro60-0175 on NAc DA release (Di Matteo et al., 2002). The ability of clozapine to act as an antagonist at 5-HT_{2A} receptors may help explain its greater efficacy. However, α1-adrenoceptor antagonist activity of clozapine could not be disregarded since prazosin, an α1 receptor antagonist, reversed the disruptive effects of PCP on PPI and locomotion (Mathe et al., 1996; Bakshi and Geyer, 1997).

Interestingly, the efficacy of typical versus atypical antipsychotics such as haloperidol and clozapine, respectively to counteract the behavioural syndrome induced by NMDA antagonists is debated. For example, haloperidol abolished hyperlocomotion (Swanson and Schoepp, 2002) and it partially restored short-term memory deficits (Schroeder et al., 2000). Disruption of pre-pulse inhibition (PPI) of startle response induced by non-competitive NMDA receptor antagonists is not affected by haloperidol (Swerdlow et al., 1996; Bast et al., 2001; Linn et al., 2003) whereas haloperidol was effective against the effects of competitive NMDA receptor

antagonist on PPI (Bakshi et al., 1999). The ability of clozapine to reverse the PPI deficits induced by NMDA receptor antagonists (Bakshi et al., 1994; Zhang et al., 1999; Linn et al., 2003) has been suggested to depend on its antagonist activity at the 5-HT$_{2A}$ receptor, as M100907 was shown to have similar effects (Varty et al., 1999). Interestingly, haloperidol (0.1 mg/kg) and clozapine (5 mg/kg) administered during conditioning had no effect on dizocilpine induced latent inhibition (LI) perseveration. The later has been argued to represent a deficit in attentional set shifting. However, when administered during the pre-exposure stage, clozapine but not haloperidol reversed the effects of dizolcipine on LI perseveration (Gaisler-Salomon and Weiner, 2003).

That concomitant 5-HT$_{2A}$ receptor antagonism may confer additional properties to DA D$_2$ receptor antagonists (particularly to those with low D$_2$ potency) has been shown by a series of microdialysis studies measuring extracellular DA in mPFC and NAc (Meltzer et al., 2003). Clozapine (5 mg/kg) preferentially increased DA efflux in the mPFC but not NAc while haloperidol (0.1 mg/kg) induced DA efflux was higher in the NAC than in mPFC (Meltzer et al., 2003). M100907 potentiated haloperidol and l-sulpiride-induced DA release in the mPFC and inhibited that in the NAc (Ichikawa et al., 2001; Liegeois et al., 2002). These neurochemical findings are consistent with behavioural studies showing that ritanserin a 5-HT$_{2A}$/5-HT$_{2C}$ receptor antagonist, augments the effects of raclopride on conditioned avoidance (Wadenberg et al., 1996) and the addition of ritanserin to low-dose haloperidol treatment improved negative symptoms (Duinkerke et al., 1993).

The CPP-induced increase in anticipatory and perseverative responding on the 5-CSRT task was reduced by intra-mPFC l-sulpiride and systemic haloperidol suggesting that DA D$_2$ mechanisms in the mPFC importantly modulate inhibitory

response control. As haloperidol was given systemically the precise locus of its action is unclear, however it could not be excluded that it exerted its effect by blocking DA D_2 receptors in the mPFC. However, in the study by Passetti et al. (Passetti et al., 2003b) systemic l-sulpiride (15 and 30 mg/kg IP) had no effect on perseverative over-responding induced by mPFC or PrL-IL lesions. These data add to the previous findings showing that DA mechanisms are importantly involved in the modulation of frontal executive functions (inhibitory response control) (Robbins, 2000a). For example, in the 5-CSRT task, d-amphetamine injected into the NAc increased anticipatory responses (Cole and Robbins, 1987) while DA depletion in ventral striatum had opposite effects (Cole and Robbins, 1989). Dorsal striatal DA depletion but also systemic d-amphetamine greatly increased perseverative responding (Baunez and Robbins, 1999). However, depletion of DA from the PFC or intra-mPFC l-sulpiride in rats had little or no effect on the performance of the 5-CSRT task (Robbins, 2002), while stimulation of D_1 receptors improved performance when the baseline performance was low (Granon et al., 2000). It should be noted that mPFC l-sulpiride had no effect on baseline performance of control rats thus replicating the findings by Granon et al. (Granon et al., 2000). In comparison, depletion of DA in the dorsolateral PFC of marmosets improved attentional set shifting, impaired spatial delayed response (a test of working memory) (Roberts et al., 1994) and had no effect on sequencing of spatial responses in a different working memory paradigm (Collins et al., 1998). Several recent studies have reported that treatments that increase DA release in the mPFC improve rather than impair set-shifting (Tunbridge et al., 2004; Hatcher et al., 2005). However, activation of DA transmission may improve performance when it is low but impair it when it is high. In fact, the D_1 agonists enhanced poor working memory in difficult

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conditions but impaired good memory in easy condition (Zahrt et al., 1997; Chudasama and Robbins, 2004). Blockade of DA D_2 receptors in the mPFC impaired attentional set shifting by increasing perseverative errors whereas agonists at D_2 receptors had no effect (Ragozzino, 2002; Floresco et al., 2005). Similarly Mehta et al. (Mehta et al., 2004) showed a trend for attentional set shifting to be impaired following l-sulpiride in human volunteers.

The effects of l-sulpiride and haloperidol on perseverative responding were similar to those found after stimulation of $5-HT_{1A}$ receptors in the mPFC (Chapter 5). As discussed in Chapter 5, 8-OH-DPAT could have decreased perseverative responding by an interaction with DA system. Moreover, 8-OH-DPAT significantly increased the ability of l-sulpiride to increase DA release in the mPFC (Ichikawa and Meltzer, 1999b) and adding a $5-HT_{1A}$ receptor agonist, tandospirone, to typical antipsychotics treatment in patients improved their performance in a test of executive functioning such as Wisconsin Card Sorting Test and in a test of verbal memory compared to those that did not receive tandospirone (Sumiyoshi et al., 2001a).

The fact that clozapine reduced anticipatory, but not perseverative, responding induced by CPP could suggest that non-D_2 receptor mechanisms may have played a role in its effects. Clozapine show a high $5-HT_{2A}/D_2$ affinity ratio *in-vitro* (Table B) and a high potency in occupying $5-HT_{2A}$ receptors in the cortex versus D_2 receptors in the striatum and the limbic regions in *in-vivo* binding studies (Stockmeier et al., 1993; Sumiyoshi et al., 1995). Thus, at the dose used in this study the effects of clozapine may be mediated via its interaction with $5-HT_{2A}$ receptors in the mPFC. This is consistent with findings showing that blockade of $5-HT_{2A}$ receptors in the mPFC has no effects on CPP-induced perseverative responses but reduce anticipatory responding (Chapter 5). Comparing the effects of clozapine with those

of 8-OH-DPAT suggests that the effects of clozapine are unlikely to be due to its agonist activity at 5-HT$_{1A}$ receptors since 8-OH-DPAT reduced CPP-induced perseverative responses (Chapter 5).

The neurochemical mechanisms responsible for the effects of these antipsychotic drugs observed in the present study are unclear. As reviewed in the General Introduction, like other NMDA receptor antagonists CPP increase glutamate, DA and 5-HT release in the mPFC. Activation of DA and 5-HT mechanisms, in conjunction with increased transmission at glutamate non-NMDA receptors, has been associated with the behavioural syndrome induced by NMDA antagonists. In fact, bearing in mind all the limitations discussed previously, antagonists at AMPA, DA D$_2$ and 5-HT$_{2A}$ receptors were able to reduce the behavioural deficits. Moreover, decreasing glutamate efflux in the mPFC induced by NMDA antagonists has been suggested to be a viable means of abolishing the behavioural deficits (discussed in Chapter 4). However, microdialysis studies in awake rats show that haloperidol (0.1 mg/kg) and clozapine (5 mg/kg) had no effect on PCP-induced glutamate efflux in the mPFC (Adams and Moghaddam, 2001). Moreover, as shown in Chapter 5 and 6 the ability of M100907 and 8-OH-DPAT to reduce CPP-induced glutamate and 5-HT efflux appears to be independent of their effects on anticipatory and perseverative responding. The present findings with DA D$_2$ antagonists might suggest an interaction between 5-HT and DA mechanisms in the in the modulation of PFC functions. However, it should be noted that catecholamine-independent mechanisms might be involved in the behavioural effects of NMDA antagonists. Thus the locomotor stimulant effects of PCP and dizolcipine are still present in monoamine-depleted rats (Carlsson and Carlsson, 1989; Martin et al., 1998b; Swanson and Schoepp, 2002) and in dopamine-deficient (DD) mice in which tyrosine hydroxylase

is selectively inactivated in DA neurons (Chartoff et al., 2005). Nevertheless in DA-depleted rats PCP-induced effects on locomotion are decreased (Swanson and Schoepp, 2002) and restoration of DA transmission in DD mice enhanced their locomotor response to PCP and dizocilpine suggesting that glutamate and DA can act cooperatively to induce behavioural deficits. The fact that the ability of clozapine to reduce PCP-induced locomotion was not dependent on endogenous DA or 5-HT whereas the effects of ketanserine or M100907 were abolished in PCPA-pretreated rats adds further complexity to the mechanisms of action of clozapine. In contrast, the effects of haloperidol were reduced in DA but not 5-HT-depleted rats (Swanson and Schoepp, 2002). Nevertheless, the specific alterations in mesocortical DA function induced by CPP in the mPFC and its modulation by antipsychotic drugs deserve further characterization.

Finally, a direct interaction of D_2 with NMDA and GABA receptor mechanisms in the PFC could not be disregarded as blockade of D_2 receptors in the mPFC by l-sulpiride reduced the effects of CPP. In the mPFC, DA D_2 receptors are present on pyramidal cells, local circuit interneurons, and presynaptic terminals (Vincent et al., 1993; Sesack et al., 1995). The data in-vitro suggest that high micromolar levels of DA are required to activate D_2 receptors in the PFC that subsequently decrease NMDA and GABA currents (Zheng et al., 1999; Seamans et al., 2001; Trantham et al., 2003 ; Trantham-Davidson et al., 2004). It could be speculated that elevated and sustained DA release induced by NMDA antagonists activate D_2 receptors and further reduce NMDA and GABA transmission. Thus blocking these DA D_2 receptors might prevent the inhibition of NMDA and GABA transmission and re-establish normal PFC functioning.

CHAPTER 8. CONCLUDING DISCUSSION

The goal of this thesis was to provide a better understanding of the role played by 5-HT receptor mechanisms in cognitive functions of the PFC. The methodological approach adopted was to study to what extent the impairments in attention and inhibitory response control induced by manipulations of PFC glutamate NMDA transmission function may actually depend on altered 5-HT neuromodulation occurring in the PFC. To suggest neuropharmacological specificity of this neuromodulation of attention and inhibitory response control the experimental work compared the effects of relatively specific pharmacological agents on 5-HT, glutamate and DA systems on a common behavioural paradigm, the 5-CSRT task, in which attentional functioning and different aspects of inhibitory response control are relatively independent (Robbins, 2002). An important aspect of these studies was to examine whether and how the NMDA receptor antagonists-induced behavioural deficits in the 5-CSRT task may be accounted for by enhanced glutamate and 5-HT release in the PFC. Thus, *in-vivo* neurochemistry has attempted to provide some insight into the causal relationship between extracellular changes in glutamate and 5-HT and the behavioural effects induced by 5-HT receptors agents. Therefore, the experimental work contained in this thesis investigated: first, the behavioural and neurochemical effects of blockade of NMDA receptors in the mPFC. Second, it examined whether activation of mechanisms capable of limiting excitatory transmission might prevent the behavioural impairments. Third, the effects of 5-HT receptor agents on CPP-induced behavioural deficit and changes in glutamate and 5-HT release were studied. Fourth, the effects of DA D_2 receptor antagonists on CPP-induced deficit in attentional performance were examined.

The main points that have emerged from this experimental work are schematically presented in Table 1 and are as follows:

- Blockade of glutamate NMDA receptors in the mPFC by a competitive NMDA receptor antagonist, CPP, impairs attentional functioning, inhibitory response control and increases glutamate and 5-HT release (Chapter 3).

- Activation of $mGlu_{2/3}$ receptors in the mPFC by local application of LY379268 by presumably suppressing excitatory glutamate transmission abolishes the accuracy deficit induced by blockade of NMDA receptors in the same area (Chapter 4). Blockade of AMPA receptors in the mPFC by a competitive antagonist, NBQX, does not antagonise the effects of CPP, suggesting that concomitant activation of AMPA receptors is not necessary for the effects of NMDA receptor antagonists on attentional performance deficits (Chapter 4).

- An especially important finding is that $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptors regulate in opposite fashion attentional functioning while having dissociable roles on inhibitory response control (Chapter 5). Stimulation of $5\text{-}HT_{1A}$ receptors reduces "compulsive" perseverative responding but has no effect on impulsivity and *vice-versa* blockade of $5\text{-}HT_{2A}$ receptors reduces "impulsive" anticipatory responses but not perseveration (Chapter 5). Agonists at $5\text{-}HT_{1A}$ or antagonists at $5\text{-}HT_{2A}$ receptors such as 8-OH-DPAT and M100907 respectively, reduced the CPP-induced rise in glutamate and 5-HT release (Chapter 6). Together these data suggest that suppression of cortical glutamate release by these 5-HT agents hardly account for their effects on anticipatory and perseverative responding. Agonist at $5\text{-}HT_{2C}$ receptors ameliorated accuracy and decreased impulsivity but have no effect on

perseveration suggesting that $5\text{-}HT_{2C}$ receptors exert a functionally opposing role to $5\text{-}HT_{2A}$ receptors in the control of attention and "impulsive", anticipatory responding (Chapter 5).

- Blockade of DA D_2 receptors in the mPFC reduced CPP-induced attentional impairment and anticipatory and perseverative over-responding. Intriguingly, a mixed $5\text{-}HT_{2A}/D_2$ antagonist clozapine ameliorated accuracy and reduced "impulsive", anticipatory responding but had no effect on perseveration. These effects of clozapine were similar to those found after blockade of $5\text{-}HT_{2A}$ receptors by M100907 which does not have appreciable affinity for DA D2 receptors, suggesting that the effects of clozapine may be best explained as resulting from blockade of $5\text{-}HT_{2A}$ receptors in the mPFC (Chapter 7).

- Activation of $5\text{-}HT_{1A}$ or blockade of DA D_2 receptor mechanisms in the mPFC prevented compulsive perseveration while $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors appeared particularly involved in the control of impulsivity.

- Although hyperactivity of glutamatergic neurotransmission by itself is not sufficient to explain the loss of inhibitory response control the data reveal a likely association between increased glutamate release in the mPFC and attentional dysfunctioning.

TABLE 1.
Effects of CPP plus glutamate (+ GLU), serotonin (+ 5-HT) and dopamine (+ DA) agents on accuracy, "impulsivity", "compulsivity" and GLU and 5-HT release

	Accuracy	Impulsivity	Compulsivity	GLU	5-HT
CPP	⇓	⇑	⇑	⇑	⇑
+ GLU agents					
LY379268 sys	0	0	=		
LY379268 mPFC	0	=	=		
NBQX mPFC	=	=	=		
+ 5-HT agents					
M100907 sys	0	0	=	0	0
M100907 mPFC	0	0	=	0	
8-OH-DPAT mPFC	0	=	0	0	0
Ro 60-0175 sys	0	0	=		
+ DA agents					
Haloperidol sys	=	0	0		
Clozapine sys	0	0	=		
l-Sulpiride mPFC	0	0	0		

⇓ reduction; ⇑ increase; 0 reversal; = no effect;

Abbreviations: sys=systemic administration; mPFC=injected into the mPFC

- **Methodological issues**

In this thesis pharmacological, behavioural and neurochemical techniques have been used. The advantages and limits of techniques employed have been discussed in Chapter 2 (General Methods). The behavioural studies presented in this thesis have used the 5-CSRT task to study the role of 5-HT and other neurochemical mechanisms in attentional and executive function. The value of this task is that is an analogue of the human Continuous Performance Test and thus allows a substantial link to human measures of attention. Furthermore, the various measures of

performance such as attentional accuracy and different aspects of inhibitory response control (i.e. *executive function*) such as "impulsive" anticipatory responses and "compulsive" perseveration are relatively independent. This property of the task permits identification of neuromodulatory mechanisms that selectively affect these different processes of attention and response control. The additional advantage of employing this task is that it has been extensively used for measuring the effects of drugs and other manipulations such as selective lesions of monoaminergic and cholinergic systems and excitotoxic lesions of various cortical regions among which mPFC and its different sub-regions (Robbins, 2002). These data form the basis to which present findings may be compared and have been reviewed in the Introduction (Chapter 1). This comparison may provide significant insights into the role that different neuromodulatory mechanisms in the mPFC may play in various aspects of attentional performance in this task.

A constraint of the present studies is the fact that only the 5-CSRT task has been used. This limit interpretation as it does not enable generalization to be made about the underlying psychological processes. There are many other tests of attention for rodents that tap into different aspects of attentional and executive functions such as test of vigilance and divided attention (McGaughy et al., 1994; McGaughy and Sarter, 1995) and extradimentional set shifting (Birrell and Brown, 2000) which may be particularly well suited to study executive function (Dias et al., 1997; Robbins, 1998). A comparison with these tasks in the same experimental design might reveal similarities and differences in the neuromodulation of vigilance, divided attention and attentional-set shifting and thus provide a more adequate representation of the complexity of attentional dysfunction in schizophrenia.

A central issue in applying the microdialysis technique to the study of drug effects on neurotransmitter release in general, and glutamate release in particular, is whether the recovered neurotransmitters reflect "true" neuronal release. This issue was discussed at length in Chapter 2 (General Methods). Here it is important to note that although the basal levels of glutamate were insensitive to the effects of TTX the effects of CPP on extracellular glutamate were TTX-dependent and thus may reflect neuronal release (Chapter 3). It should be noted that the effects of various pharmacological agents on attentional performance and extracellular glutamate and 5-HT were measured in different groups of rats. Rats used in microdialysis experiments were not food deprived and were not performing the task. Experimental conditions, such as food deprivation and behavioural arousal, may be important determinants of neurotransmitter release (Ungerstedt, 1991; Di Ciara, 2005). Additionally in microdialysis experiments drugs were administered through the probe for long periods of time (about 120 min) and thus the brain concentration of drugs in microdialysis and behavioural studies (where drugs were administered by relatively rapid infusion lasting 2 min) are not directly comparable. Therefore, inferences about causal relationships between neurotransmitter release and behaviour are only indirect and are based on the demonstration of similar or dissociable effects of various drugs on behaviour and glutamate and 5-HT release.

- **Role of glutamate transmission in the mPFC: attentional performance and glutamate release**

From this work (Table 1), it appears clear that glutamate transmission in the mPFC is particularly important for the control of performance on the 5-CSRT task. This is supported by data shown in Chapter 3 and Chapter 4 that blockade of glutamate

NMDA or AMPA receptors in the mPFC is sufficient to impair attentional functioning (indexed by accuracy) and to cause a loss of inhibitory response control as shown by increased "impulsivity" and "compulsive" perseveration. The effects of NMDA receptor antagonists in the mPFC are remarkably similar in nature and magnitude to the deficits observed in the same task after either systemic administration of NMDA receptor antagonists (Higgins et al., 2003b; Le Pen et al., 2003) or the effects of excitotoxic lesions of the mPFC (Muir et al., 1996; Passetti et al., 2002). Previous evidence has suggested distinct anatomical substrate in the PFC for attention (anterior cingulate, ACg) and impulsive (infralimbic, IL) and compulsive behaviours (prelimbic, PrL and orbitofrontal, OF) in this task (Passetti et al., 2002; Chudasama et al., 2003) and a specific role of glutamate transmission in the IL in the modulation of impulsivity (Murphy et al., 2005). Thus the pattern of deficits observed in this study after CPP injections into a single site in the mPFC may be the sum of somewhat independent deficits in more "dorsal" attentional and "ventral" inhibitory response control functions of the prefrontal cortex.

The present results parallel those showing that intra-mPFC NMDA and AMPA receptor antagonists disrupt attentional set shifting which requires several similar components of cognition to those involved in the 5-CSRT task such as selective and sustained attention, attentional disengagements, response selection and inhibition (Stefani and Moghaddam, 2002; Stefani et al., 2003; Stefani and Moghaddam, 2003). Together, they provide strong evidence for the involvement of mPFC glutamate system in executive attentional processes that enable accurate response selection in the face of distraction and interference (Shallice, 1982; Robbins, 1998).

The NMDA receptor antagonist CPP had no effect on acquisition of a spatial discrimination (Appendix 1), which is particularly sensitive to disruption of

glutamate NMDA transmission in the hippocampus (Carli et al., 1999) whereas manipulation of hippocampus had little effects on 5-CSRT task performance (Kirkby and Higgins, 1998) thus suggesting a degree of behavioural and anatomical selectivity.

Hypoactivity of glutamate transmission due to postsynaptic blockade of glutamate NMDA receptors on pyramidal neurons may account for the observed deficits. However, various findings demonstrate that the NMDA receptor antagonists may exert at least in part their effects by *hyperactivation* of glutamate neurotransmission in the mPFC as measured by firing activity of pyramidal neurons (Jackson et al., 2004) and extracellular glutamate (Moghaddam et al., 1997; Moghaddam and Adams, 1998). Indeed, disinhibition of glutamate transmission may account for the increase in human frontal cortex metabolism and resting cerebral blood flow after ketamine (Breier et al., 1997; Vollenweider et al., 1997; Holcomb et al., 2005). Consistent with these studies are findings presented in Chapter 3 showing that the competitive NMDA receptor antagonist CPP administered into the mPFC enhanced glutamate release. As discussed in Chapter 3 the effect of CPP on extracellular glutamate may be mediated by direct or indirect suppression of cortical GABArgic transmission, which in turn enhances the release of glutamate from glutamate neurons under control of GABA receptors. These "disinhibited" neurons proceed to release abnormally high levels of glutamate (Olney et al., 1991). However, it could not be excluded that extracellular glutamate in the mPFC is increased, but glutamatergic transmission along corticostriatal efferent systems, is reduced. The striatum has been involved in the performance of a 5-CSRT task (Rogers et al., 2001) and the broad behavioural syndrome is consistent with some convergence of input into the medial striatum from the mPFC system (Christakou et al., 2001) that

clearly control separate elements of performance such as accuracy, "impulsivity" and perseveration.

In a preliminary experiment using immunocytochemistry it was found that blockade of NMDA receptors in the mPFC increased phosphorylation of the transcriptional factor CREB locally whereas in the medio-dorsal striatum the phospo-positive CREB cells were significantly reduced (Appendix, 2). Other studies are needed to associate the changes in CREB phosphorylation to changes in behaviour induced by disregulation of glutamate NMDA transmission.

- **Role of enhanced glutamate release and stimulation of AMPA receptors in attentional performance deficit**

Intriguingly, activation of $mGlu_{2/3}$ receptors in the mPFC by LY379268 prevented the accuracy deficit but had no effect on the loss of inhibitory control such as "impulsivity" and perseveration (Table 1). However, systemic LY379268 had some additional effects. Specifically it reduced CPP-induced impulsivity but not perseverative responding. The findings that systemic LY379268 reduced impulsivity in CPP-injected rats present an interesting parallel with those reported after systemic 5-HT$_{2A}$ receptor antagonist, M100907 (Table 1). Despite the suggested functional analogy between mGlu$_{2/3}$ agonists and 5-HT$_{2A}$ antagonists (Aghajanian and Marek, 1997) the underlying cellular mechanisms may be different. Moreover, the effects of M100907 may be fully explained by blockade of 5-HT$_{2A}$ receptors in the mPFC while the effects of LY379268 are presumably due to activation of mGlu$_{2/3}$ receptor mechanisms in the mPFC. The effects of LY379268 are compatible with previous studies showing that $mGlu_{2/3}$ agonists such as LY379268 and LY354740 reduced behavioural deficits induced by NMDA receptor

antagonists such as impulsivity in mice performing a 5-CSRT task (Greco et al., 2005) and impairments in working memory in rats (Moghaddam and Adams, 1998). In the present study the ability of LY379268 to prevent CPP-induced increase in glutamate release was not determined but LY379268 injected systemically or delivered locally in the mPFC prevented ketamine-induced raise in extracellular glutamate (Lorrain et al., 2003a). Other studies have also shown that a structurally related mGlu$_{2/3}$ agonist LY354740 prevented the increase in firing rate and extracellular glutamate induced by NMDA receptor antagonists (Moghaddam and Adams, 1998; Homayoun et al., 2005). Thus it could not be excluded that LY379268 reduced accuracy deficit by preventing glutamate release.

As reviewed in Chapter 4, at the synaptic level mGlu$_{2/3}$ receptor agonists act at presynaptic mGlu$_2$ autoreceptors and limit the excitatory glutamate transmission and glutamate release (Anwyl, 1999; Cartmell and Schoepp, 2000; Schoepp, 2001) but they may also influence postsynaptic excitability in glutamatergic neurons (Tyszkiewicz et al., 2004). Their presence in glial wrappings of synapses (Petralia et al., 1996) might also contribute to the regulation of synaptic efficacy (Oliet et al., 2001). Thus activation of these receptors may limit the excessive glutamate release by actions on multiple mechanisms.

The mGlu$_{2/3}$ receptors are also present at hetero-synapses, where they control the release of neurotransmitters such as GABA, monoamine and ACh (Cartmell and Schoepp, 2000). Although, the effects of LY379268 may be mediated through activation of GABA release (Greenslade et al., 1999) a significant involvement of monoamine release in its present effects is unlikely, as LY379268 injected into the mPFC has no effect on 5-HT or DA release. Doses 30 to 100-fold higher than those used in the present studies were reported to increase 5-HT and DA release. In

addition, the behavioural syndrome elicited by NMDA receptor antagonists is independent of monoamine levels and LY379268 is still able to prevent behavioural deficits in monoamine-depleted rats (Swanson and Schoepp, 2002).

That the negative impact of NMDA receptor blockade on attention and inhibitory response control were not caused by stimulation of AMPA receptors (due to enhanced extracellular glutamate) (Moghaddam et al., 1997) is shown by the lack of effects of a competitive AMPA receptor antagonist NBQX injected into the mPFC. As discussed in the Chapter 4, the ability of AMPA receptor antagonists to reduce the effects of NMDA receptor antagonists is not clearly established; antagonism, synergistic and no effects have been reported. However, these data do not confirm the hypothesis advanced by Moghaddam et al. (Moghaddam et al., 1997) that over-stimulation of AMPA receptors under condition of blocked NMDA receptors may account for all behavioural deficits.

- **Role of 5-HT neuromodulation in attentional performance and glutamate release**

The experiments schematically illustrated in Table 1 provide considerable and novel evidence that through a complex neuromodulation of the glutamatergic mPFC functions the 5-HT system participate in the control of attention and inhibitory response control. This complex 5-HT neuromodulation is demonstrated by opposing role of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors on attentional functioning and their dissociable role in different aspects of inhibitory response control. In fact impairments in attentional functioning induced by NMDA receptor antagonist were completely abolished by systemic M100907 and intra-mPFC 8-OH-DPAT and M100907. Clearly, the opposition between the 5-HT_{1A} and 5-HT_{2A} receptor

subtypes suggests that the improvement produced by M100907 and 8-OH-DPAT on CPP-induced accuracy deficit may have resulted from a functionally antagonistic activity of these receptors on a potentially common mechanism.

These results are generally compatible with previous pharmacological studies in normal animals that suggested an opposing role of 5-HT_{1A} and 5-HT_{2A} receptors in the mPFC in attentional functioning (Winstanley et al., 2003b). However, 5-HT_{1A} receptors appear to impair or facilitate attentional functioning depending on their localization in the DR where they function as presynaptic autoreceptors or on their postsynaptic localization in the mPFC (Carli and Samanin, 2000; Winstanley et al., 2003b) where they directly inhibit pyramidal neurons (Nicoll et al., 1990). Accordingly, opposite behavioural effects of 8-OH-DPAT were often reported depending on whether the drug was administered directly into the DR or into its projecting areas (Carli et al., 1995a; Warburton et al., 1997; Carli et al., 1998).

Clinical and experimental evidence shows that the tendency to act without foresight, i.e. impulsivity may manifest itself in several ways (Evenden, 1999, 1999a). Specifically, in reaction time task such as the 5-CSRT task, impulsivity may take the form of increased anticipatory responding (Evenden, 1999a). As discussed extensively in the General Introduction, pharmacological and lesion studies show that 5-HT system exert a definite, albeit complex, action on impulsivity depending upon the importance of various behavioural factors, receptor sub-types and brain areas involved. As shown in Table 1, "impulsivity" induced by blockade of NMDA receptors in the mPFC was reduced by M100907 and Ro60-0175 but not 8-OH-DPAT. In addition M100907 and Ro60-0175 reduced the anticipatory responding under conditions of increased demands on response inhibition such as when inter trial intervals (ITI) of increasing durations were presented unpredictably (Chapter 5).

In accordance with previous pharmacological studies (Evenden, 1999a; Robbins, 2002; Higgins et al., 2003a; Winstanley et al., 2004b) these results demonstrate that by playing an opposing role the $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors are particularly involved in mechanisms that control the inhibition of highly prepotent responses in anticipation of reward. The functional opposition of $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors extends also to attentional accuracy. Consistently, stimulation or blockade of $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors have been shown to exert opposite effects on DA release (Di Matteo et al., 1998; Millan et al., 1998; Di Matteo et al., 2001), locomotor and motivational effects of cocaine (Fletcher et al., 2002) and behavioural effects of NMDA receptor antagonists (Martin et al., 1998b; Hutson et al., 2000; Higgins et al., 2003a).

Perseverative behaviour represents another form of inhibitory deficit (more akin to "compulsive" rather than "impulsive" behaviour), in which rats continue to respond in the holes even following signals that food is available. The enhanced tendency to perseverate is presumably an expression of behavioural inflexibility after blockade of NMDA receptors in the mPFC, preventing the rats from suppressing irrelevant responses and shifting their attention to the next relevant response in a well-learned sequence. This perseverative behaviour is akin to that reported in frontal-lobe and schizophrenic patients (Owen et al., 1993; Lyon et al., 1988; Goldberg et al., 1994), and animals with PFC lesions (Mishkin, 1964; Chudasama et al., 2003).

As shown in Table 1, activation of $5\text{-}HT_{1A}$ receptors in the mPFC had no effect on impulsivity but reduced "compulsive" perseveration whereas blockade of $5\text{-}HT_{2A}$ receptors in the mPFC had no effect. Similarly to M100907, systemic Ro60-0175 had no effect on perseverative over-responding. Previous pharmacological studies with $5\text{-}HT_{1A}$ agonists (Carli and Samanin, 2000; Winstanley et al., 2003b) or with

other 5-HT ligands (Robbins, 2002) have never reported effects on perseverative responding in this task. Although no increase in perseverative responding has been reported in rats depleted of 5-HT (Harrison et al., 1997a; Carli and Samanin, 2000), Winstanley et al. (Winstanley et al., 2004b) showed an increase in perseveration but in their conditions perseverative responses were not punished.

These results clearly demonstrate the selectivity of executive control processes and indicate that impulsivity and perseveration may be dissociated by 5-HT$_{1A}$ and 5-HT$_{2A}$/5-HT$_{2C}$ receptor mechanisms in the PFC. Evidently response inhibition operates independently for preparing responses and for monitoring performance, thus providing behavioural flexibility. This conclusion is generally consistent with emerging evidence of distinct neural systems in different sub-regions of the PFC associated with the control of "impulsive" behaviour and "compulsive" behaviours.

The 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors are highly co-localised in glutamatergic pyramidal neurons in ACg and IL region of the mPFC. As reviewed in the Introduction they appear to be confined to different domains of pyramidal neurons. Thus, it can be speculated that the segregation of 5-HT$_{2A}$ receptors to apical dendrites of glutamatergic pyramidal neurons (Jakab and Goldman-Rakic, 1998) and to GABAergic interneurons specialised in the perisomatic inhibition of pyramidal cells (Jakab and Goldman-Rakic, 2000) affects excitatory glutamate input (Aghajanian and Marek, 1997) whereas 5-HT$_{1A}$ receptors in the axon hillock (DeFelipe et al., 2001; Czyrak et al., 2003) can suppress the generation of action potentials along the axon and influence the activity in their subcortical projection areas.

That enhanced glutamate and 5-HT release may not be involved in CPP's effects on anticipatory and perseverative responses is demonstrated by findings showing that while both M100907 and 8-OH-DPAT prevented CPP-induced glutamate and 5-HT

release their effects on impulsivity and "compulsive" perseverative responding were completely dissociated (Table 1). This suggestion is further supported by data showing that activation of $mGlu_{2/3}$ receptors locally in the mPFC which as discussed previously inhibits glutamate release has no effect on impulsivity and perseveration induced by NMDA receptor blockade in the same area.

The data show that the ability of CPP to increase anticipatory responding is independent of its effects on 5-HT release and suggest that various mechanisms may be involved in the control of "impulsivity" in the 5-CSRT task. However, impulsivity in a 5-CSRT task is positively associated not only with high 5-HT turnover and release in the mPFC but DA mechanisms has also been shown to be importantly involved (Cole and Robbins, 1987; Puumala and Sirvio, 1998; Baunez and Robbins, 1999; Dalley et al., 2002). As discussed in General Introduction the role of 5-HT and DA mechanisms in "impulsivity" is complex and the various ways these might be involved in this multifaceted phenomena remain to be fully elucidated. As shown repeatedly in previous pharmacological experiments but also in this study the $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors are definitely involved in anticipatory responding in this task. However, more problematic appears the exact role of endogenous 5-HT as lesion studies show that depletion of 5-HT, causes a clear increase in impulsivity. The present and other studies (Winstanley et al., 2004b) may suggest that through stimulation of $5\text{-}HT_{2C}$ receptors 5-HT exerts tonic inhibitory control over impulsive anticipatory responding. Interestingly, M100907 does not reduce anticipatory responding in animals depleted of 5-HT (Winstanley et al., 2004b) and the ability of $5\text{-}HT_{2A}$ antagonists to reduce the effects of NMDA antagonists is prevented in animals depleted of 5-HT but not DA (Martin et al., 1998b; Swanson and Schoepp, 2002). Thus, it could be suggested that blockade of

$5\text{-}HT_{2A}$ receptors may unveil the participation of other 5-HT receptors in control of impulsivity. As shown in these experiments, activation of $5\text{-}HT_{1A}$ receptors had no effect while activation of $5\text{-}HT_{2C}$ receptors decreased anticipatory responding.

The exact mechanism by which 8-OH-DPAT might reduce CPP-induced perseverative responding is at present unclear. However, it does not do it by reducing CPP-induced glutamate or 5-HT release. As discussed in Chapter 5 reducing but not increasing 5-HT function in the PFC leads to response perseveration in tasks such as reversal learning (Clarke et al., 2004; Clarke et al., 2005), decision-making (Rogers et al., 1999a; Rogers et al., 1999b) and in some instances in a 5-CSRT (Winstanley et al., 2004b). However, inflexible responding on a discrimination reversal task is one example of response rigidity seen after frontal lobe damage and may not correspond to perseverative responding in the 5-CSRT task. Moreover, these two types of response perseveration may differ in their modulation by ascending monoamine systems innervating the PFC.

Interestingly, recent studies show that $5\text{-}HT_{1A}$ receptors modulate the AMPA receptor currents through inhibition of Ca2+ Calmodulin-dependent Kinase II (CaMKII) (Cai et al., 2002). However, it is unlikely that 8-OH-DPAT by decreasing AMPA receptor currents, reversed the behavioural effects of CPP, since the AMPA receptor antagonist, NBQX had no effect. In the discussion of these data in Chapter 5 it has been suggested that 8-OH-DPAT might control perseverative responding through some interaction with DA mechanisms. This suggestion is supported by the analogous effects of DA D_2 receptor antagonists l-sulpiride and haloperidol (Table 1).

- **Role of DA D$_2$ neuromodulation in attentional performance**

The major findings of the experiments with haloperidol, clozapine and l-sulpiride are summarized in Table 1. l-Sulpiride was administered into the mPFC. The most important finding of these experiments is that blockade of DA D$_2$ receptors in the mPFC reduced CPP-induced "compulsive" perseverative responding. However, systemic l-sulpiride had no effect on perseveration in mPFC lesioned rats (Passetti et al., 2003b) whereas in the present study systemic haloperidol reduced perseverative over-responding of CPP-injected rats. Intriguingly, systemic clozapine, which is also a DA D$_2$ receptor antagonist, did not reduce perseverative responding.

These data add to the previous findings showing that DA mechanisms are importantly involved in the modulation of frontal "executive" functions (reviewed in General Introduction). The mesocortical DA system which does not appear to participate in the control of perseverative responding in a 5-CSRT task is particularly involved in the executive control of attention in task such as attentional set formation and set shifting (Robbins, 2000b; Crofts et al., 2001) and in working memory, which may be viewed as a component of executive function (Goldman-Rakic, 1987). As reviewed in Chapter 7, pharmacological studies manipulating DA release or DA receptors in the PFC have often reported conflicting results in tasks dependent on PFC functions. An analysis of these studies suggests a rather complex modulation of executive function by mesocortical DA system depending on various factors such as the underlying state of animal, the nature of the task and baseline levels of performance (Robbins, 2005). All these studies were performed in normal animals and it could be speculated that under different conditions of PFC functions such as those caused by blockade of glutamate NMDA receptors and a consequently highly enhanced DA release in this area, blocking these DA receptors might

ameliorate performance. In the mPFC, DA D_2 receptors are present on pyramidal cells, local circuit interneurons, and presynaptic terminals (Vincent et al., 1993; Sesack et al., 1995). The data *in-vitro* suggest that high micromolar levels of DA are required to activate D_2 receptors in the PFC that subsequently decrease NMDA and GABA currents (Zheng et al., 1999; Seamans et al., 2001; Trantham et al., 2003 ; Trantham-Davidson et al., 2004). It could be speculated that elevated and sustained DA release induced by NMDA antagonists activate D_2 receptors and further reduce NMDA and GABA transmission. Thus blocking these DA D_2 receptors might prevent the inhibition of NMDA and GABA transmission and re-establish normal PFC functions. In this study the animals displayed a dramatic impairment in performance caused by dysfunctional NMDA receptor function in the mPFC. Thus there was the potential for DA D_2 antagonists to improve performance.

Clozapine reduced anticipatory but not perseverative responding induced by CPP (Table 1). Moreover, clozapine completely reversed the effects of CPP on attentional accuracy whereas haloperidol had no effect and l-sulpiride only partially reduced the accuracy deficit (Chapter 7). Thus it could be suggest that non-D_2 receptor mechanisms may have played a role in its effects. In fact, clozapine show a high 5-HT_{2A}/D_2 affinity ratio *in-vitro* and a high potency in *in-vivo* binding studies in occupying 5-HT_{2A} versus D_2 receptors in the cortex, in the striatum and the limbic regions (Stockmeier et al., 1993; Sumiyoshi et al., 1994). Thus, it is conceivable that the effects of clozapine may be mediated via its interaction with 5-HT_{2A} receptors in the mPFC. The lack of effects of clozapine on perseverative responses is interesting since it has been shown that clozapine exerts some effects through 5-HT_{1A} receptors (Meltzer et al., 2003). However, being a partial agonist at 5-HT_{1A} receptors clozapine could have some antagonistic activity at 5-HT_{1A} receptors and as

shown in Chapter 5 the 5-HT$_{1A}$ antagonist WAY100635 had no effect by itself on CPP-induced perseverative over-responding but reduced the effects of 8-OH-DPAT on these responses.

- **Concluding remarks**

The data clearly demonstrate that glutamate transmission in the mPFC is involved in the control of attentional performance on the 5-CSRT task. The data also show for the first time that enhanced glutamate and 5-HT release induced by blockade of NMDA receptors in the mPFC may not be causally related to aspects of inhibitory response control as assessed in the 5-CSRT task by anticipatory and perseverative responses.

The important suggestion emerging from this study is that of distinct neuromodulation in the control of "impulsive" behaviour by 5-HT$_{2A}$/5-HT$_{2C}$ receptors and in "compulsive" behaviours by 5-HT$_{1A}$ and DA D$_2$ receptors in the prefrontal cortex.

Intriguingly deficits in attentional accuracy might be associated with increased glutamate release in the mPFC. This is based on two main findings. First, 8-OH-DPAT and M100907 both reversed the deficit in attentional accuracy and reduced glutamate release. Second, activation of mGlu$_{2/3}$ receptors specifically in the mPFC reversed the accuracy deficit but had no effect on aspects of inhibitory response control.

Thus behavioural evidence obtained in animals with dysfunctional glutamate transmission performing a 5-CSRT task, as well as a series of pharmacological manipulations of 5-HT, DA and mGlu$_{2/3}$ receptor mechanisms have been presented that are consistent with the hypothesis that these mechanisms in the prefrontal cortex

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play specific roles in attention and response selection particularly when these have a possible function in the strategic control of responding. These response selection mechanisms are sensitive to inhibitory influences at several functional levels, including mechanisms for preparing responses and for monitoring performance to enable behavioural flexibility. These dissociable aspects of inhibitory response control appear sensitive to differential neuromodulation by 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and DA D_2 receptor mechanisms in the prefrontal cortex.

A number of neurochemical and behavioural studies have highlighted the importance of 5-HT modulation of DA within various brain areas and they have been reviewed in the General Introduction. From this review it is clear that ligands at 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors all modulate DA function in the mPFC. Thus the effects of agonists and antagonists at these 5-HT receptors may be due at least in part on their ability to interact with mesocortical DA system. Several lines of evidence (reviewed in the Introduction) and the findings presented in this thesis make it clear that the outcome of this 5-HT/DA interaction depends on the particular behavioural process that is activated. In the present study the interaction between 5-HT and DA receptor mechanisms has not been studied directly. However, comparing the results of 5-HT receptor agents and DA D_2 receptor antagonists shows clearly that this interaction might be more important for some, but not other, aspects of performance.

- **Clinical Implications**

Although there is little doubt that patients with schizophrenia have attentional impairment and deficits in executive function it is quite difficult to specify their underlying neural and neurochemical basis. Abnormal glutamatergic transmission in

the prefrontal cortex (PFC) has been associated with schizophrenia (Krystal et al., 2003). However, this suggestion is largely based upon psychotomimetic effects of PCP and the discovery that it induces its behavioural effects by blocking glutamate NMDA receptors. The advantage of the glutamate model of schizophrenia over the DAergic model (amphetamine-induced psychosis) is the ability of NMDA antagonists to induce schizophrenia-like cognitive and neuropsychological deficits (Javitt and Zukin, 1991; Krystal et al., 2003). However, the associations between positive and negative dimensions of symptoms and neurocognitive functioning of schizophrenic patients were found to be weak, suggesting a relative independence of these disease processes (Nieuwenstein et al., 2001). This may suggest that not a single neurobiological hypothesis may explain the complexities of psychopathology of schizophrenia. The fact that psychotic symptoms of schizophrenic patients respond adequately to treatments with DA antagonists has been and still remains a powerful neurochemical hypothesis of schizophrenia. Some neurochemical findings have suggested a role for 5-HT mechanisms (Breier, 1995) but it should be made clear that no unequivocal data support this 5-HT hypothesis of psychosis. The 5-HT hypothesis is best viewed as complementary to the dopaminergic hypothesis, rather than as an alternative to it, since these systems are anatomically connected and functionally interactive (Kapur and Remington, 1996). However, the focus on the role of 5-HT system is the result of the therapeutic action of so called "atypical" antipsychotic drugs, the prototype being clozapine. Of particular interest was the discovery that atypical antipsychotics may possess direct or indirect effects on various 5-HT receptors, especially $5-HT_{1A}$, $5-HT_{2A}$ and $5-HT_{2C}$ (Meltzer et al., 2003). The fact that atypical antipsychotics are more consistent than typical antipsychotics (haloperidol) in improving some aspects of attention such as vigilance and in

favouring executive functions in patients with schizophrenia (Meltzer and McGurk, 1999; Harvey and Keefe, 2001; Harvey et al., 2003a; Harvey et al., 2003b) has suggested that the 5-HT receptor mechanisms may importantly contribute to cognitive functions.

The data from the present study complement these findings and provide valuable insight into the neurochemical mechanisms that mediate the cognitive impairments in schizophrenia. The finding that blockade of D_2 and 5-HT_{2A} receptor or stimulation of 5-HT_{1A} and 5-HT_{2C} may importantly and differently control aspects of attentional performance may be valuable for the development of new pharmacotherapeutic strategies. These data may also suggest that diverse pharmacological activities of a given drug may be better suited for the treatment of complex disorders such as schizophrenia, which may result from dysfunctions in multiple mechanisms.

The findings suggest that a complex interaction of glutamatergic NMDA receptor mechanisms with the 5-HT and DA systems in the PFC may be essential for preserving attentional selectivity and executive function, whose main purpose is to orchestrate the function of other systems in the performance of complex cognitive tasks such as comprehension, learning, planning and reasoning.

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APPENDIX 1. EFFECTS OF CPP ON ACQUISITION OF SPATIAL DISCRIMINATION

Male Lister hooded rats were housed in groups of two in standard laboratory conditions (temperature 20 ± 1°C and 60% relative humidity) in a room with the light on from 07.00 to 19.00 h. Food and water were freely available. Implantation of bilateral guide cannulae made of 23-gauge stainless steel tubing and intracortical injection procedures were as described in General Methods.

Apparatus A circular "swimming pool" was used, 1.5 m in diameter and 0.5 m high. The pool was filled to a depth of 0.29 m with water at 26 ± 1°C, rendered opaque by the addition of a food dye (coffee color, Bayo, Italy). The water was changed daily. The pool was placed in the middle of a large room and was surrounded by various visual cues: a blackened window with a big white cross, a white wall with a big black cross, a long table, a door and a picture-covered wall with a rack for cages. The objects could be covered, when required, by black curtains around the maze. When open, the curtains were collected together at one corner of the room, forming another prominent visual cue. The room was lit by a 100 W light bulb in the center of the ceiling, 2.4 m above the water surface. The light intensity at the water surface was 80 lux (measured by an Illuminometer, Mod 5200, Kyoritsu, Japan).

Two visible grey platforms were used in the spatial discrimination task. The fixed one protruded 1.5 - 2.0 cm above the water. Its top was square (11 x 11 cm) and made of Perspex. The second platform was also grey with square top (11 x 11 cm) and protruded 1.5 - 2.0 cm above the water. It was made of the same material but was filled with expanded polystyrene. It was "anchored" by thread to a solid movable base on the bottom of the pool. Thus one platform was rigid and provided support, and the other sank when the rats tried to climb onto it. The size, shape and color of the second platform were identical to those of the rigid escape platform.

Spatial discrimination training. The black curtains were drawn together to allow a full view of extra-maze cues. Rats were trained to swim to the rigid grey escape platform while avoiding the floating grey platform. For all rats, the fixed escape platform (correct) was always in the same place at the centre of one of the eight sectors. The floating platform (incorrect) was positioned over successive trials in a quasi-random sequence of eight locations around the pool, subject to the constraint that the spatial relationship between the platform and the starting position did not consistently reward either right- or left-turning tendencies. Both platforms, the fixed and the floating one were at approximately the same distance away from the pool wall. No apparent intra-maze cues were available to the rats to distinguish the correct from the incorrect platform.

The rats were trained with ten trials a day for five days. A trial began with the rat being placed in the pool while held at, and facing, the side wall. Eight possible starting locations were used in quasi-random sequence across trials. A trial ended when the rat escaped onto the rigid platform, where it was allowed to sit for 15 s before being returned to a holding cage until the next trial. The rats were trained in squads of four. Inter-trial intervals were approximately 2-4 min so each rat's daily testing lasted approximately 30 min. A correct trial was one in which the rat climbed onto the rigid platform without touching the floating platform with its forepaws or snout. The occasional incident of brushing past the floating platform in passing was not considered an error. If the rat did not choose to escape onto either platform (correct or incorrect) in 60 s it was taken out of the pool and an omission error was scored. We measured (1) the first choice in each trial (correct/incorrect), (2) the latency to escape (i.e. the time of releasing the rat into the pool till it climbs onto the correct or incorrect platform) (sec), and (3) the number of omissions.

Treatment schedules and statistical analysis After recovery from surgery and adaptation to the injection procedure rats were allocated to different treatment groups. On each acquisition day vehicle or CPP at doses of 10 and 50 ng/µL were injected 10 min before testing to different rats. Statistical analysis of data was made by between subjects one-way ANOVA.
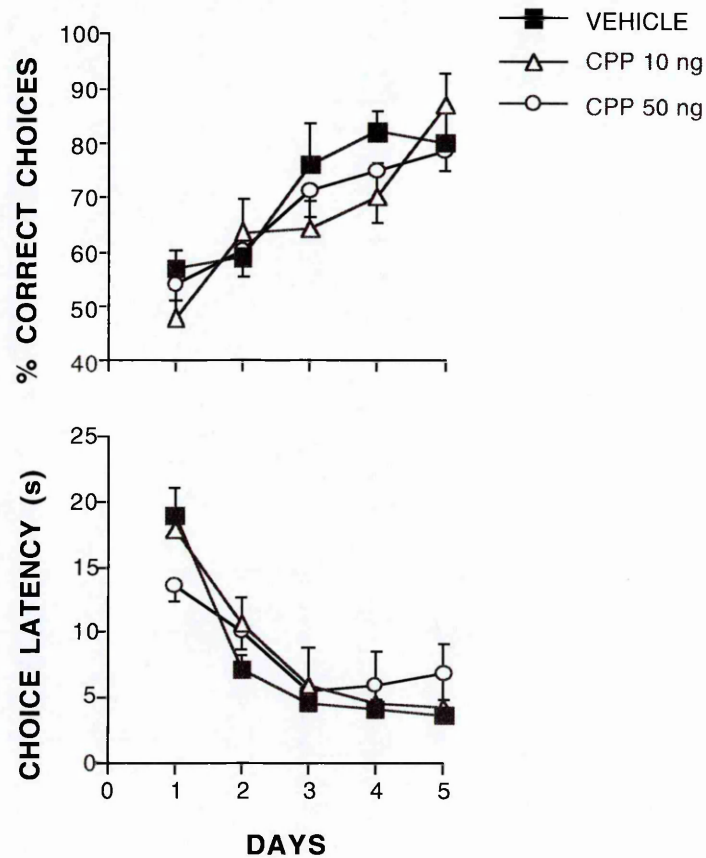
Effects of blockade of NMDA receptors in the mPFC on acquisition of a two-platform spatial discrimination task. On each acquisition day rats were injected with of vehicle (1 µL) and CPP (10 and 50 ng/µL) into the mPFC and 10 min later tested in the water maze.

APPENDIX 2. EFFECTS OF CPP ON phospho-CREB IN mPFC AND
CAUDATE NUCLEUS

Lister hooded male rats (250 – 300 gr) were implanted monolaterally with a permanent cannula as described in the General Methods. Rats were allowed few days of recovery from surgery after which they were injected into the mPFC with 50 ng/μL CPP. The injection procedure and the rate of infusion was the same as in behavioural experiments.

Determination of phospho-Ser133-CREB by Immunohistochemistry

Animals were deeply anesthetized with equitesin (3 ml/kg, IP) and transcardially perfused with cold, fresh 4% (w/v) paraformaldehyde (PAF) in PBS, pH 7.4 via a peristaltic pump (flow rate 30 ml/min). Brains were postfixed for 2 hr in the same fixative solution followed by 48 hr in 20% sucrose in PBS at 4°C.Postfixed brains were sectioned coronally on a freezing microtome at a thickness of 20 μm. Sections were collected in 24-well plates containing PBS and sodium azide (0.01%). Free floating sections were then washed three times in PBS and immersed for 30 min in H_2O_2, followed by three washing in PBS. The sections were then blocked with 2% normal goat serum (NGS-Vector Laboratories) in PBS for 1 hr, followed by O/N incubation with the phospho-ser133-CREB antibody (1:500) in 2% NGS in PBS at 4°C. The sections were then washed with PBS and incubated with biotinylated goat anti-rabbit antibody (1:200) in PBS for 1 hr at room temperature, followed by two PBS washes. Finally, avidin-biotin horseradish peroxidase complex (1%, ABC Vector Kit) was added for 1 hr. Peroxidase activity was determined by reaction for few minutes with a solution containing 0.5 mg/ml DAB and 10 mg/ml nickel ammonium sulphate in TBS. The sections were washed three times with PBS. They were then mounted on slides, dried, dehydrated and covered with Permount. Phospho-CREB immunoreactivity was found to be exclusively nuclear.

Statistical analysis

Data are expressed as number of phosho-Ser133-CREB-positive cells in a fixed area of 1 mm^2 (means ± SE of arbitrary optical density units). Data were analyzed by one-way ANOVA followed by *post-hoc* comparisons made by Tukey/Kramer's test. Statistical analysis was performed using the StatView 5.0 (SAS Institute Inc., Cary NC, USA) statistical package for Macintosh computer.

TABLE 1. Number of phospho-CREB positive cells in the prefrontal cortex and caudate nucleous after intra-mPFC injection of CPP

Region	Vehicle	CPP 10 min	CPP 40 min
PFC (IS)	197 ± 41	650 ± 68 *	491 ± 78 *
PFC (CS)	112 ± 27	158 ± 53	146 ± 22
CAU N (IS)	1951 ± 153	765 ± 197 *	nd
CAU N (CS)	1732 ± 99	1073 ± 144*	nd

Data are expressed as mean ± S.E.M. (4 – 10 rats per group) of phospho-CREB positive cells detected in a fixed area of 1 mm^2 and are representative of two independent experiments.
*P<0.05 vs. Vehicle (Tukey's HSD test)
Abr. PFC= prefrontal cortex; CAU N = caudate nucleous; IS = injection side; CS = contralateral to the injection side; nd = non determined.

Fig.1

Time course of the effect of intracortical CPP injection on CREB phosphorylation in prefrontal cortex and caudate nucleus of rats. (a) Stereotaxic atlas representation of the sections corresponding to AP +3.2 mm for the prefrontal cortex and AP+0.20 mm for the caudate nucleus from bregma (Paxinos and Watson atlas, Academic Press, 1997).

Frames indicates the area were cells were counted.Histograms in (b) and (c) show the values as mean ± SE of the number of phospho-CREB positive cells. White bars indicates the cells counted in the injection side, grey bars indicates cells counted in the controlateral non-injected side.
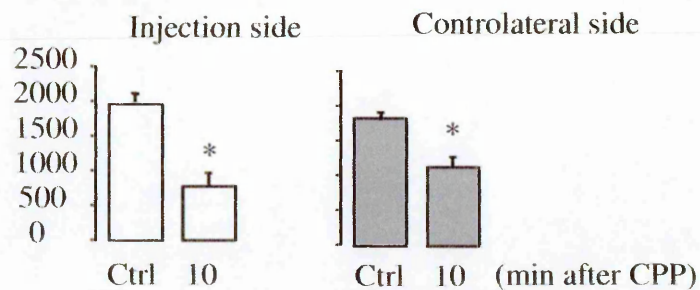* p<0.05 vs. Ctrl (Tukey/Kramer test).

a. Intracortical CPP
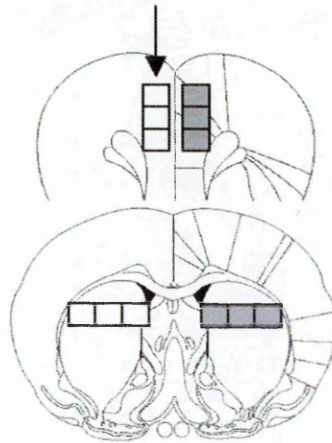
b. *prefrontal cortex*
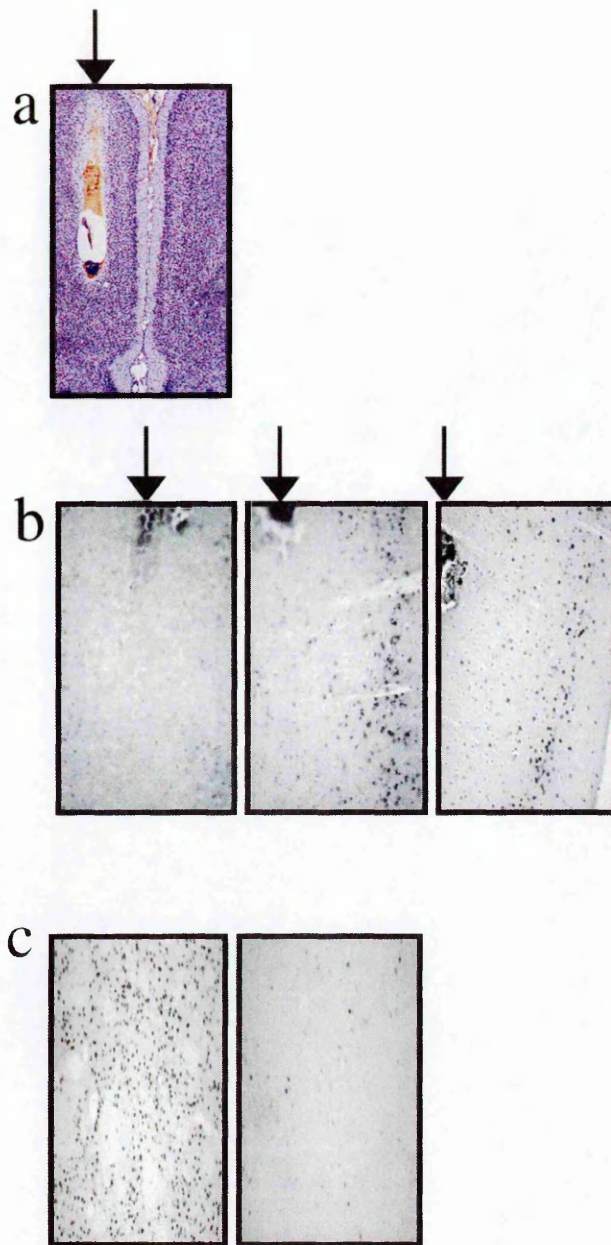

c. *caudate nucleus*

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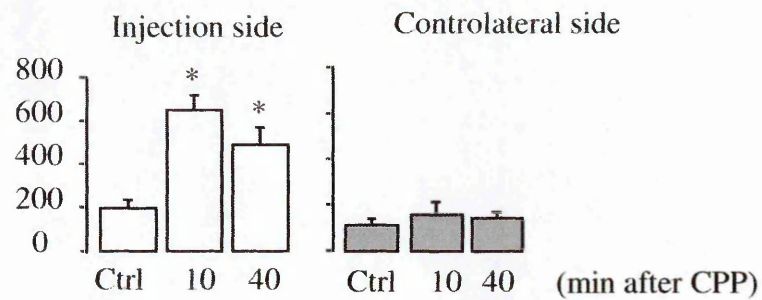
Fig.2

Rapresentative images (magnification x 20 (a) and x 100 (b, c)) showing the effect of an acute intracortical injection of PBS (**Ctrl**) or CPP on phospho-CREB-positive cells in the prefrontal cortex (**b**) and caudate nucleus (**c**) after 10 or 40 min wash-out. (**a**) Nissl staining showing the position of the cannula in the prefrontal cortex corresponding to AP +3.2 from bregma (Paxinos and Watson, Academic Press 1997).

Sections were stained using antibodies and methods as described in *Material and Methods* Neuronal nuclei were the ones subjected to semiquantitative densitometric analysis. Arrows in panels (**a**) and (**b**) indicate the position of the cannula for PBS or CPP injection.