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Role of the Transcription Factor CREB in the NMDA Receptor Antagonist-Induced Attention Deficits

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ROLE OF THE TRANSCRIPTION FACTOR CREB

IN THE NMDA RECEPTOR ANTAGONIST-INDUCED

ATTENTION DEFICITS

by

Laura Pozzi

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Open University, London, UK

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Department of Neuroscience
Istituto di Ricerche Farmacologiche "Mario Negri",
20156 Milano, IT

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—— Advanced School of Pharmacology ——
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PREFACE

The following body of work was performed at the Neuroscience Department, Institute of Pharmacological Research "Mario Negri", Milan (IT), under the direction of Dr. Roberto Invernizzi and the external supervision of Prof. Trevor Sharp, Oxford University (UK).

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. Mirjana Carli.

ABSTRACT

This thesis is the result of a multidisciplinary approach which combines behavioral, biochemical and neurochemical assays to identify and delineate the molecular mechanisms involved in the control of attention. Blockade of prefrontocortical glutamate NMDAR is associated with attentional performance deficits as assessed in a task capable of measuring selective attention and response control such as the five choice serial reaction time (5-CSRT) task.

I investigated whether phosphorylation of proteins linked to the PKA/CREB pathway in the prefrontal cortex (PFC) and in subcortical regions may be affected by NMDAR blockade. Pharmacological experiments using western blot and immunohistochemical techniques clearly demonstrated that increased CREB phosphorylation (p-CREB) in the PFC but not in subcortical regions may be associated to deficits in attention performance caused by the blockade of prefrontocortical NMDAR with the selective antagonist CPP.

Attempts has been made to identify the intracellular pathways responsible for CPP-induced p-CREB changes by examining different protein kinases upstream to CREB such as protein kinase A (PKA), extracellular regulated protein kinases 1/2 (ERK1/2) and calcium/calmodulin kinase II (CaMKII). However, the precise role of these kinases in NMDAR antagonist induced changes in p-CREB is unclear and deserves further studies.

The hypothesis that attention deficits induced by NMDAR blockade in the PFC might reflect excessive glutamate (GLU) release in the PFC was investigated by assessing the effects of intracortical CPP on attention, GLU release and p-CREB under basal conditions and in rats pre-treated with the mGlu2/3 receptor agonist LY379268. The results provide evidence that enhanced GLU release in the PFC and CREB phosphorylation are associated to attention deficit.

As there are evidences that drug responses may be modulated by the behavioural state of animals, I evaluated the effect of blockade of NMDAR in the PFC on p-CREB in rats performing the 5-CSRT task. Intriguingly, I found that in these rats CPP-induced p-CREB in the PFC was decreased as opposed to the increase found in behaviourally naïve rats at the same time point, suggesting that the direction of CPP-induced p-CREB changes critically depends on the behavioural state of animals.
Moreover CPP-induced cognitive impairments were prevented by the intracortical administration of Sp-cAMP suggesting that the activation of the PKA/CREB cascade in the PFC is required for the correct performance of the task.

In conclusion the present thesis support the role of CREB and provide new information on the mechanisms involved in the control of attention and executive functions associated with some neuropsychiatric disorders.

SUPERVISOR

Trevor Sharp, PhD
Department of Pharmacology
Oxford University,
Mansfield Road, Oxford OX1 3QT.UK
Email: trevor.sharp@pharm.ox.ac.uk

EXAMINERS

Jeff Dalley, PhD
Department of Experimental Psychology and Department of Psychiatry
University of Cambridge, UK
Email: jwd20@cam.ac.uk

Tiziana Borsello, PhD
Department of Neuroscience
Mario Negri Institute for Pharmacological Research
Milan, IT
Email: tiziana.borsello@marionegri.it

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Distinct changes in CREB phosphorylation in frontal cortex and striatum during contingent and noncontingent performance of a visual attention task
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Frontiers in Neuroscience_ Special Topic: Neuronal Cell Signalling and Behaviour
In Press

Attention deficit induced by blockade of NMDA receptors in the prefrontal cortex is associated with enhanced glutamate release and CREB phosphorylation: Role of mGluR2/3
Laura Pozzi, Marta Baviera, Giuseppina Sacchetti, Eleonora Calcagno, Claudia Balducci, Roberto W Invernizzi and Mirjana Carli.
Neuroscience. 2011 Mar 10;176:336-48.

Blockade of serotonin 2A receptors prevents PCP-induced attentional performance deficit and CREB phosphorylation in the dorsal striatum of DBA/2 mice.
Laura Pozzi, Barbara Greco, Giuseppina Sacchetti, Giampaolo Leoni, Roberto W Invernizzi and Mirjana Carli.
Psychopharmacology (Berl). 2010 Feb;208(3):387-99.

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CHAPTER 1

INTRODUCTION

1. COGNITIVE FUNCTIONS

Defining cognitive deficits is not easy, because of the complexity of the phenomenon and its poor comprehension. Cognition literally means "the act or process of perceiving or knowing". Consequently, this description tends to apply to processes such as memory, attention, perception, action, problem solving and mental imagery, abstract concepts such as reasoning, intelligence, learning, and many others that are related to numerous capabilities of the human brain.

Clinical diagnoses of cognitive disabilities have been described in schizophrenia, autism, attention deficit and hyperactivity disorder (ADHD), Down's syndrome, traumatic brain injury and Alzheimer's disease. Cognitive deficits in schizophrenia have been documented in numerous studies. The following paragraphs contain a brief description of cognitive symptoms in schizophrenia, with particular emphasis on deficits in executive functions including attention.

Symptoms of schizophrenia can be separated into *positive* (acquired through the illness process), *negative* (symptoms that reflect functions and behaviours that have been lost due to the illness), and *cognitive* symptoms (deficits in thinking, present from the onset of the disorder; Weickert et al. 2000).

The diagnosis of schizophrenia according to DSM-IV (Diagnostic and Statistical Manual of Mental Disorders; Cooper 1995) criteria is based on the concomitant appearance of at least two of the following symptoms each present for a significant portion of time during a 6-month period: delusions, hallucinations, disorganized speech (e.g., frequent derailment or incoherence), grossly disorganized or catatonic behavior and negative

symptoms. Although psychosis is usually the most striking clinical aspect of the illness, disturbances in specific cognitive processes, such as attention, certain types of memory, and executive functions, are considered to be the core features of the illness (Elvevag and Goldberg 2000; Birkett et al. 2007; Harvey et al. 2001), and are associated with impaired quality of life and poor outcome (Green et al. 2000; Luck and Gold 2008). Cognitive deficits are fundamental aspects of schizophrenia that may account for much of the long-term social disability associated with the disorder. In particular, cognitive symptoms, which cannot be regarded as unitary, include: impaired executive functions and attention (inability to focus/sustain attention), impaired memory, reduced ability to learn from mistakes or feedback, reduced capacity to form new concepts and poor problem-solving. Although cognitive symptoms have several similar features with negative symptoms (onset, temporal course, correlations with other aspects of the illness, and prognostic importance), negative and cognitive symptoms may be separable (Harvey, 2006).

1.1 Executive Functions and Attention

Executive functions (EF) could perhaps be viewed as the quintessence of higher-order cognition. Although there is no agreement upon definitions of EF in the literature, even after decades of extensive research, the majority of authors concur that EF are related to higher-order control processes involved in the goal-directed thought and actions (Barkley 1997; Miyake et al. 2000). EF are defined as the ability to initiate and stop actions, to monitor and adapt behavior as needed, to anticipate outcomes and to plan future behaviors when faced with unpredictable novel tasks and situations. For example, on being presented with a potentially rewarding stimulus, the automatic response might be to

take it. However, where this behavior conflicts with internal/external plans the executive functions might be engaged to inhibit this response.

The prefrontal cortex (PFC) plays a key monitoring role in EF, but other brain areas are also involved (Elliott 2003). Attempts to localize executive functioning to discrete frontal areas have been inconclusive. The emerging view is that executive function is mediated by dynamic and flexible networks.

Executive processes have been primarily linked to the prefrontal and cortico striatal thalamic circuits (Roberts and Bell 2002; Andres 2003).

The PFC is, however, a multifaceted area of the brain, and in recent years, the evidence for regional specialization within the PFC for different EF components has accumulated (Bunge et al. 2001; Ridderinkhof et al. 2004a,b; Shallice et al. 1994).

Attention is defined as the cognitive process of selectively concentrating on one aspect of the environment while filtering or ignoring extraneous information. It is a very basic function that often is a precursor to all other neuropsychological/cognitive functions.

Many forms of attention can be described: (1) *Focused Attention*, which is the ability to respond discretely to specific visual, auditory or tactile stimuli, (2) *Sustained Attention* which is the ability to maintain a consistent behavioral response during continuous and repetitive activity, (3) *Selective Attention* which is the capacity to maintain a behavioral or cognitive set in the face of distracting or competing stimuli, (4) *Alternating Attention*, which is the capacity for mental flexibility that allows individuals to shift their focus of attention and move between tasks having different cognitive requirements, and (5) *Divided Attention*, which is the ability to respond simultaneously to multiple tasks or multiple task demands.

Attention is closely related to and often intimately involved in executive control and working memory (Luck and Gold 2008). Abnormalities in attention are one of the frequently studied cognitive deficits in schizophrenic patients (Braff 1993), and were described in the early clinical accounts of schizophrenia (Groom et al. 2008). Attention is apparently dysfunctional in schizophrenia in several ways, including sustained focused attention (Cornblatt and Keilp 1994), selective attention (Nestor et al. 2001), and cognitive control of attention (Cohen et al. 1996).

Cognitive disturbances in patients can be present for years before the onset of psychosis and persist throughout the illness, exist in milder forms in unaffected relatives of individuals with schizophrenia, and are the best predictor of long-term functional outcome (Gold 2004). Their presence in attenuated form among the first-degree relatives of schizophrenic patients suggests their promise as components of genetic susceptibility to schizophrenia and related disorders (Gur et al. 2007; Snitz et al. 2006). In adults with schizophrenia, the stability of neurocognitive deficits across different phases of illness has been described (Heaton et al. 2001; Hoff et al. 1999).

1.2 Neuroanatomy of Cognitive Functions

Schizophrenia involves an imbalance in circuits distributed throughout the brain, including multiple cortical and subcortical regions, that leads to impairment in the ability to set priorities, to process and produce information, and to turn them into meaningful thoughts and behaviour. Many studies of cognitive deficits in schizophrenic patients have focused on the prefrontal cortex (PFC) because of its demonstrated importance in executive functions (Fuster 1991). However, the role of the striatum, which belongs to the cortico-striatal circuitry has also recently received some consideration. In the following

paragraphs I will briefly outline the pathological findings related to excitatory (glutamatergic) and inhibitory (GABA) neurotransmission in patients. Afterwards the anatomical circuitry involved in the control of executive functions with particular emphasis on attention will be briefly reviewed. Evidence from human, monkeys and rodents will be presented.

1.3 Cortical and Striatal Abnormalities in Schizophrenic Subjects

The Wisconsin Card Sorting Test (WCST) is a neuropsychological test of "set-shifting", i.e. the ability to display flexibility in the face of changing schedule demands. Imaging studies have shown that this task involves significant activation of the dorsolateral PFC (Berman et al. 1995; Cabeza et al. 2000). However, more recent fMRI studies have shown that the ventrolateral PFC (Konishi et al. 1998) together with the caudate nucleus (Monchi et al. 2001), may be the regions importantly involved in the set-shifting processes required during the WCST.

It was found that 75% of schizophrenic patients performing the WCST exhibit deficits thus implicating dysfunctions of the cortico-striatal circuit in schizophrenia. In addition, schizophrenic patients show an abnormally low glucose utilization rate in the PFC and right temporal cortex during a test of attention and vigilance such as the Continuous Performance Test (CTP) (Buchsbaum et al. 1990).

It has been reported that the failure of intracortical connectivity and abnormalities of presynaptic terminals of the PFC accounts for both cognitive and psychotic

manifestations of the illness (Dorph-Petersen et al. 2009; Sweet et al. 2009; Weinberger et al. 1994).

The question of whether there are histo-pathological changes in the brains of schizophrenic patients generated reports of volume loss (Bogerts et al.1985), increased $GABA_A$ receptor binding in superficial layers of cingulate cortex (Benes et al. 1992a), increased density of glutamate-immunoreactive in cingulate cortex (Benes et al. 1987a,b; Benes et al. 1992b) and other subtle cytoarchitectural variations (Kovelman and Scheibel 1984) in corticolimbic brain regions of individuals with schizophrenia. A reduced number of neurons expressing the enzyme nicotinamide-adenine dinucleotide phosphate-diaphorase (NADPH-d: the enzyme that catalyzes the reaction of nitric oxide) (Akbarian et al. 1993a,b) in the dorsolateral prefrontal area of subjects with schizophrenia was also reported.

The presynaptic terminals in the striatum of schizophrenic patients examined post-mortem also showed a lower level of excitatory amino acid transporters 3 (EAAT 3) and significant deficits in the vesicular glutamate transporter 1 (VGluT1) compared to healthy controls (McCullumsmith and Meador-Woodruff 2002; Nudmamud-Thanoi et al. 2007). These latter data provide additional evidence for a glutamatergic synaptic pathology in the caudate nucleus in schizophrenia, likely reflecting a loss of glutamatergic cortico-striatal neurons.

Current pathophysiological models of functional disturbances in schizophrenia emphasize disinhibitory processes, acting through GABA, glutamate (GLU), and dopamine (DA) neurotransmission to affect the firing patterns of local networks of cortical neurons (Lisman et al. 2008; Lewis and Sweet 2009).

A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia has recently emerged (Simpson et al. 2010). Using functional magnetic resonance (fMRI) Manoach and coworkers found an aberrant activation of the PFC and basal ganglia (including the head of the caudate and the lentiform nucleus) during the performance of tasks involving working memory (Manoach et al. 2000; Tost et al. 2005). The role of the striatum in attentional processes and cognitive control is further suggested by functional neuroimaging in healthy volunteers showing that selective attention condition activate the head of caudate nucleus and globus pallidus (Corbetta et al. 1991). In addition, attentional set shifting during the Wisconsin Card Sorting Task activates the caudate nucleus in healthy human subjects (Rogers et al. 2000). In monkeys lesions of the medial striatum cause behavioral inflexibility (Clarke et al. 2008).

1.4 The Prefrontal Cortex: functional anatomy

The PFC is the anterior part of the frontal lobes of the brain, lying in front of the motor and premotor areas. This brain region has been implicated in planning complex cognitive behaviors, personality expression, decision making and moderating correct social behavior (Fuster 1997; Miller and Cohen 2001).

It is well established that anatomically distinct regions of the PFC play a critical role in facilitating different forms of behavioral flexibility: orbital regions of the PFC mediate simpler forms of behavioral flexibility such as shifts between different stimulus-reward associations, while the medial or dorso-lateral regions of the PFC are involved in the control of more complex behaviors (Birrell and Brown 2000; Dias et al. 1996a). The ventromedial and dorsolateral prefrontal cortices exhibit reciprocal connectivity with

different posterior brain regions, with ventromedial prefrontal regions being associated with emotional processing areas (for example, amygdala) and dorsolateral prefrontal regions with non-emotional sensory and motor areas (for example, basal ganglia and parietal cortex).

However, patients with frontal lesions do not always show executive deficits (Andres 2003; Ornstein et al. 2008). Indeed, patients with lesions restricted to frontal regions and presenting impaired short-term memory storage, showed normal performance in measures of executive processes such as the capacity to undertake two tasks simultaneously and the capacity to inhibit no-longer relevant information (Andres and Van der Linden 2002).

Whether there is a homolog of primate PFC in the rodent brain is an issue of continuing debate (Povinelli and Preuss 1995; Uylings et al. 2003). The PFC of the rat has been divided into medial, ventral and lateral (figure 1.1). The _medial_ PFC is subdivided into a dorsal region including pre-central (PrC) and anterior cingulated (ACg) cortices and a ventral component that comprises prelimbic (PrL), infralimbic (IL) and medial orbital (MO) areas (Berendse and Groenewegen 1991; Hoover and Vertes 2007). Each part of the medial PFC communicates with its adjacent areas and, with the possible exception of IL, each division interconnects with the others as well as with brain regions projecting to the PFC. However, from an anatomical perspective the medial regions of the PFC in rodents (i.e. IL, PL and ACg) share similar patterns of efferent and afferent connectivity with the dorsolateral PFC in primates and humans (Ongur and Price 2000).

The _lateral region_ of the PFC includes dorsal and ventral agranular insular (AID,AIV) and lateral orbital (LO) areas. The _ventral region_ of the PFC encompasses the ventral orbital (VO) and ventral lateral orbital areas (VLO) (figure 1.1).

An alternative approach to the issue is to consider the function, rather than anatomy, of prefrontal areas. The evolution of PFC in the primates is, presumably, to support a degree of behavioural complexity that might not be found in the rat. However, if the rodent does demonstrate behaviour dependent on functions associated with primate PFC, the neural substrate of such behaviour could be regarded as at least analogous, if not homologous, to primate PFC. Accordingly, the medial PFC of rats posses a degree of functional equivalence with the dorsolateral PFC of non human primates as lesions of this region causes deficits in similar functions; impairs working memory, and temporal sequencing of behavior and causes behavioral inflexibility and inattention (Heidbreder and Groenewegen 2003; Dalley et al. 2004). Previous studies have suggested separate roles in different aspects of cognitive flexibility for the PL and IL cortex, as well as the orbitofrontal cortex (Boulougouris et al. 2007; Dalley et al. 2004; Ragozzino and Rozman 2007; Sun et al. 2010).
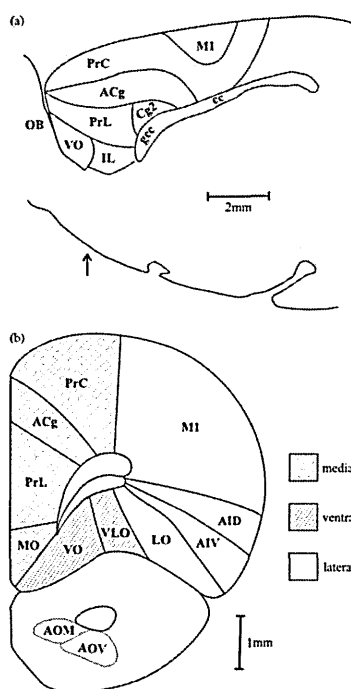
Fig.. 1.1 Illustrative diagrams of the rat prefrontal cortex

a. Lateral view, 0.9 mm from the midline.
b.Unilateral coronal section, approximately 3.5 mm forward of bregma (depicted by the arrow above). The different shadings represent the three major subdivisions of the prefrontal cortex (medial, ventral and lateral).
From Dalley et al, 2004

Abbreviations:
ACg, anterior cingulate cortex; AID, dorsal agranular insular cortex; AIV, ventral agranular insular cortex; AOM, medial anterior olfactory nucleus; AOV, ventral anterior olfactory nucleus; cc, corpus callosum; Cg2, cingulate cortex area 2; gcc, genu of 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.

Although the PFC has been linked to cognitive functions for a number of reasons, unequivocal evidence linking one specific region of the brain to schizophrenia has yet to be established. Recently, using positron emission tomography (PET) it has been shown that the frontal and parietal lobes are activated in response to attention effort in both visual and somatosensory modalities, thus confirming that these cortical areas are anatomical substrates for the processes involved in sustained attention (Pardo et al. 1991; Granon et al. 1998). Imaging studies in human subjects have also described the involvement of the anterior cingulate cortex (ACg) and dorsolateral PFC in cognitive control (Kerns et al. 2004). The PFC is activated during ketamine-induced psychosis in healthy individuals (Breier et al. 1997). Moreover, the metabolic rate of glucose utilization and the regional cerebral blood flow (rCBF) in the PFC were increased after ketamine much more in schizophrenic patients than in healthy controls (Vollenweider et al. 1997a,b; Holcomb et al. 2005).

Interestingly, Everitt and Robbins (2005) showed that the change from a voluntary drug use to more habitual and compulsive use caused a transition at the neural level from PFC to striatal control. In agreement, human imaging studies show the involvement of prefrontal regions early in instrumental and motor learning, but not after the tasks had been learned (Jenkins et al. 1994; Raichle et al. 1994; Jueptner et al. 1997; Duncan et al. 2000; Doyon et al. 2002).

1.5 The corticostriatal cognitive circuit: excitatory striatal afferent projections

The PFC sends its excitatory afferents to the striatum, the biggest structure of the basal ganglia. The striatum consists of the dorsal (caudate putamen or CPu), ventral (nucleus accumbens or NAC) striatum and the striatal elements of the olfactory tubercle (OT). Although the terms 'dorsal' and 'ventral' striatum are widely used, an unequivocal boundary between these main striatal parts it's hard to define. The medial territory of the CPu, is considered part of a "prefrontal cognitive circuit" and has been implicated in cognitive control over actions (Owen 1997), action-outcome learning (Yin et al. 2005) and continuous visuospatial task performance as reported by a PET activation study in humans (Corbetta et al. 1991). The CPu is unique in its complete lack of intrinsic glutamatergic neurons. Instead, 95% of striatal neurons are GABAergic medium-sized spiny neurons (Kawaguchi 199; Tepper and Bolam 2004). The striatum has been divided into three areas on the basis of their afferents and functions (Voorn et al. 2004) (fig. 1.2). The *dorsolateral striatum* (fig. 1.2 green) which receives predominantly sensorimotor-related information (Brown et al. 1998) from the sensorimotor area of the frontal cortex, posterior and lateral intralaminar thalamic nuclei, nuclei of the intermediate basal amygdaloid complex (Wright and Groenewegen 1996), dorsal hippocampus and dopaminergic (DA) projections from the substantia nigra compacta (SNc; A9 cell group); the *ventromedial striatum* (fig. 1.2 red and pink) that collects visceral-related afferents from ventral prelimbic (PLv) and infralimbic cortex (IL) (Vertes 2004), paraventricular nucleus (PV) of midline thalamic nuclei, caudal basal amygdaloid nuclei, subiculum and CA1 region of the hippocampal formation and DA projections from ventral tegmental area (VTA) (A10 cell group). Finally, the striatal area lying between these extremes

called the *dorsocentral striatum* (fig. 1.2: blue and purple), which receive higher order 'associative' information (McGeorge and Faull 1989; Reep et al. 2003) from the dorsal anterior cingulate cortex (ACd) and dorsal prelimbic cortex (PLd), nuclei of the rostral basal amygdaloid complex (Wright and Groenewegen 1996) and DA innervations arising from the VTA and SNc.

Recently, the dorsomedial and dorsolateral part of the striatum have been implicated specifically in the learning and performance of goal-directed actions (White 1989; Packard and McGaugh 1996; Devan et al. 1999a; Devan and White 1999b; Featherstone and McDonald 2004) as well as in habit learning respectively (Featherstone and McDonald 2005; Winocur 1974; Packard and White 1990; Robbins et al. 1990; Reading et al. 1991; McDonald and White 1993).
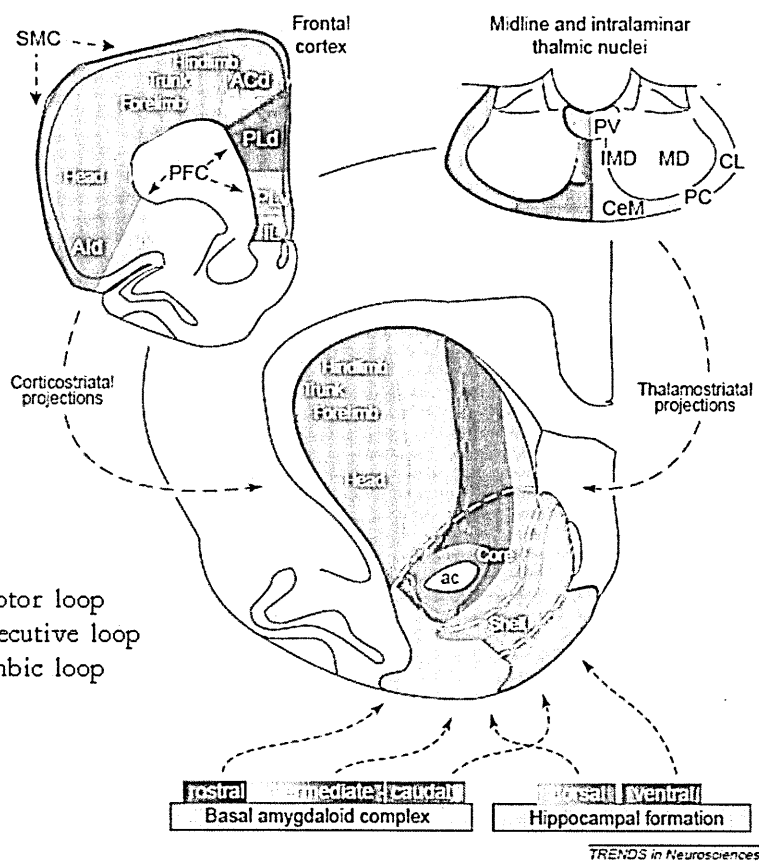
Fig. 1.2

Excitatory Striatal Afferent Projections

The topographical arrangement of striatal afferents originating in the frontal cortex (upper left), midline and intralaminar thalamic nuclei (upper right), basal amygdaloid complex (lower left) and hippocampal formation (lower right) are illustrated. Frontal cortical areas and their corresponding striatal projection zones are shown in the same colors. The dorsolateral striatum receives somatotopically organized sensorimotor information (green), the most ventromedial part of the striatum collects cortical afferents (red and pink), and striatal areas between these extremes receive information from higher associational cortical areas (blue and purple).

Abbreviations: ac, anterior commissure; ACd, dorsal anterior cingulate cortex; AId, dorsal agranular insular cortex; AIv, ventral agranular insular cortex; CeM, central medial thalamic nucleus; CL, central lateral thalamic nucleus; IL, infralimbic cortex; IMD, intermediodorsal thalamic nucleus; MD, mediodorsal thalamic nucleus; PC, paracentral thalamic nucleus; PFC, prefrontal cortex; PLd, dorsal prelimbic cortex; PLv, ventral prelimbic cortex; PV, paraventricular thalamic nucleus; SMC, sensorimotor cortex. (Adapted from Voorn P. et al., 2004).

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The ventral striatum, which comprises the *nucleus accumbens* (NAC) has been described as a 'limbic–motor interface' (Mogenson et al. 1980) receiving inputs from the PrL and IL areas and from distal limbic structures such as the hippocampus and amygdala. The PFC is one of the major sources of excitatory amino acid input to the NAC (Sesack and Pickel 1992). Specifically, the *core region* of NAC (NACc) receive glutamatergic afferents from the PrL region of the PFC, while the *shell region* of NAC (NACs) receive afferents from the IL subregions of the PFC (Berendse et al. 1992; Brog et al. 1993; Groenewegen et al. 1999; Vertes 2004; Voorn et al. 2004). A schematic diagram is shown in fig.1.3.
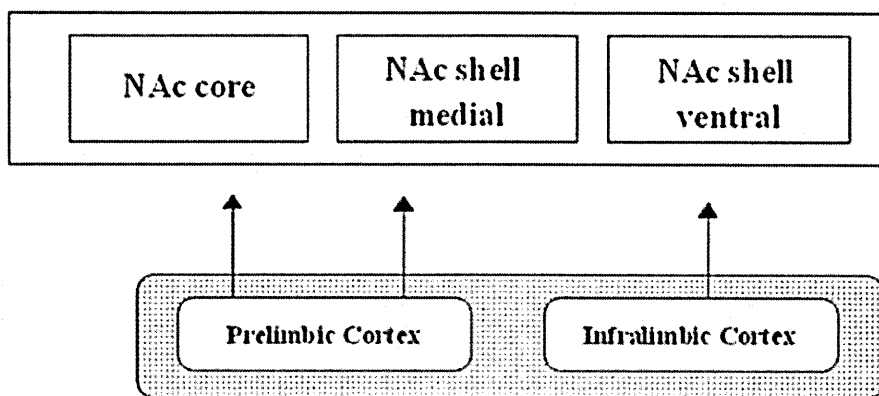
Fig. 1.3
Cortico-accumbal connections
Schematic illustration of connections between the three portions of the NAC shell and core regions with sub-regions of the PFC.
Modified from Shirayama and Chaki 2006.

It has been reported that glutamatergic afferents from the PFC modulate the neuronal activity in the NAC directly or via dopaminergic neurons in the ventral tegmental area (VTA) (Kalivas and Stewart 1991; Kalivas et al. 1993). The activity of the NAC is thus the results of the balance between limbic and cortical inputs and therefore both increases and decreases in neurotransmission in this region exert a potent effect on information processing (Goto and Grace 2005). Interestingly, disruption of this balance may be one of the central components in the pathophysiology of schizophrenia (Csernansky and Bardgett 1998; Meyer-Lindenberg et al. 2002).

The CPu and the NAC contain a small populations of GABA-containing and cholinergic interneurons, in addition to a large number of efferent GABA-ergic medium spiny projecting neurons (Meredith et al. 1993). The activity of projecting medium spiny neurons is regulated by glutamatergic afferents arising from the PFC, hippocampus and amygdala, by dopaminergic afferents from the substantia nigra (SN) and the VTA (Pennartz et al. 1992) and by local GABA and Ach interneurons. These afferents converge primarily on medium spiny GABA neurons, the main output cells of this region (Meredith et al. 1993).

2. CAN COGNITIVE DEFICITS BE MIRRORED IN ANIMAL MODELS?

2.1 Tests of Attention in Rodents

A model is defined as any experimental preparation developed to study a particular condition or phenomenon in the same or different species. Typically, in preclinical research, models are cellular preparations or living animals that are used to mimic human condition. Animal models include both models of the full syndrome and models of specific signs or symptoms.

Certainly, the primary difficulty in modeling cognitive deficits in rodents is that they cannot self-report the features of the disease. The most common approach for developing animal models has been to exploit drug-induced states that produce disease-like symptoms in healthy humans (pharmacological models). These models generally have face or construct validity (only if the drug reproduce the pathological state) and have lead to the formulation of prominent theories of schizophrenia such as the dopaminergic and more recently the glutamatergic hypotheses.

2.2 The 5-CSRT Task

As with any unobservable cognitive process, assessment of attention requires quantification of an observable phenomenon, such as the behaviour of the animal or the activity of its nervous system. Three main tasks have been developed to study attention in rodents:

(1) The *attentional set-shifting procedure* to study the ability to shift attention (Birrell and Brown 2000).

(2) The *signal detection task* to study the ability to attend selectively to a subset of environmental information while filtering out extraneous stimuli (McGaughy and Sarter 1995).

(3) The *five-choice serial reaction time* (5-CSRT) task that can be arranged to assess both sustained and selective attention, but also to study the ability to sustain attention over time (Carli et al. 1983).

The 5-CSRT task was developed based on Leonard's choice reaction time task (Carli et al. 1983; Leonard 1969; Robbins 2002). It constitutes a rodent analogue of the continuous performance task (CPT) that is used to quantify attention in humans. The apparatus and the training procedure are described in the general methods. Here, it will be discussed how various measures of performance may represent the executive functions in their various aspects such as attentional functioning and inhibitory response control.

The 5-CSRT task essentially tests the ability of rats to sustain spatial attention divided among a number of locations over a large amount of trials. This capacity is measured by *accuracy* of visual discrimination (number of correct responses over a total number of correct plus incorrect responses) expressed as the percentage of correct responses. Changes in accuracy could not be explained as a simple motor effect since both correct and incorrect responses have equivalent motor requirements.

Omissions, not included in the accuracy measure are expressed 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* (the latency to make a correct response) that may reflect decision time but also motor and motivational factors. These different

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interpretations may be disambiguated by observing the overall pattern of effects on omission and other measures such as latency to make a correct response and latency to collect food. The number of anticipatory (occurring during the inter-trial interval) and perseverative responses (repeated responses in the holes after a correct response) are measures of different aspects of inhibitory response control.

Anticipatory responses occur inappropriately when the rat is waiting for the target stimulus. An increase in these responses might signal that the inhibitory control is lost, and, as such, might represent a form of impulsivity (Evenden 1999).

By contrast, *perseverative responses* represent a different form of deficit in inhibitory response control. This deficit has been suggested to be more in accordance 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 magazines (Chudasama et al. 2003). The great advantage of this task is that it provides largely independent measures of accuracy, speed, impulsivity, compulsive perseveration and motivation (Robbins 2002). A detailed description of the procedures and the training schedule for the 5-CSRT task is reported in the general methods section (Chapter 2).

2.3 The 5-CSRT Task: Behavioural Pharmacology and Functional Neurochemistry

The fact that the 5-CSRT task includes various independent performance variables has revealed dissociations between underlying neuronal substrates of various aspects of performance. 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 and sub-cortical regions among which mPFC and its different sub-regions as well as dorsal and ventral striatum (Robbins 2002). This impressive achievement has been accomplished over three decade of systematic analysis of the task, using lesion, metabolic and pharmacological methods. Traditionally the neural mechanisms underlying performance on the 5-CSRT task have been mostly studied in non-human primates. From the 1980' however, the majority of studies have been employing rats. The reasons are multiple; a better knowledge of the neural organization and projections of the rats' PFC as well as its cellular and sub-cellular structure and neurochemistry.

The level of functioning of the PFC is highly dependent upon serotonin (5-HT), dopamine (DA), noradrenaline (NE) and acetylcholine (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. In particular the orbital and medial PFC in primates and the medial PFC in rats are the regions that have direct projections back to cholinergic basal nuclei while monoaminergic neurons receive efferents mainly from the dorsolateral PFC in primates and the medial PFC in rats (Uylings et al. 2003). This bi-directional connection of PFC with cholinergic and monoaminergic systems, indicate that the PFC might influence its own cholinergic and monoaminergic neurotransmission (Groenewegen and Uylings 2000). Numerous evidence suggest that these neurochemical systems although all implicated in functions associated with PFC mediate different forms of neuromodulation, which is known 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 (Aston-Jones et al. 1996; Goldman-Rakic 1998; Robbins 2000; Sarter et al. 2001). Interestingly distinct contributions to attentional functioning and inhibitory response control measured in the 5-CSRT task revealed that an impaired noradrenergic transmission induced attention deficits in rats (Carli et al. 1983).

Excitotoxic or immunotoxic lesions of Ach-ergic 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. 1997; Carli et al. 2001). Manipulation of dopaminergic system had distinct effects. DA depletion in the NAC 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 stimuli were presented unpredictably in time (Robbins et al. 1990).

These studies have been extended and include studies examining the selective manipulations of specific receptors in particular brain regions and more conventional pharmacological studies (Robbins 2002), and due to amount of data that have been gathered they cannot be reviewed here. However, although lesions and pharmacological manipulations are useful in delineating the cognitive functions of cortical and striatal regions, they do not address the molecular mechanisms that occur in each region or whether these mechanisms may interact to influence behaviour.

Relevant for this project is a study by Muir et al. (1996a) showing that the mPFC but not post-genual cingulate, anterior dorsolateral and parietal cortex is crucial for attentional functioning in the 5-CSRT task in rats. Bilateral excitotoxic lesions of the mPFC caused increased perseverative responding whereas post-genual cingulate cortex lesions specifically increased anticipatory responses (Muir et al. 1996a). 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).

Recent studies by Chudasama et al. (2003) with lesions restricted to sub-regions of the mPFC such as ACg and IL and to orbitofrontal (OFC) sectors of PFC showed that in a 5-CSRT task attentional selectively is related to ACg, impulsive responding to IL and perseveration to the OFC suggesting that impulsiveness and compulsiveness can be anatomically distinguished. Lesions of the PrL-IL sector of the mPFC impaired attentional selectivity and increased impulsivity and perseverative responding (Passetti et al. 2002). Therefore it could be suggested that visual selectivity and the ability to use temporal cues to guide performance might reside in the dorsal areas of the mPFC (ACg) whereas ventral regions of the mPFC and OFC appear critical for different aspects of inhibitory response control such as impulsivity (IL) and compulsive perseveration (PrL and OFC).

The dorsal and ventral regions of the mPFC have been shown to be involved in different behavioral 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 2000) and temporal patterning of behavioral sequences (Delatour and Gisquet-Verrier 2001), timing of extrinsic stimuli (Dietrich and Allen 1998), egocentric memory (Ragozzino and Kesner 2001) and spatial win-shift behavior

(Seamans et al. 1995). In contrast, the ventral part of the mPFC comprising ventral PrL and IL as well as the motor cortex appears critical for the flexible shifting to new strategies or rules in spatial and visual discrimination (Ragozzino et al. 1998 and 1999). 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).

The striatum is as an obvious component of the functional neural system engaged by 5-CSRT task performance, considering not only its connectivity with the mPFC, but also the effects of excitotoxic striatal lesions on 5-CSRT's task performance. The dorsal CPu for instance is the main striatal projection area of ACg and has been implicated in the formation of the response required to perform an attentional task (Brown and Robbins 1991a). Excitotoxic lesions of medial region of the CPu in rats caused impairment in accuracy and deficits in response control in the 5-CSRT task reminiscent of the effects of excitotoxic lesions of the mPFC (Muir et al. 1996a,b) or blockade of NMDARin the mPFC (Mirjana et al. 2004; Murphy et al. 2005). In addition, in rats damage to the CPu impairs acquisition of associations between stimuli and motor responses (Colombo et al. 1989; Packard and White 1990; Packard and McGaugh 1992; McDonald and White 1994).

The striatal projection area of the PrL-IL includes also the NAC, which is important for the control of behaviour by the reward-associated stimuli (Cardinal et al. 2002). In addition, lesions of the core region of the NAC affect some aspects of performance, such

as an elevation in premature responses, but do not affect response accuracy (Christakou et al. 2004) consistent with the hypothesis that several components of 5-CSRT task performance are mediated by somewhat distinct cortico-striatal circuits.

3. NMDAR AND COGNITIVE FUNCTIONS

Glutamate (GLU) serves as the major excitatory neurotransmitter in the central nervous system. Given the multiplicity of receptor subtypes that have been described for GLU, a particular neuron's response to this excitatory neurotransmitter will be determined by the presence and organization of diverse receptor types. GLU receptors have been classified as ionotropic and metabotropic (fig.1.4). Ionotropic N-methyl-D-aspartate receptor (NMDA), α-amino-3-hydroxy-5-methyl-4-propionate (AMPA), and kainate (KA), receptors are ligand-gated cation channels, whereas metabotropic GLU receptors (mGluRs) are coupled to various signal transduction systems (Hollmann and Heinemann 1994a; Hollmann et al. 1994b; Monaghan et al. 1989; Nakanishi 1992).

Glutamate Receptor Subtypes

	Ionotropic			Metabotropic		
Functional classes	NMDA	AMPA	Kainate	Class I	Class II	Class III
Gene families	NR1	GluR1	GluR5	mGluR1	mGluR2	mGluR4
		GluR2	GluR6	mGluR5	mGluR3	mGluR6
	NR2A	GluR3	GluR7			mGluR7
	NR2B	GluR4				mGluR8
	NR2C		KA1			
	NR2D		KA2			
	NR3A			↑IP$_3$ Ca^{2+}	↓cAMP	
	Ligand-gated ion channels			Second messenger systems		
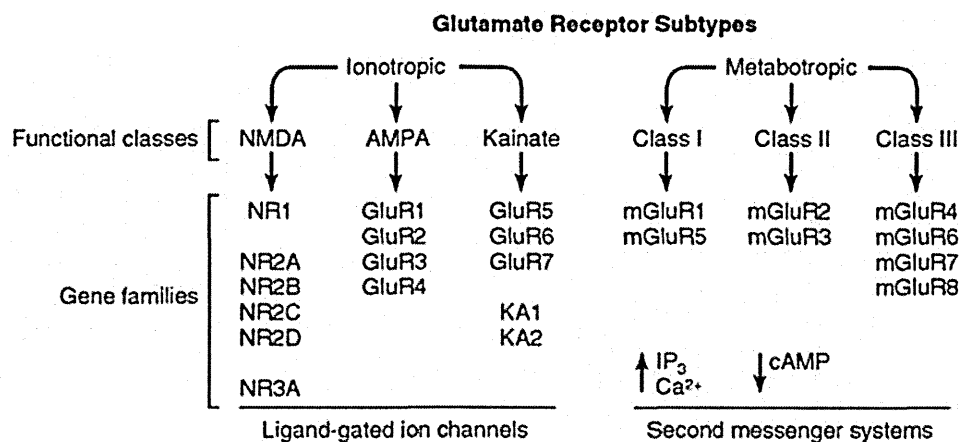
Fig.1.4
Molecular families of glutamate receptors.
Each of the two main glutamate receptor divisions comprises three functionally defined groups (classes) of receptor. These are made up of numerous individual subunits, each encoded by a different gene.

The glutamate NMDAR receptor is a tetramer consisting of different subunits, NR1, NR2 (A, B, C, D) and NR3 (A and B) which affect the biophysical characteristics of the receptor and the sensitivity to drugs (Lynch and Guttmann 2001). All NMDAR types appear to function as heteromeric assemblies composed of multiple NR1 subunits in combinations with at least one type of NR2 subunit. The NR3 subunits do not form functional receptors alone, but can co-assemble with NR1/NR2 complexes (Hirai et al. 1996; Laube et al. 1997; Perez-Otano et al. 2001). Different properties of the NMDAR depend on the subunit composition (Nakanishi 1992). Moreover, NMDAR subunits interact with various intracellular scaffolding, anchoring and signaling proteins and molecules associated with the post-synaptic densities (Cull-Candy et al. 2001). A distinctive feature of this receptor is that it is relatively inactivated under normal physiological conditions (Scatton 1993).

Autoradiographic studies have shown that NMDAR are widely distributed in the rat brain (Buller et al. 1994). *In situ* hybridization has revealed overlapping but different quantitative expression of the various NR2 subunits. In particular, NR2A mRNA is distributed ubiquitously like NR1 with highest densities occurring in hippocampal regions while NR2B subunit mRNA is expressed predominantly in the forebrain but not in the cerebellum where NR2C subunit predominates. In the cerebral cortex NMDAR are particularly enriched in the layers II/III and V/VI where they are preferentially localized on dendritic spines of pyramidal neurons but also present on excitatory and inhibitory axon terminals (Conti et al. 1997).

The NMDARs beside participating in excitatory synaptic transmission have been shown

to play a critical roles in many functions such as learning and memory, neuronal plasticity

but also in excitotoxicity (Dingledine et al. 1999; Li and Tsien 2009; O'Brien et al. 1998).

The involvement of NMDARs in these diverse processes, reflect their unique features, i.e.

(1) they are blocked by extracellular Mg^{2+} in a voltage dependent manner, (2) they have a

high permeability to Ca^{2+}, Na^+ and K^+ and, (3) they have slow activation/deactivation

kinetics.

Fig. 1.5
Activation and inactivation of the NMDAR
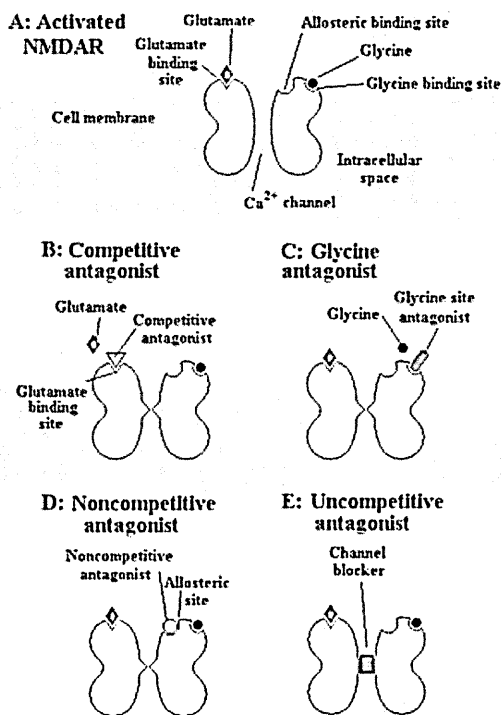
For electrical signals to pass, the NMDAR must be open. To remain open, an NMDAR must bind to GLU and to glycine. An NMDAR that is bound to GLU and glycine has an open ion channel and is activated.

Chemicals that deactivate the NMDAR are called antagonist. NMDAR antagonists fall into four categories: (1) competitive antagonists, which bind to and block the binding site of the neurotransmitter glutamate; (2) glycine antagonists, which bind to and block the glycine site; (3) noncompetitive antagonists (such as phencyclidine; PCP), ketamine and dizocilpine or MK-801), which inhibit NMDARs by binding to allosteric

sites; and (4) uncompetitive antagonists, which block the ion channel by binding to a site within it (fig. 1.5)

3.1 NMDAR Antagonists and Cortical Functions

Neuroimaging studies have provided some initial insights into the target brain regions of NMDAR antagonists in the human brain. Earlier studies reported that low doses of ketamine affect selectively the areas that are thought to be dysfunctional in schizophrenia such as the limbic cortex and basal ganglia (Morris et al. 2005; Soyka et al. 2005; Tamminga et al. 2003). A robust dose-dependent increase in cerebral blood flow (rCBF) was reported in the anterior cingulate, thalamus, putamen, and frontal cortex in healthy volunteers receiving ketamine (Holcomb et al. 2005; Langsjo et al. 2003).

Neuroimaging studies have also examined the regional effects of NMDAR antagonists in the brain of rodents. Ketamine has been shown to increase the blood oxygenation in the hippocampus, retrosplenial and orbital cortex, nucleus accumbens and ventral pallidum of anesthetized rats (Littlewood et al. 2006a,b). Risterucci et al (2005) examined the effects of PCP in two different animal models of schizophrenia (i.e. neonatal ventral hippocampal lesion and acute administration of PCP) using perfusion imaging. They found a significant increase in blood perfusion in the enthorhinal cortex, NAC, thalamus, and ventral pallidum, but a decreased blood perfusion in the PFC, temporal cortices and dorsal striatum. In contrast, Gozzi et al. (2008a) showed that PCP increased regional cerebral blood flow (rCBV) in the rat PFC and cingulate cortex (fig. 1.6).
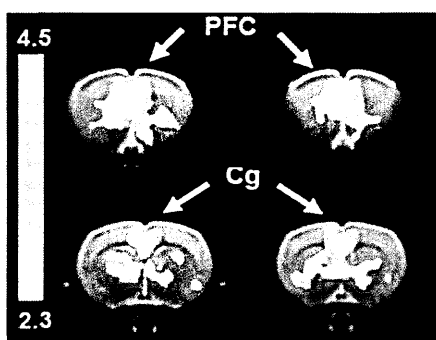
Fig. 1.6
Maps of regional cerebral blood flow (rCBV) response following acute PCP
Orange/yellow indicated increased rCBV.
Abbreviations: PFC, prefrontal cortex and Cg, cingulate cortex.

Several lines of investigation have implicated dysfunction of NMDAR in the pathophysiology of cognitive functions in schizophrenia (Anand et al. 2000; Goff and Coyle 2001; Meador-Woodruff and Healy 2000). A role for these receptors was suggested when NMDAR antagonists, such as phencyclidine (PCP) and ketamine, were found to induce a syndrome resembling the positive and negative symptoms of schizophrenia in healthy subjects and exacerbate such symptoms in patients with schizophrenia (Lahti et al. 1995; Luby et al. 1962; Javitt and Zukin 1991; Krystal et al. 1994; Malhotra et al. 1997). This discovery prompted a number of investigators to suggest that reduced NMDAR function is a core feature of the disease and subsequent studies with ketamine and dizocilpine (MK-801) have led to the hypothesis of an NMDAR dysfunction in schizophrenia (Javitt and Zukin 1991; Olney et al. 1999).

In animals systemic or intracortical NMDAR antagonists have been shown to cause hyperactivity (O'Neill, 1987; Jentsch et al. 1998), impairments in sensory-motor gating (Jentsch and Roth 1999; Yee et al. 2004) and deficits reminiscent of frontal lobe dysfunctions such as deficits in working memory in T-maze (Moghaddam and Adams 1998; Moghaddam et al. 1997; Romanides et al. 1999; Verma and Moghaddam 1996;

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Wesierska et al. 1990) and in a Delayed Matching-to-Position task (Aura and Riekkinen 1999) as well as impairments in attentional functioning (Greco et al. 2005; Le Pen et al. 2003; Mirjana et al. 2004; Pozzi et al. 2010). The selective NMDA-R2B receptor antagonist (Ro 63-1908) has been shown to induce deficits in attentional performance in the 5-CSRT task (Higgins et al. 2003a). Administration of NMDAR antagonists has become a widely used pharmacological model of schizophrenic-like cognitive deficit in rodents.

3.2 NMDAR and Glutamate Release

The behavioural effects of NMDAR antagonists in humans and experimental animals have been mostly attributed to hypoactivity of glutamatergic neurotransmission due to postsynaptic blockade of NMDAR (Javitt and Zukin 1991; Jentsch and Roth 1999). However, NMDAR antagonists, induce cortical excitation in humans (Breier et al. 1997; Holcomb et al. 2005; Vollenweider et al. 1997b) and in behaving animals (Gozzi et al. 2008b) and increase GLU release in rats (Moghaddam and Adams 1998). Thus, the NMDAR hypofunction may paradoxically cause an increase in GLU release and hypermetabolism (Gozzi et al. 2008a; Homayoun and Moghaddam 2007; Jackson et al. 2004; Takita et al. 2002) in corticolimbic regions.

How can NMDA receptor hypofunction lead to increased glutamatergic transmission?

In the intact brain, although locally applied NMDAR antagonists do not affect extracellular glutamate levels (Lopez-Gil et al, 2007; Lorrain et al 2003b), systemic administration leads to increased extracellular GLU in the mPFC.

NMDAR are preferentially expressed by pyramidal neurons (almost 70%) but are also present on non-pyramidal cells in the rat cortex such as astrocytes (Aoki et al. 1994; Conti et al. 1997; DeBiasi et al. 1996). Thus, NMDAR expressed by both cells populations may have contributed to NMDAR antagonists effects on extracellular GLU.

In addition, PCP markedly reduced bursting activity of cortical pyramidal neurons and it has been suggested that the response of GABA-ergic interneurons depends on the firing pattern of pyramidal cells (Shi and Zhang 2003). Thus, the enhanced GLU release in response to blockade of NMDAR likely reflects the reduced drive of cortical inhibitory interneurons by NMDAR antagonists (Homayoun and Moghaddam 2007; Jackson et al. 2004), which in turn causes GLU release and cortical excitation by removing the inhibitory tone on pyramidal neurons resulting in the dishinibition of cortical circuits. Indeed, NMDA blockade was associated with approximately 10-fold greater effects on GABAergic cells than on glutamatergic cells (Grunze et al.,1996). It follows that excitatory projections to the mPFC that are under GABAergic control could be more strongly affected by systemic NMDA antagonism than the pyramidal cells themselves, thereby leading to increased glutamate release by mPFC pyramidal cells.

These findings suggest that interactions between excitatory and inhibitory neurons play an important role in the response to systemically applied NMDAR antagonists in vivo. NMDAR antagonists may exert at least in part, their effects by disinhibiting GLU non-NMDA neurotransmission (Lewis and Moghaddam 2006; Moghaddam 2003a; Moghaddam and Jackson 2003b).

Thus, the effect of CPP on extracellular GLU may be mediated by direct or indirect suppression of cortical GABA-ergic transmission, which in turn enhances the release of

GLU from afferents to the mPFC. In particular, it can be suggested that NMDAR located on cortical GABA-ergic interneurons, precisely the fast spiking interneurons, are very sensitive to the effect of NMDAR antagonists in the mPFC (Homayoun and Moghaddam 2007) and this cause a disinhibition of postsynaptic GLU neurons and increase GLU release. These findings confirm the previous evidence corroborating the hypothesis of a hyper-activation of cortico-striatal transmission after the blockade of NMDAR in the PFC.

In addition NMDAR antagonist increase a variety of neurotransmitters in the PFC, dorsal and ventral striatum and hippocampus (Del Arco et al. 2008a; Martin et al. 1998; Matsumoto et al. 1992). Acute injection of NMDAR antagonists increased DA release and utilization in the PFC, as well as NE, Ach and 5-HT (Adams and Moghaddam 1998; Deutch et al. 1987; Hondo et al. 1994; Knauber et al. 1999; Krystal et al. 1994; Mathe et al. 1996; Rao et al. 1990; Wedzony and Golembiowska 1993; Yonezawa et al. 1998; Jentsch et al. 1998; Martin et al. 1998; Moghaddam and Adams 1998).
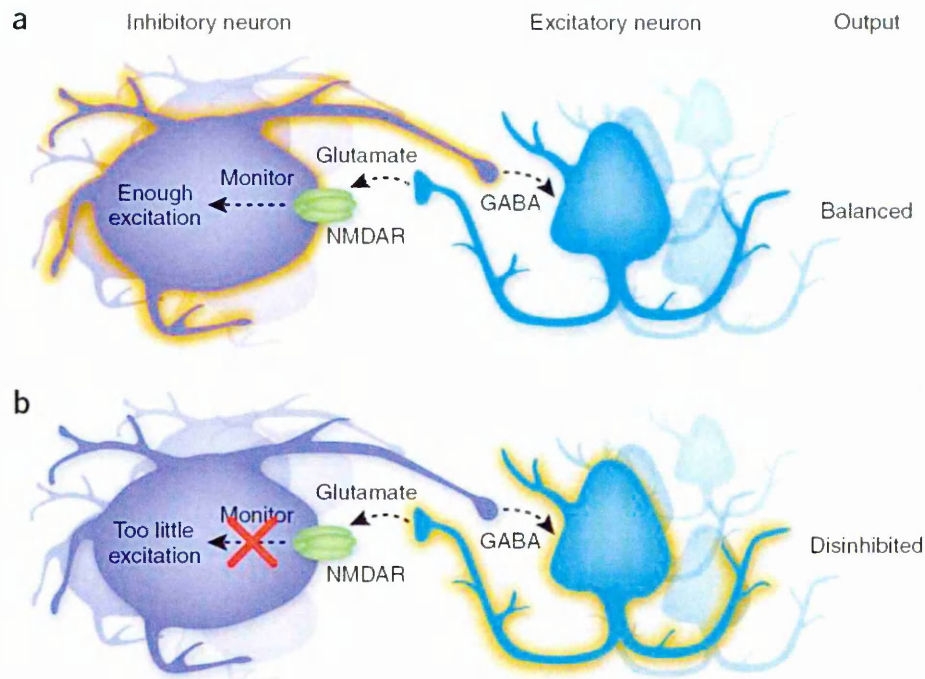
Fig. 1.7
The hyper-activation of cortico-striatal transmission after the blockade of NMDAR in the PFC.
a) Inhibitory neurons monitor levels of excitatory activity via NMDAR signalling. Normally, the inhibitory neuron maintains sufficient GABA release to balance inhibition with excitation. (**b**) In the cortex of individuals with schizophrenia, decreased NMDAR signalling disrupts this monitoring function, fooling inhibitory neurons into acting as if there is insufficient excitatory activity. The inhibitory neurons downregulate their output, disinhibiting the excitatory neurons.

4. CREB AND COGNITIVE FUNCTIONS

An exhaustive cellular and molecular explanation of cognitive processes is far from clear however, it is possible to identify synaptic plasticity, the ability of the brain to change the strength of synaptic connections in response to different stimuli, local protein synthesis in neurones and modifications of relevant genes as a common mechanism that may sustain cognitive performances. It appears clear that cognitive processes are complex phenomena in which interactions between different neuronal districts and brain areas need to be extremely fine regulated and integrated. Hence, the alteration in one of the numerous cellular pathways implicated may lead to significant changes in the overall cognitive capability.

4.1 The Transcription Factor CREB

Second messengers are molecules that convey signals from receptors on the cell surface to target molecules inside the cell, in the cytoplasm or nucleus. They rely on and greatly amplify the signals of hormones, growth factors and neurotransmitters, and cause changes in the activity of the cell. Second messengers are a component of signal transduction cascades.

Cyclic adenosine monophosphate (cAMP) is a second messenger important in many biological processes. cAMP is derived from adenosine triphosphate (ATP) and is used for intracellular signal transduction in many different organisms (Lalli and Sassone-Corsi 1994). It is involved in the activation of protein kinases and genes. It also regulates the passage of Ca^{2+} through ion channels. All genes regulated by cAMP contain a cis-acting DNA sequence, called cAMP-response element (CRE), which binds the phosphorylated

form of a transcription factor called CRE-binding (CREB, c-AMP Response Element-Binding) protein. A transcription factor is a protein that binds to specific DNA sequences thus controlling the transcription of genetic information from DNA to mRNA. It can act alone or in conjunction with other proteins in a complex, promoting (as activators) or blocking (as repressors) the recruitment of RNA polymerase (the enzyme that performs the transcription of the genetic information from DNA to mRNA) to specific genes (Latchman 1997; Mayr and Montminy 2001).

CREB is constitutively synthesized so that it exists in neurons under basal conditions. In its basal state, non-phosphorylated CREB is localized in the nucleus where it is bound to its response element CRE without considerable transcriptional activity. Indeed, CREB is unable to promote the transcription when not phosphorylated, whereas phosphorylated CREB strongly stimulates transcription. Binding of neurotransmitters and hormones to G_s protein−coupled receptors activates adenylyl cyclase, leading to an increase in cAMP and subsequent activation of the catalytic subunit of cAMP-dependent protein kinase (cAPK or PKA). The catalytic subunit of PKA then translocates into the nucleus where it phosphorylates CREB protein on serine 133 (S133) residue. Only phosphorylated CREB can interact with CREB binding protein (CBP). The CREB-CBP complex can recruit basal transcription factors and RNA polymerase on the promoter and initiate transcription.
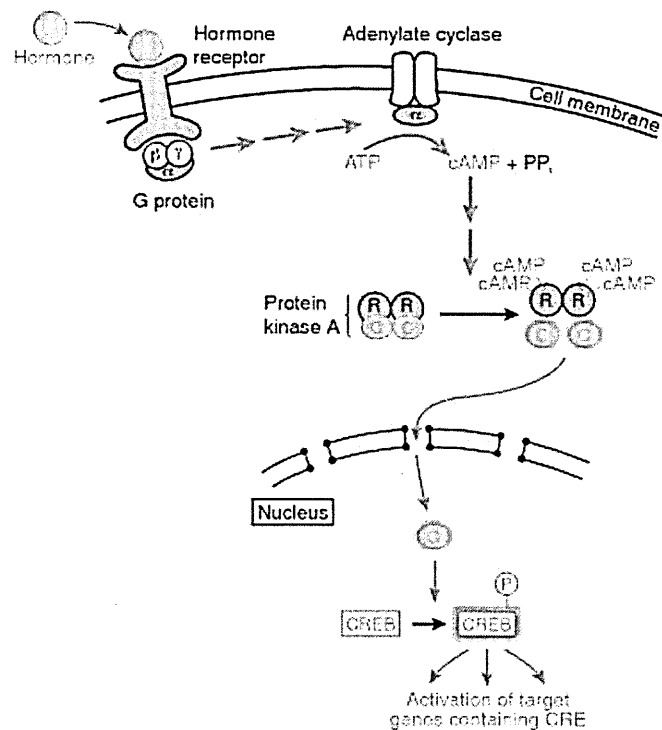
Fig. 1.8
Signaling through the cyclic AMP-protein kinase A signal transduction pathway phosphorylates CREB.
Binding of hormone to a specific cell surface receptor promotes the interaction of the receptor with a G protein. The activated G protein α subunit dissociates from the receptor and stimulates the membrane-bound adenylate cyclase to synthesize cAMP. The latter phosphorylates the regulatory subunits of protein kinase A, enabling release of the catalytic subunits which migrate to the nucleus and activate the transcription factor CREB (CRE- binding protein) by phosphorylation. Activated CREB binds to cAMP response elements in the promoters of target genes.

4.2 CREB Regulation

Phosphorylation of serine 133 is generally accepted to be a key event in the regulation of

CREB as mutation of this serine to alanine (S-CREB→A) abolishes the stimulus-induced

transcriptional activation by CREB (Gonzalez and Montminy 1989).

Protein phosphorylation is catalyzed by *protein kinases* which are enzymes that

phosphorylate proteins by transferring a phosphate group from ATP to the hydroxyl

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groups of the side chains of serine, threonine, or tyrosine residues. Most protein kinases phosphorylate serine, threonine or tyrosine residues. These enzymes are called protein-serine/threonine kinases or protein-tyrosine kinases, respectively. Protein phosphorylation is reversed by *protein phosphatases,* which are enzymes that reverse the action of protein kinases by catalyzing the hydrolysis of phosphate groups from phosphorylated amino acid residues. Like protein kinases, most protein phosphatases are specific either for serine and threonine or for tyrosine residues, although some protein phosphatases recognize all three phospho-amino acids.
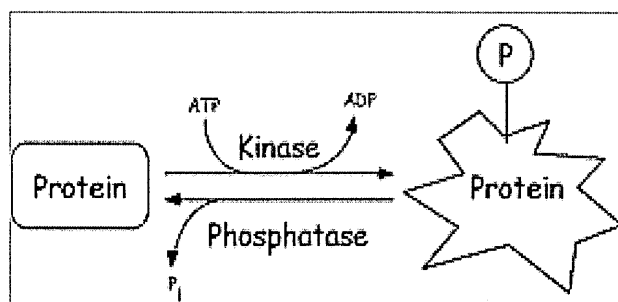
Fig. 1.9
Basic steps in protein phosphorylation
The combined action of protein kinases and protein phosphatases mediates the reversible phosphorylation of many cellular proteins.

The phosphorylation of CREB on S^{133} is regulated by a balance between the action of proteins kinases and phosphatases. As already mentioned, upon activation of the adenylate cyclase (AC) pathway, the serine at position 133 of CREB is phosphorylated by PKA, which enhances the transcriptional activity of the protein. Two other important protein kinases strictly associated with phosphorylation of CREB on S^{133}, are the dual phosphorylated mitogen activated protein (MAPK; i.e. ERK1 and ERK2) kinase and the calcium/calmodulin-dependent protein kinase II (CaMKII), which is a crucial mediator of

synaptic plasticity (Choe and Wang 2001b; Colbran and Brown 2004; Dash et al. 1991; Sheng et al. 1991).

Because of the complexity of Ca^{2+} signal transduction, it is still unclear how Ca^{2+} signals are propagated to the nucleus to regulate CREB S^{133} phosphorylation. Ca^{2+} directly influences the activity of many key regulatory enzymes, such as Ca^{2+}/ CaMKs, protein kinase C, and Ca^{2+}-calmodulin-dependent AC, which in turn may activate PKA. Similar to growth factor signals, Ca^{2+} can also activate the MAPK pathway (Finkbeiner and Greenberg 1996), which involves Ras, raf kinases, MAP kinase kinase (MEK), MAPK, and p90 ribosomal S6 kinase (RSK).

Despite phosphorylation on S^{133} represent the key event in CREB activation, CREB can be phosphorylated at a number of other sites including S^{129}, S^{142} and S^{143}, however, the physiological significance of phosphorylation at these sites is unknown (Johannessen et al. 2004; Johannessen and Moens 2007).

Several protein phosphatases have been shown to regulate de-phosphorylation of S^{133}CREB. They act either directly on CREB, or they may govern the phosphorylation status of CREB indirectly by controlling the enzymatic activity of CREB's kinases.

The serine–threonine protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) directly dephosphorylates CREB at S^{133} (Alberts et al. 1994; Hagiwara et al. 1992). Studies in rat hippocampal neurons suggest an indirect role for the protein phosphatase 2B (PP2B also known as calcineurine) in CREB dephosphorylation, as it potentiated the activity of PP1, presumably by dephosphorylating the PP1 inhibitor I-1 (Bito et al. 1996; Johannessen et al. 2004). PP1 is involved in the dephosphorylation of calcium-activated CREB phosphorylation *in vitro* (Bito et al. 1996) and *in vivo* (Genoux et al. 2002). PP1

and PP2A gave similar rates of *in vitro* dephosphorylation of both S^{133} and S^{142} phosphoserines, while PP2B was most active towards phosphoserine S^{133}.

4.3   Stimulus-induced   activation   of   CREB-dependent transcription requires the concerted action of multiple kinases

Multiple stimulus-dependent protein kinases have been implicated in CREB phosphorylation in neurons. Often, components of different molecular pathways interact, resulting in signaling networks. Indeed, protein kinases function as components of signal transduction pathways in which one kinase activates a second kinase, which may act on yet another kinase. An example of this cascade of events is represented by the MAPK pathways (Orton et al. 2005). The recruitment of CREB by a variety of intracellular pathways allows the integration of different transduction mechanism involved in cellular functions.

Strikingly, the same stimuli, such as PKA, may activate both CREB kinases and CREB phosphatases (Ahn et al. 2007). Furthermore, it is important to note that the final effect of CREB activity depends not only on the nature of the stimulus but also on the cellular context where the stimulation takes place. For example, activation of synaptic NMDAR induces CREB phosphorylation and CREB-dependent gene expression, while activation of extra synaptic NMDAR shut off CREB activity by increasing its dephosphorylation (Hardingham and Bading 2002a; Hardingham et al. 2002b; Riccio and Ginty 2002). The convergence of multiple intracellular signalling cascades on CREB, place this transcription factor in an ideal situation to integrate different stimuli and regulate

neuronal responses. The next cartoon (Fig.1.10) illustrates signalling to CREB via multiple pathways.
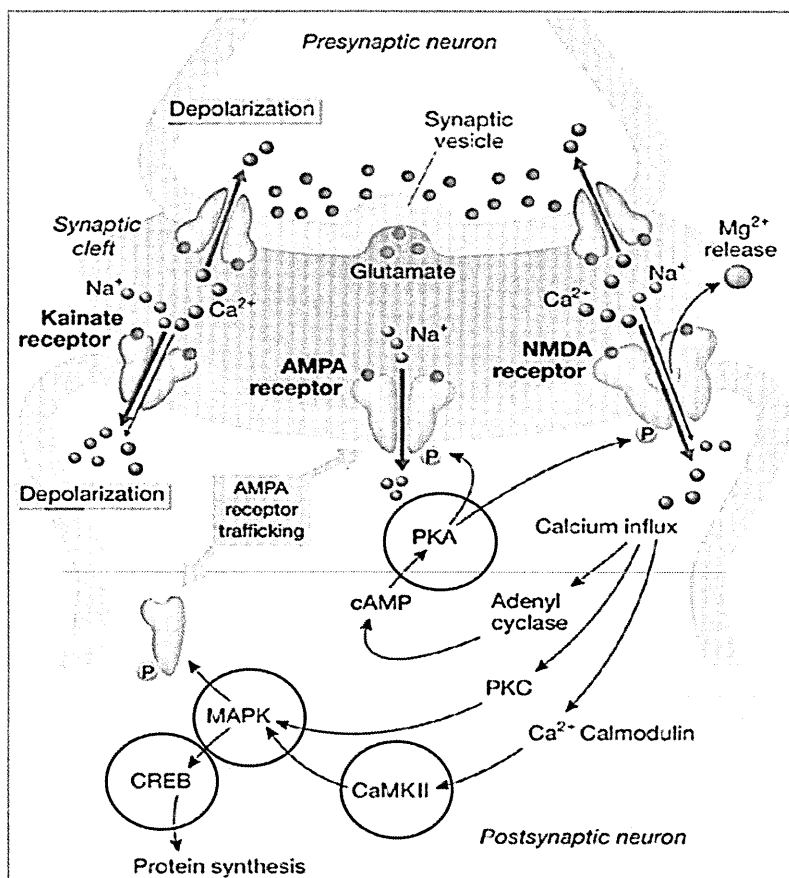
Fig. 1.10
Multiple pathways regulate signalling to CREB.
CREB activates protein synthesis and transcription of target genes in response to a vast array of stimuli, including glutamate. These stimuli activate a variety of intracellular signalling pathways, leading to activation of a number of protein kinases such as PKA, Ca2+/CaMKII and MAPK.
Abbreviations AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CaMKII, calcium/calmodulin-dependent kinase II; CREB, cAMP response element binding protein; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C.

4.4 CREB and Cognitive Functions

CREB is a component of intracellular signalling events that has been linked to a number of cellular processes and regulates a wide range of biological functions, from spermatogenesis to circadian rhythms as well as memory formation. CREB is expressed during development, so structural deficits caused by deregulation of these early processes could lead to memory abnormalities. Because CREB is central not only to cell survival but also to many other physiological processes, it is not surprising that the consequences of disrupting CREB function in vivo are quite severe. A numbers of "loss-of-function" and "gain-of-function" strategies have been designed to discern the necessity for CREB in learning, memory, and plasticity in vertebrates.

The evidence that supports a role for CREB in cognition falls into two experimental categories: negative manipulations (where the levels of CREB are lowered) and positive manipulations (where the levels of CREB are increased).

4.5 CREB loss-of-function studies

Genetic studies using recombinant viruses or mutant mice have shown that inhibition of CREB leads to deficits in object recognition (Pittenger et al. 2002), socially transmitted food preference (Brightwell et al. 2005), and conditional taste or odour preferences (Balschun et al. 2003; Brightwell et al. 2005).

Furthermore, fear-associated learning has been shown to increase phosphorylated CREB levels and CRE-mediated transcription in the hippocampus (Bernabeu et al. 1997; Impey et al. 1998; Taubenfeld et al. 1999). Dysregulation of CREB pathway has also been

associated with age-dependent memory impairments (Brightwell et al. 2004; Chung et al. 2002).

A line of mice with a targeted disruption of the α and δ isoforms of CREB showed intact short-term memory, but disrupted long-term memory in several behavioural tasks, including contextual conditioning and spatial learning in the water maze, two hippocampal-dependent learning tasks (Bourtchuladze et al. 1994; Guzowski and McGaugh 1997). These studies clearly provide evidence for a direct role of CREB in memory formation in mice, and they were soon confirmed in rats (Josselyn et al. 2001; Pittenger et al. 2002; Silva et al. 1998).

Interruption of CREB signalling in rat hippocampus by infusion of *antisense oligo-deoxynucleotides* resulted in impairment of memory formation for water maze training (Guzowski and McGaugh 1997). Antisense oligonucleotides against hippocampal CREB mRNA can lower the levels of CREB and impair spatial memory. Interestingly, tests given immediately after training showed that the oligonucleotides against CREB do not disrupt short-term memory (Guzowski and McGaugh 1997). Also, injection of *small interfering RNAs* (siRNAs) directly into the hippocampus of wild-type mice, knocked down CREB's expression and disrupted long-term, but not short-term, memory (Peters et al. 2009). Electrophysiological studies in *Aplysia* indicated that decreasing CREB function blocked long, but not short-term changes in synaptic function (Dash et al. 1990).

Another strategy for interfering with CREB function is the use of a *dominant negative transgenic* strategy. In this strategy, a modified fragment of the CREB gene can be inserted in specific regions of the mouse brain (Kida et al. 2002). Using a microelectroporation technique to overexpress dominant negative mutant CREB

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(mCREB) in the adult mouse brain, it has been found that overexpression of mCREB in the anterior cingulate cortex induced neuronal degeneration (Ao et al. 2006). These findings were surprisingly, and suggested that a balanced constitutively active CREB phosphorylation is important for the survival of mammalian cells in the brain (Ao et al. 2006).

Recently, it has been shown that preventing CREB phosphorylation by viral-vector gene transfer in the PFC impaired attention (Paine et al. 2009) suggesting that a decreased activity of CREB has a negative influence on cognitive processes. These data are supported by findings showing that the PKA inhibitor Rp-cAMP disrupted attention in the same task (Paine et al. 2009). By contrast PCP-induced attention deficit was associated with increased p-CREB in the striatum (Pozzi et al. 2010).

While the complete disruption of CREB is lethal in mice (Rudolph et al. 1998) and is therefore not likely to be compatible with life, several pathological conditions exist in humans in which CREB function may be disrupted subtly or incompletely. In humans, some genetic disorders leading to mental retardation have been directly related to the CREB pathway. The gene encoding RSK-2 (p90 ribosomal S6 protein kinase 2), one of several putative CREB kinases, for instance, is mutated in Coffin-Lowry syndrome, a complex disorder characterized by multiple physical abnormalities and mental retardation (Trivier et al. 1996). In addition, a heterozygous mutation in the CREB binding protein (CBP) gene produces Rubenstein-Taybi Syndrome, which is similarly characterized by multiple deficits including mental retardation (Petrij et al. 1995).

4.6 CREB gain-of-function studies

The role of over expression of CREB has not been examined systematically. While viral CREB rescued conditioning deficits in CREB knockout animals, additional CREB did not seem to enhance memory of the wild-type controls. Chronic enhancement of CREB, using genetic manipulations in mice, did not seem to enhance memory in a water maze task (Viosca et al. 2009). While additional CREB did not enhance acquisition, it interfered with retrieval, suggesting that there may be an optimal level of CREB activation that is compatible with normal memory function (Viosca et al. 2009).

Over expression of CREB can enhance the formation of long-term memory (Josselyn et al. 2001). Recent papers suggest that CREB is one factor that controls intrinsic excitability which provides an additional mechanism by which CREB can contribute to a gating function on memory acquisition and expression (Lopez de Armentia et al. 2007; Viosca et al. 2009; Zhou et al. 2009).

Some studies have also evaluated the role of CREB in response to performance or training on behavioural task. In particular evidences indicate that CREB pathway is a core component of the molecular switch that converts short- to long-term memory. Genetic studies in *Drosophila* also uncovered a role for CREB in memory, suggesting that this transcription factor has an evolutionary conserved role in memory (Yin et al. 1994). CREB was first implicated in learning and memory in flies by experiments in which inducible expression of a CREB activator was found to enhance and inducible expression of a CREB repressor was found to block the formation of long-term memory in the olfactory task (Yin et al. 1994 and 1995).

Formation of memory for a place strategy, appears related to sustained phosphorylation of CREB in the hippocampus, whereas formation of memory for a response strategy is related to phosphorylation of CREB in the striatum (Colombo et al. 2003). CREB phosphorylation is increased by exposure to a novel environment (Vianna et al. 2000), contextual fear conditioning (Stanciu et al. 2001), inhibitory avoidance (Bernabeu et al. 1997); (Cammarota et al. 2000; Taubenfeld et al. 2001), and radial arm maze training (Mizuno et al. 2002).

In conclusion, inhibition of CREB activity impairs behavioural performance in various memory tests across different species, while over expression of CREB had no effect or facilitated long-term memory. These studies indicate that memory formation require CREB and support its importance in complex cognitive processes.

4.7 Glutamatergic regulation of CREB

CREB activity has been demonstrated to be regulated by glutamate. Activation of glutamate receptors result in Ca^{2+} influx through *ionotropic* NMDA, AMPA/kainate receptors and voltage-gated Ca^{2+} channels. Ca^{2+} then activates kinases such as Ca^{2+}/calmodulin-dependent kinases (CaMK) and mitogen-activated protein kinases (MAPK), resulting in activation of transcription factors including CREB, which mediate long lasting structural changes and function (Ghosh and Greenberg 1995; Mattson et al. 1988; Soderling et al. 1994). Interestingly, it was found that neither AMPA/kainate receptors nor NMDAR were able to stimulate independently S^{133} CREB phosphorylation (Vanhoutte et al. 1999). Instead a series of connected events from AMPA/kainate receptors to NMDAR and from NMDAR to L-type Ca^{2+} channels was shown to be

necessary. The AMPA/kainate receptors are involved in relieving the Mg^{2+} block of

NMDAR, which in turn trigger the opening of L-type Ca^{2+} channels. The Ca^{2+} entry

through L-type Ca^{2+} channels (Rajadhyaksha et al. 1999) most likely activates S^{133} CREB

phosphorylation (Fig 1.11).

Fig. 1.11
Model of the interaction of glutamate receptors and CREB
(1) The activation of AMPA/kainite receptors causes Na^+ influx and a local depolarization that relieves the Mg^{2+} block of the NMDAR. **(2a)** Activation of the NMDAR via ligand binding and depolarization leads to Na+ as well as Ca^{2+} influx. Unlike the AMPA/kainate receptors, NMDAR do not rapidly desensitize and allow for a depolarization that is strong enough to trigger the opening of L-type Ca^{2+} channels. **(3)** L-type Ca^{2+} channels allow for Ca^{2+} influx and activation of a kinase pathway that translocates to the nucleus to phosphorylate S^{133} CREB. **(2b)** A L-type Ca^{2+} channels-independent signal transduction cascade from NMDAR to CREB phosphorylation is negligible (From Rajadhyaksha, 1999).

Synapse

L-type Ca^{2+} channel

AMPA/kainate

NMDA

1.

2a.

L-type Ca^{2+} channel

2b.

3.

CREB kinase

^{133}Ser-phospho-CREB

gene expression
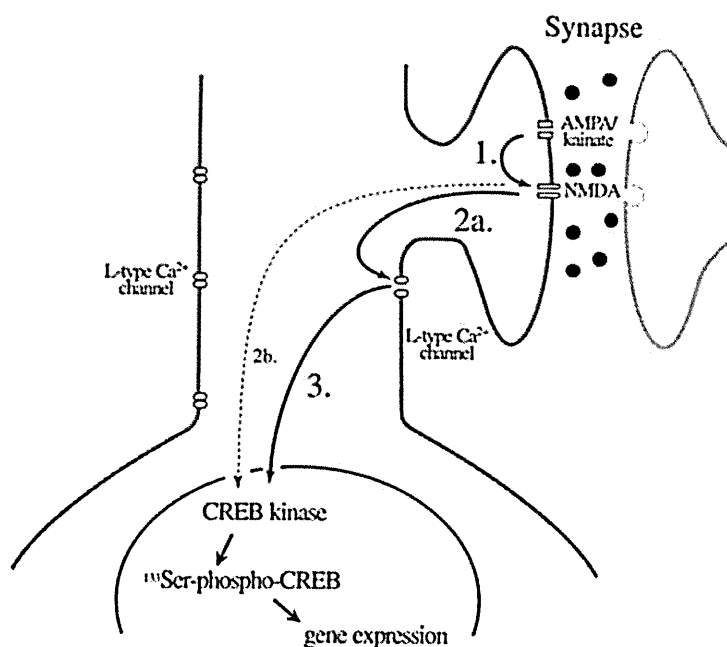
This NMDAR-mediated rise in postsynaptic Ca^{2+} concentrations activates a network of kinases and phosphatases that modulate CREB phosphorylation (Nijholt et al. 2002). Thus, it is conceivable that blockade of these receptors by competitive or non competitive antagonists should reduce the Ca^{2+} influx into the cell. As reported in fig. 1.10, Ca^{2+} influx trough NMDAR influenced the activity of various intracellular pathways such as CaMK, PKC and cAMP/PKA thus a decrease in Ca^{2+} concentration should lead to a decreased activity of these intracellular pathways and possibly to a decrease in CREB phosphorylation.

Accordingly, NMDAR antagonist decreased p-CREB on the active site S^{133} both *in-vitro* and *in-vivo*. In cells, MK-801 inhibited the p-CREB and the expression of CREB-regulated genes (Stepulak et al. 2005). Blocking the NMDAR in corticostriatal organotypic cultures with PCP also decreased p-CREB (Xia et al. 2010). Chronic blocked of NMDA receptor through systemic administration of the competitive antagonist, CGP 39551, decreased p-CREB in the cerebellum of rat pups (Monti et al. 2002) and repeated PCP injections decreased of p-CREB levels in the rat PFC (Molteni et al. 2008).

Interestingly, NMDAR antagonists at doses that impair cognitive functions in rodents increased the phosphorylation of CREB on S^{133}. For instance, it has been reported that acute PCP increases p-CREB in the FC and CPu of mice (Svenningsson et al. 2003). Accordingly, we found that that the dose of PCP and MK-801 that caused attentional and performance deficit in the 5-CSRT task increased p-CREB in the CPu of DBA mice (Pozzi et al. 2010) and rats (Pozzi, unpublished).

Although the mechanisms leading to increased S^{133}CREB phosphorylation after blockade of NMDAR is not yet clear, it might likely be the consequence of activation of non-

NMDAR by glutamate (such as mGluRs) or might involve NMDAR located extrasynaptically. Indeed, the localization of NMDAR on the synapses further complicates the relationship between NMDAR and CREB. While the activation of synaptic NMDAR supports robust phosphorylation of CREB on its critical transcriptional regulatory residue, S^{133}, in dramatic contrast, when extrasynaptic NMDAR are activated, CREB is only transiently phosphorylated and CRE-dependent gene expression is not activated (Riccio and Ginty 2002). Thus, a 'shut-off' signal from extrasynaptic NMDAR seems to selectively inhibit CREB-dependent transcription.

Glutamate also activates a different group of receptors i.e. the *metabotropic* glutamate receptors (mGluRs) that are G-protein receptors (GPCR) coupled via second messenger systems to biochemical pathways and ion channels (Pin and Acher 2002; Platt 2007). Specifically: *group I (mGluR1 and mGluR5)* receptors stimulate inositol trisphosphate IP3 hydrolysis/Ca^{2+} signal transduction; *group II (mGluR2 and mGluR3)* receptors inhibit adenylyl cyclase and decrease cAMP (Platt 2007); on the other hand, it was also shown that stimulation of mGluR group II could increase cAMP accumulation in astrocytes (Moldrich et al. 2002). Glutamate *group III (mGluR4, mGluR6, mGluR7 and mGluR8)* receptors are negatively linked to AC activity (Platt 2007).

All these events might affect S^{133} CREB phosphorylation in the postsynaptic neurons. Furthermore, CREB phosphorylation may be also regulated by the interactions between both ionotropic and mGlu receptors. In cultured striatal neurons for instance, activation of NMDAR with NMDA increased the number of neurons expressing phosphorylated p-CREB. Co-incubation of a group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG)

that itself did not alter basal p-CREB expression augmented NMDA-induced CREB

phosphorylation (Mao and Wang 2002).

5. AIMS OF THE PROJECT

This introduction has set out the general background to this work. This has included material from neuropsychology, pharmacology and behavioural psychopharmacology. With such diverse background it was essential to frame specific questions to which experiments were addressed. The main question was "does p-CREB play a role in the mechanisms that govern NMDAR antagonists-induced impairments in executive function and attention?".

5.1 Statement of the Problem

Recent finding from my lab showed that blockade of NMDAR in the PFC, by infusion of the competitive NMDAR antagonist (R)-CPP, was sufficient to cause behavioural impairments in the 5-CSRT task. Thus, the first question to be asked was whether and how (R)-CPP (at the dose that impaired 5-CSRT task's performance) affected p-CREB in the cortico-striatal circuit. The second question to be asked was whether the mGlu2/3 agonist (LY379268) that prevented the (R)-CPP-induced attentional deficits in the behavioural task, also prevents the (R)-CPP-induced p-CREB changes and the (R)-CPP-induced release of glutamate in the PFC. Third question was whether and how performance on the 5-CSRT task may affect (R)-CPP-induced p-CREB changes.

5.2 Aims

It is possible to summarize this PhD project by the following three aims:

AIM 1: To study the time course of the effects of the competitive NMDAR antagonist, (R)-CPP microinjected into the mPFC on levels of p-CREB, and its upstream kinases; p-ERK1/2, p-CaMKII, and p-PKA in the FC, CPu and NAC.

AIM 2: To test the hypothesis that cognitive deficit induced by blockade of NMDAR in the mPFC might be associated with increased glutamate release and changes in CREB phosphorylation I studied the ability of a mGluR2/3 agonist: (1) to reverse the (R)-CPP-induced attention performance deficit; (2) to interfere with CREB phosphorylation in FC, CPu and NAC, and (3) to prevent the (R)-CPP-induced rise in extracellular GLU in the PFC.

AIM 3: To investigate more directly the causal relationship between CREB phosphorylation and attention performance in the 5-CSRT task, I measured CREB phosphorylation in rats that were performing the task in condition of impaired attentional performance induced by intra mPFC infusion of (R)-CPP.

The experimental methods have concentrated on behavioural, neurochemical and immunoblotting techniques. It was hoped that these studies could provide new information on the molecular 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

This chapter contains a description of the general methods and protocols used in this thesis. All behavioural experiments were supervised by Dr. Carli Mirjana head of the Pharmacology of Cognitive Behaviour Unit of the Neurochemistry and Behaviour Laboratory, at the Mario Negri Institute for Pharmacological Research.

Intracerebral microdialysis was done in collaboration with Dr. Calcagno Eleonora and supervised by Dr Roberto Invernizzi head of the Neurochemistry and Behaviour Laboratory, at the Mario Negri Institute for Pharmacological Research.

Some of the data presented in the thesis have already been reported and they were showed to support the hypothesis.

I contributed to the planning and execution of all the experiments discussed in this thesis.
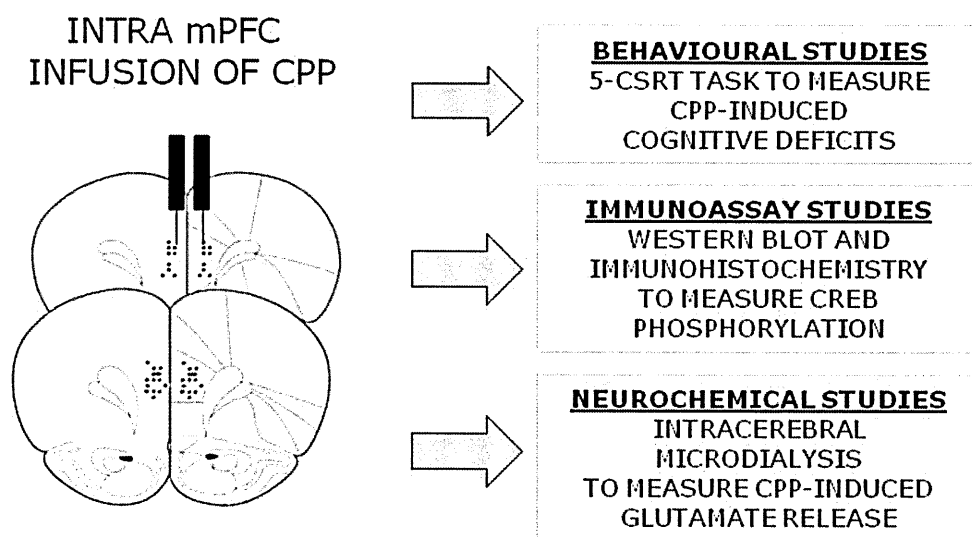
Fig. 2.1
General design of the study
This thesis is the result of a multidisciplinary approach which combines behavioral, biochemical and neurochemical assays to evaluate the role of the transcription factor CREB and cortical glutamate release in the ability of (R)-CPP to impair attention and response control in rats performing the 5-CSRT task.

2.1. ANIMALS

Ethical Issues In Animal Research

The work described in this thesis involves experimentation in living animals. For decades rats have been the species of choice in experimental neuropharmacology research. This is in part due to the reliability and relative ease with which invasive, such as site-specific brain micro-infusion, and molecular techniques, can be applied, in combination with a wide range of well established behavioral models. Of course rodents are not miniature humans so it's impossible to fully model the complexity of human neuropsychiatric diseases in experimental animals. Therefore the utility and validity of rodent's models must be evaluated based on objective criteria such those first proposed by Robbins (Robbins and Sahakian 1979), and that cannot be established in a single experiment but are the results of many studies over time:

- Face Validity: the ability to mirror the human deficit.

- Construct Validity: comply with the fundamental or hypothesized pathophysiological basis of the disorder.

- Predictive Validity: the most relevant case of predictive validity in research using animal models is pharmacological validity, which is the ability of a drug effect in the animal model to predict similar effects in humans.

Moreover, when animals are used for the benefit of humans, such as is the case in research, it is crucial that this is done in the most ethical manner possible. The *"three Rs"*, which were first described by Russell (1995), form the basis of the ethical laws: R as reduce the number of animal tested, or obtains more information from the same animal; R as replace the animal models with alternative methods whenever possible, to achieve

same scientific aim; <u>R as refine</u> the methods used in order to reduce or eliminate discomfort and distress for the animals.

Animals   All the rats used in these studies were male Lister Hooded weighing 250–350 g (Charles River, Italy). They were housed under temperature-controlled conditions (21 ± 2 °C) and constant humidity (50 ± 10%) with a 12-h light/dark cycle (light on 7:00 am–7:00 pm). Rats used in behavioral experiments had limited access to food (pellets for rats, Altromin Riper, Italy) at the end of each day's testing to keep them at 85-90% of their initial free-feeding weight. Rats used in biochemical and microdialysis experiments had free access to food. For all rats water was available *ad libitum*.

Laws And Policies   Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with the national (Decreto Legislativo n. 116, Gazzetta Ufficiale, suppl., 40, 18 Febbraio 1992, Circolare No. 8, Gazzetta Ufficiale, 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, US National Research Council, 1996). All efforts were made to minimize animal suffering and to reduce the number of animals employed in the study.

2.2 SURGICAL PROCEDURES

Cannulae Implantation   The Kopf stereotaxic frame (model 900, David Kopf, Tujunga, CA, USA) (Fig. 2.2) consists of a 3-D system based on the Cartesian coordinates system. Three arms move along the anterior–posterior (AP), lateral (L) and dorso-ventral (DV) axis and each is provided with a micrometric scale. Using a brain atlas (Paxinos and Watson, 2007) mapping rat's central nervous system by coronal, sagittal and horizontal brain's sections, it is possible to determine the exact position of the brain area of interest and the coordinate to follow in order to reach a desired locus. Such an approach allows the injection of drug solutions directly into the specific brain areas.

Rats were anesthetized with 3 mL/kg Equithesin (composition in mM: pentobarbital 39, chloral hydrate 256, $MgSO_4.7H_2O$ 86, ethanol 10% v/v, propylene glycol 39.6% v/v). The rat head was shaved and secured in the stereotaxic frame with the incisor bar set at -3.3 mm relative to the interaural line. Next, the scalp was incised on the midline of the occipito-frontal plane, thus the surface of the cranium was exposed. Bilateral 23-gauge, stainless-steel guide cannulae (Cooper's Needles, UK) were implanted in the medial region of the prefrontal cortex (mPFC) (Fig 2.3) using standard stereotaxic techniques and secured to the skull using three bone screws and dental cement. The coordinates used were: anterior–posterior (AP)+3.8 mm from bregma, lateral (L)±0.8mm from midline, and dorsal–ventral (DV)-3.2 from dura (Paxinos and Watson, 2007). Thirty-gauge stainless-steel stylets were inserted in the guide cannulae. After surgery rats were housed singly and had few days of recovery.

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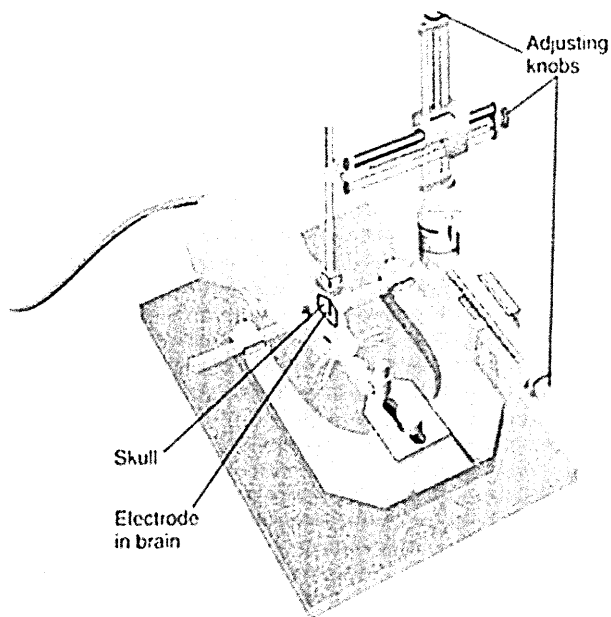
Fig. 2.2
Stereotaxic apparatus to performing brain surgery in rodents.

Recovery and post-operative care

Checks were made for any weight loss and rats that showed some discomfort were excluded from the experiment.

The rats used for the behavioral experiment and that were food-deprived during the experiments 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.

A.

B.

C.
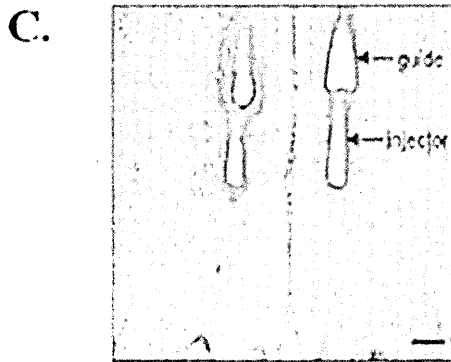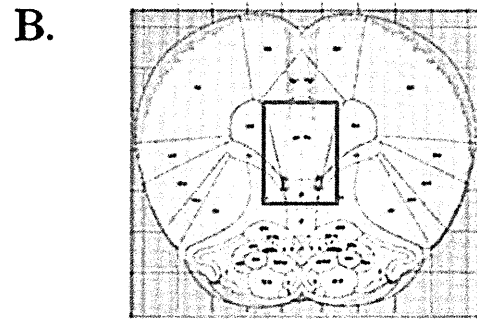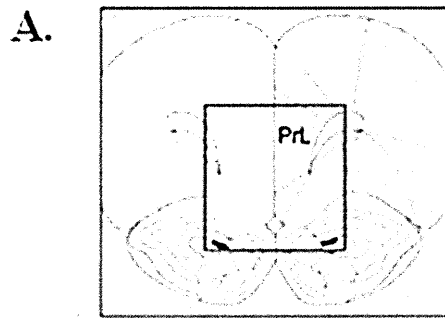
Fig. 2.3
A. Schematic representation of the prelimbic (PrL) region in the medial prefrontal cortex.
B. Example of slice stained with Cresyl violet to visualize the location of the injection tips
C. High magnification of the injection sites.

2.3 BEHAVIORAL STUDIES

The "5-choice serial reaction time" (5-CSRT) task for rats was first developed by Robbins and co-workers as an analogous of the Continuous Performance Test used with humans subjects (Carli et al. 1983; Robbins 2002).

Apparatus

The apparatus consisted of four specially designed boxes (Campden Ins. UK) controlled on-line by Whisker software. It consisted of two 25x25 cm aluminum chambers built in the Department 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 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, UK).
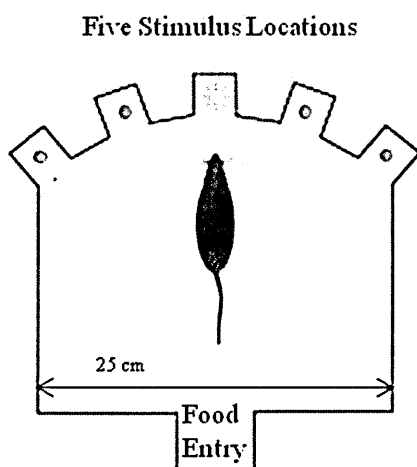
In this procedure, hungry rats are trained to monitor five locations for brief, randomly presented visual targets. Rats are placed in an operant chamber with a wall that has five nosepoke holes with stimulus lights inside. The rat must detect a light stimulus that appears randomly in one of the five nosepoke holes.

Fig. 2.4
Schematic apparatus for the 5-CSRT task.

Behavioral Procedures

Animals were trained on the 5-CSRT task to a stable performance. 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 numbers of times during the course of a complete session, with the order of presentation randomized by the computer.
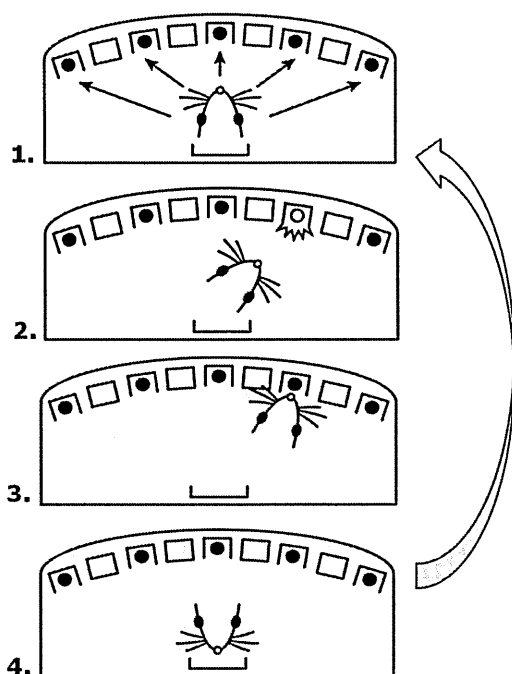
Fig. 2.5
Scheme of the different steps required to perform the 5-CSRT task.
Steps are: **(1)** scan for stimulus presentation, **(2)** Stimulus presentation, **(3)** nose poke at stimulus location and, **(4)** panel press to collect reward. *From Amitai and Markou, 2010*

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 s.

The ITI and time out both lasted 2 s during the first session and the ITI was raised to 5 s 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.

The variables recorded and analysed were:

• The percentage of *correct responses* (total number of correct responses/total number of corrects+total number of incorrect responses×100).

• The number of *anticipatory responses* (responses made in the holes during the waiting period before the presentation of the target)

• The number of *perseverative responses* (responses made in the holes after a correct response but before a nose-poke into the receptacle hole to collect the food reward).

• The number of *omissions* (total number of omissions/total number of correct+total number of incorrect+total number of omissions)

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The animal must be mildly hungry at the time of testing. Following extensive training (i.e. 60-80 days) on the task, performance generally reaches high levels, with low within- and between subject variance. Nevertheless, at 80% correct, performance is not at ceiling and it is still possible in theory to detect improvements in accuracy. The difficulty of the 5-CSRT task can be varied by reducing or increasing the brightness, duration or frequency of the target visual stimuli. Such manipulations clearly test the ability of the animal to "screen out" irrelevant stimuli and thus constitute tests of selective attention (reviewed by Robbins 2002).

The 5-CSRT task has been used in these studies to quantify cognitive deficits in rats treated with (R)-CPP alone or in combination with LY379268 or Sp-cAMP. Protocols and parameters for the 5-CSRT task have been extensively validated in our laboratory.

2.4 IMMUNOASSAYS

An immunoassay is a biochemical test that measures the amount of a substance in a biological sample. To identify target proteins among a number of proteins that are not the target of interest, these assays take advantage of the specific binding of an antibody to its antigen (Fig. 2.6).

Immunoassays, such as western blot and immunohystochemistry share several fundamental steps: preparation of sample, blocking of unspecific proteins, binding with primary and secondary antibodies, and protein detection. Proteins can be separated by electrophoresis or stained on brain slices properly treated to avoid protein degradation. In this thesis both Western blot and immunohystochemistry techniques were used.
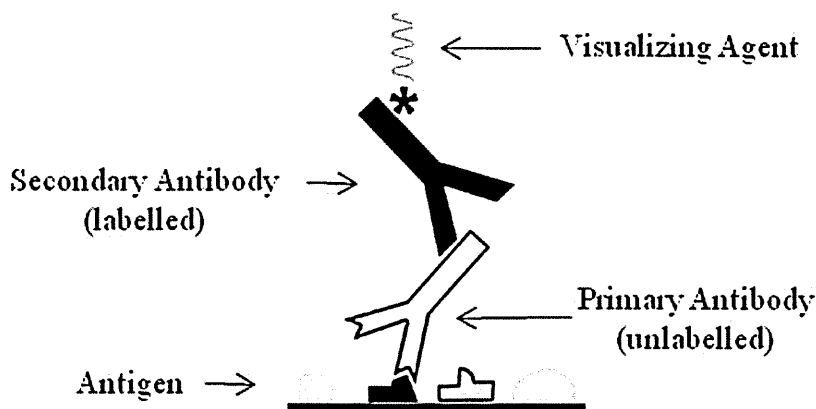
Fig. 2.6
Antigen-antibodies binding
Schematic representation of a conventional competition immunoassay with primary and labelled secondary antibodies used in immunoblotting detection.

2.4.1 WESTERN BLOT

General Principles   Western blot is an efficiently and useful method to detect and characterize proteins in the cerebral tissue even in small amounts (Towbin et al. 1979). Compared to other immunoassays approaches, it has higher specificity due to the identification of the single protein by its molecular weight.

Western blot is a very delicate process requiring the correct amounts of each component in order for successful identification of the target proteins. An imbalance in any step of the procedure may skew the entire process.

Protocol   To examine the effects of treatments on p-S^{133}CREB rats were implanted with bilateral cannulae in the mPFC as described previously. Rats were killed by decapitation; the brains were removed and immediately immersed in ice-cold saline for few seconds. The FC (comprising prefrontal, orbitofrontal, cingulate and primary motor cortex), CPu and the NAC were rapidly dissected, frozen on dry ice and stored at −70 °C for further analyses.
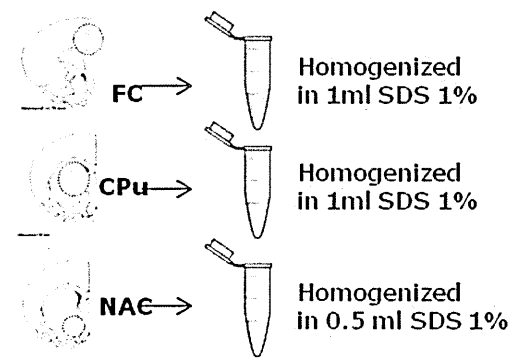
Fig. 2.7
Samples preparation for western blot analysis

Briefly, tissues samples were homogenized as indicated in figure 2.7. Twenty µg of protein were separated by 10% polyacrylamide SDS-PAGE gels, transferred onto PVDF membrane (0.45 µm, Amersham, GE Healthcare) then incubated overnight with primary antibodies against phosphorylated or total (native) protein.

The next day, after 1h incubation with appropriate horseradish peroxidase-labelled secondary antibodies, immunoreactivity was visualized with a chemiluminescent home made luminol/p-coumaric acid solution. Membranes were exposed in the dark room to autoradiography films (Hyperfilm ECL, Amersham GE Healthcare) for 30 sec, 1, 3 and 5 min then developed for 1.5 min.
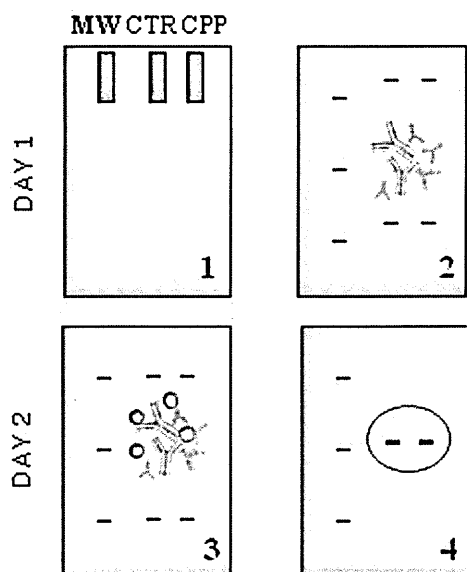
Fig. 2.8
Sequence of steps in p erformi a western blot experiment.
The western blot procedure involves samples loading onto polyacrylamide gels (1), binding with unlabelled primary antibody against the protein of interest (2), followed by HRP-peroxidase-linked secondary antibody (3). The antigen/antibodies binding is then visualised by light emission (chemiluminescence) (4).

Densitometric analysis of immunoblots was done to quantify the changes in protein levels using the public domain *ImageJ* program (Image Processing and Analysis in Java at

http://rsbweb.nih.gov/ij/). The optical density of the band for each antibody was linear in the range between 15 and 60 µg/total protein/well as calculated by rigorous linearity tests. Phosphorylated and total proteins were measured in the same samples.

Western blot has been used in this thesis to measure phosphorylated and total CREB, PKA, ERK1/2 and CaMKII in samples from FC, CPu and NAC after various treatments All the conditions used in this thesis has been validated in our laboratory as follows.

Antibodies Test Linearity   Although western blot has the advantage of using antibodies that are usually highly specific to the proteins of interest, is difficult to load a standard curve onto each gel together with the samples under analysis which are assayed, out of necessity, in separate gels. Thus, it is considered a semi quantitative technique.

We therefore developed a method that affords the standardization of the best antibody dilution for each brain region (figure 2.9). Our stringent methodology includes constructing a standard curve of at least three protein concentrations incubated with different antibody dilutions to reach the optimal ratio protein loaded/ antibody concentration with the highest signal-to-noise ratio to estimate the optical density of the samples in the best way. Such a curve serves two purposes: the first, to choose the right amount of samples to load for each brain region. The second, to choose the right dilution of both primary and secondary antibodies thus avoiding saturation of the signal with high levels of immunoreactivity that could result in a ceiling effect.

Antibodies dilutions used in this thesis fall within the linear range of the curve calculated for each antibody.

A. $p^{S133}CREB$

FC — 43 kDa
CPu
NAC
10 20 40 µg protein

B. $p^{T197}PKA\,c$

FC — 42 kDa
CPu
NAC
20 40 60 µg protein

C. $pT^{202}/Y^{204}ERK1/2$

FC — 44 kDa (ERK 1)
— 42 kDa (ERK 2)
CPu
NAC
10 20 40 60 µg protein

D. $p^{T286}CaMKII\alpha$

FC — 50 kDa
CPu
NAC
10 20 40 60 µg protein

E.

FC: 30846, 25109, 14173, 4328, r2=0.966
CPu: 32820, 22318, 12727, 5168, r2=0.996
NAC: 30736, 21534, 11512, 5854, r2=0.998

Arbitrary Optical density vs µg protein loaded
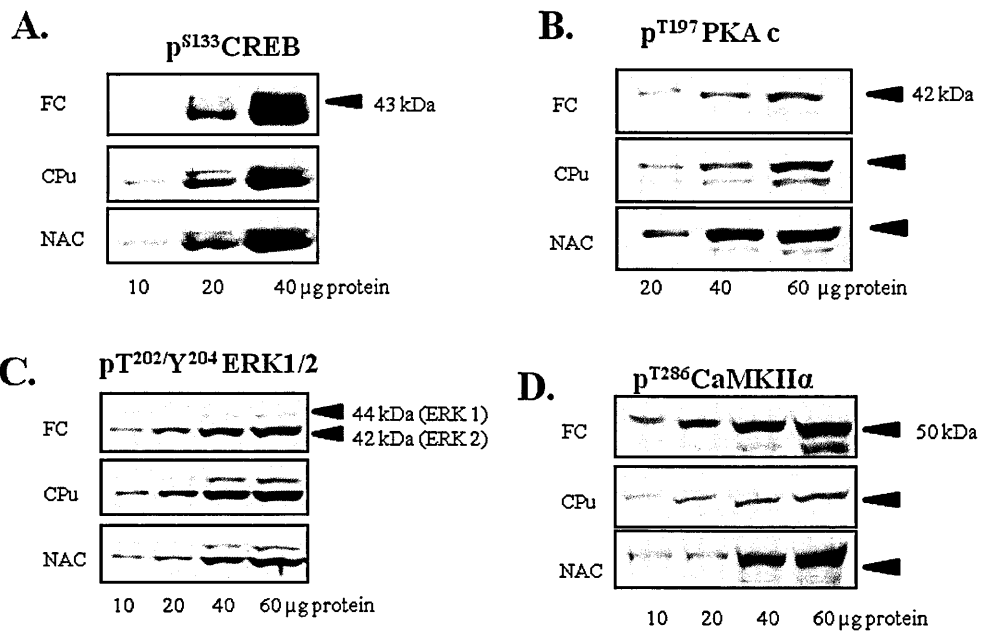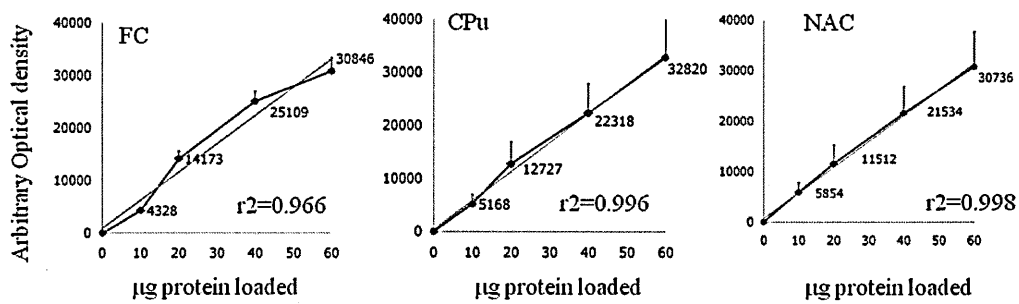
Fig 2.9
Antibodies test linearity
Representative immunoblots of p-CREB (**A**, 1:4000 dilution), pPKAc (**B**, 1:4000 dilution), pERK $_{1/2}$ (**C**, 1:8000 dilution) and pCaMKII (**D** C, 1:10000 dilution) linearity in the FC, CPu and NAC. Ten to 60 µg of total proteins were loaded in consecutive wells.
E. Example of quantification (black line) of pCREB antibody linearity test in the FC, CPu and NAC. Protein amount is plotted against the optical density measured after 1 min exposition time. Data are expressed as MEAN±SE of arbitrary optical density. N=4 per group.

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Mouse monoclonal antibodies against p-CREB (S^{133}, diluted 1:4000; Upstate Biotechnology USA; Cod. 05-667) and CREB (diluted 1:4000; Cell Signaling Technology, IT, Cod.9104) were used for western blot. Antibody binding was detected using an anti-mouse-IgG-HRP secondary antibody (diluted 1:8000; Cell Signaling Technology, IT, Cod.7174).

The following antibodies against proteins kinases were purchased from Cell Signaling Technology (IT). Dilutions of phospho primary antibodies were as follows: p-PKAc (T^{197}rabbit polyclonal, 1:4000; Cod. 4781); p-44/42MAPK (ERK1/2, $T^{202/}Y^{204}$ rabbit polyclonal, 1:10000; Cod. 9101) and p-CaMKIIα (T^{286} rabbit polyclonal, 1:10000; Cod. 3361). Antibodies against total amount of proteins (i.e. phosphorylated plus not phosphorylated) were also used and were as follows: PKAc (rabbit polyclonal, 1:4000; Cod.4782), MAPK (ERK1/2 , rabbit polyclonal, 1:10000; Cod. 9102) and CaMKII (rabbit polyclonal, 1:10000; Cod. 3362). Antibody binding was detected using anti rabbit-IgG-HRP secondary antibodies (Cell Signaling Technology, Beverly, MA, USA; Cod: 7074). Phosphorylated and total proteins were measured in the same samples.

All antibodies were dissolved in 2% albumine, 5% Tween-20 in Tris buffered saline (TBS; pH 7.6) and stored at +4°C. At these dilutions, the optical density of the band for each antibody was linear in the range between 15 and 60 μg/total protein/well as calculated by linearity tests.

2.4.2 IMMUNOHISTOCHEMISTRY

General Principles Immunohistochemistry or IHC refers to the process of localizing proteins of interest in a tissue section exploiting the same principle of antibodies-antigens binding in biological tissues (Ramos-Vara 2005). It takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue. IHC is widely used in basic research to understand the distribution and localization of proteins in different parts of a biological tissue. Immunohistochemistry is an excellent detection technique and has the tremendous advantage of being able to show exactly where a given protein is located within the tissue examined providing higher resolution imaging of antigen localization (Ramos-Vara 2005). It is also low cost, relative easy to perform, and widely used. Moreover, the possibility to label multiple antigens simultaneously is another advantage.

Its major disadvantage is that, unlike western blot, where staining is checked against a molecular weight ladder, in immunohistochemistry it's impossible to show that the staining corresponds to the protein of interest. For this reason, primary antibodies must be first well-validated before in western bot or similar procedure.

Protocol Unlike western blot, animals used in IHC are anaesthetized. Brain tissue samples require fixation procedures prior to processing. Fixing prevents enzymatic and other postmortem changes that degrade the tissue. Proper fixation facilitates immunohistochemical analyses, saves time and is an important step for histological processing.

Rats were intracardially perfused , which means that the blood is drained from the body by pumping the fixative solution into the vascular system (figure 2.10). Removing the

blood improves the results of staining, and the fixative preserves and usually hardens the

brain so that it can be sectioned (cut into thin slices) without tearing. Moreover, because

blood is responsible for the transport of myriad of other cells, and because these cells all

contain endoplasmic reticulum (Nissl substance, see Hystology section), any remaining

blood in the tissue will be deeply stained by cresyl violet and could obscure the structures

under analysis. Intracardiac perfusion is a technique that allows the delivery of a fixative

solution (e.g. paraformaldehyde – PAF) throughout the whole body, including brain.

Intracardiac perfusion requires the preparation of a fixative (4% PAF) and PBS buffer

that have to be made fresh on the day of its use or the night before and has to kept at

+4°C.
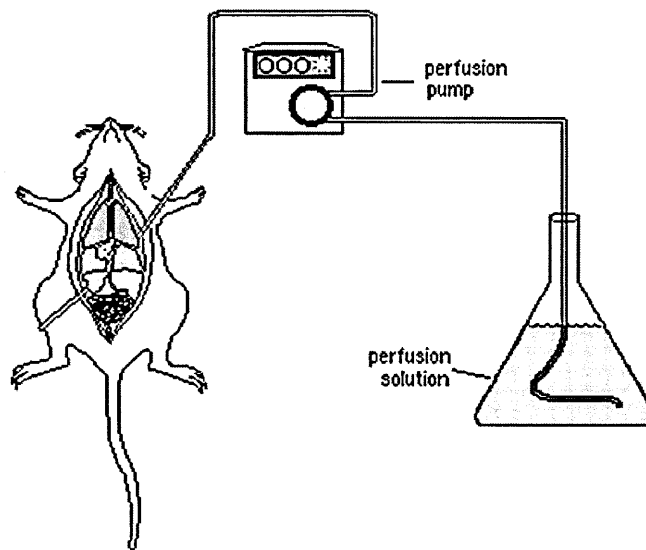
Fig. 2.10
Sequence of steps in performing immunohystochemistry experiment
Immunohystochemistry protocols require that the brain is perfused with fixative solution
by a perfusion pump via the circulatory system to achieve penetration of the fixative into
the brain.
After an incision made in the diaphragm, the sternum is clamped with a hemostat to
expose the heart. Perfusion is started very slowly (i.e., 20 to 40 mL/min). Immediately
after the peristaltic pump begins pumping the fixative, the right atrium is cut to allow an
escape route for the blood and perfusion fluid.

To examine the effects of treatments on the number of p-S^{133}CREB-positive cells, rats were implanted with bilateral cannulae in the mPFC as described previously. On the experimental day, 1 µL side vehicle or 50 ng/µL side CPP were delivered bilaterally into rats' mPFC at a rate of 0.5 µL/min. Forty minutes after vehicle or CPP injection, animals were anaesthetized with Equithesin (3 mL/kg, i.p.) and secured on a silicon support. The chest cavity was opened by cutting across the abdomen just below the sternum and then the diaphragm was carefully cut. The body cavity was exposed by clamping the ribs on each side and, if necessary, any connective tissue was cut to free the heart from the chest cavity. A butterfly needle (25 gauges) was inserted into the left ventricle, the right auricle was cut and animal was slowly perfused. The needle in fact was connected, by a plastic tube, to a peristaltic pump that allows a controlled-speed efflux of the perfusion solutions into the whole body. Ice-cold PBS was first delivered for 2 minutes to wash out blood and then 4% PAF for 4 minutes allowed the cross-linking of tissues, therefore their preservation. After perfusion was completed, the animal was decapitated and the brain quickly removed and placed in 4% PAF for 2-3 h for post-fixation.

After post-fixation procedure, brains were dipped in a solution of 20% sucrose (O/N). This step allows water removal from the brain, preventing tissue damage during freezing. Brains were frozen in n-pentane at - 45°C for 3 minutes and stored at - 80°C. The slicing of the brain is usually accomplished through the use of a cryostat that is a microtome housed in a freezing chamber that allows the sectioning to be performed at a temperature of −20° C. Brain sections for IHC analysis were obtained from frozen tissue using a cryostat (Leica Microsystems, Wetzlar, Germany). In the IHC procedure the thickness of the sections, may vary from 5 to 40 µm. Thirty µm free-floating slices (slices that are not mounted on slides) have been used in these studies.

Cells Detection And Count Protocols for IHC detection of a protein are based on the same work steps described for Western blot techniques which are: saturation of the membrane (blocking), immune detection (staining by antibodies) and visualization (figure 2.11). Free-floating sections (30 µm) were washed with TBS and then blocked with 2% normal goat serum (NGS, Vector Laboratories, UK). Sections were incubated overnight with anti mouse p-S^{133}CREB monoclonal antibody (diluted 1:500, Upstate Biotechnology, USA) at +4°C. The next day they were incubated with the secondary biotinylated goat anti-mouse antibody (diluted 1:300) for 1 hr at room temperature after which 1% avidin-biotin horseradish peroxidase complex (Vector Laboratories, UK) was added for 1 h. Peroxidase activity was determined by reaction with a solution containing diaminobenzidine (DAB, Sigma, IT) and nickel ammonium sulphate (Sigma, IT). The sections were mounted on slides, dried, dehydrated and covered with mounting medium (Permount, Fisher Scientific, USA). The procedures applied to count p-S^{133}CREB-positive cells are described below.

Because the immunohistochemical reaction and staining density may vary from time to time, all drug-treated and control were processed simultaneously with the same batch of reagents. Using a light microscope (Olympus BX51, Germany), 10X images (219x164 µm size) were captured by a video camera (Colour View, Olympus, Germany), and projected onto a computer monitor. Cell count was done using the Cell*F Image software (Olympus, Germany).
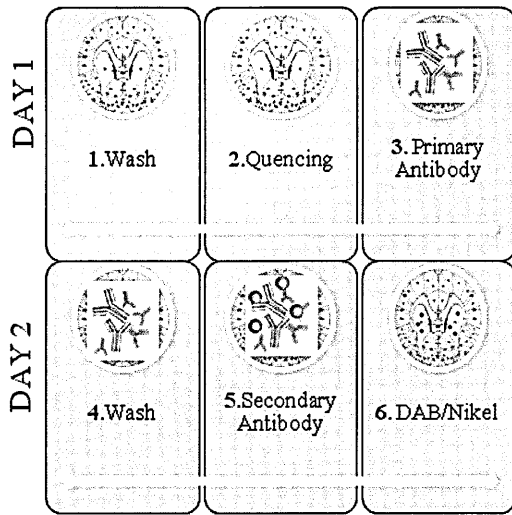
Fig. 2.11 Steps performed to detect positive cells

The immunohystochemistry procedure involves several steps: washing (1), quencing of peroxidases (2), binding with unlabelled primary antibody against the protein of interest (3) followed by washing (4) and incubation with avidin-peroxidase conjugate secondary antibody (red dots) (5). The biotinylated antigen bound to the immobilized primary antibody is then reacted with. The activity of the bound peroxidase is then visualised and the results are black-stained dots (6).

Briefly, a grid was applied onto single acquired image and cells counted in a fixed frame made of six squares of the grid (40x40 µm each) for a total area of 0.96 mm^2 (panel A in the (figure 2.12).
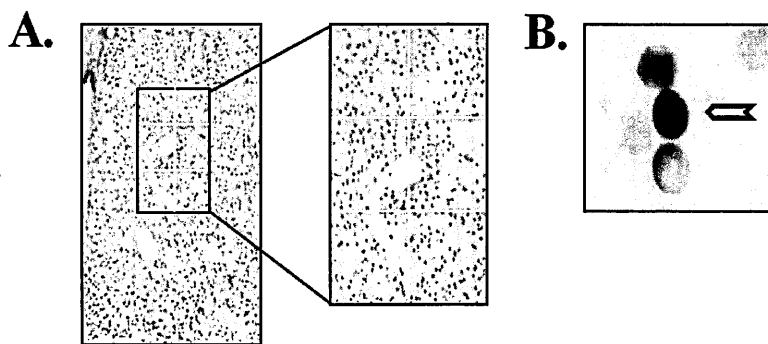
Fig. 2.12
Cells Count
(A) Representative photomicrograph (10X magnification) of p-S^{133}CREB-positive cells in the CPu of saline-infused rat. A six-framed grid was applied onto each acquired image. Inset represent positive cells counted in the sampling area (0.96 mm^2) by an automatic counting method, generated by the program after manual definition of positive nuclei by density and size criteria.
(B) High magnification (60X) of p-S^{133}CREB-positive, heavy stained, immunoreactive cell (arrow).

The number of positive cells was counted by automated selection of those cells within the unit area which fulfilled the two following criteria: (1) grey values (resolution: 256 levels) were required to be higher than the threshold value, (2) to exclude cell debris and other artifacts such as dye crystals, only nuclei with a perimeter between 0 and 5 µm, and area between 0 and 1 μm^2 were included.

Finally, the net grey values and the numbers of positive cells of all analyzed unit areas were averaged to obtain an average grey value and an average number of positive cells for each brain region of each individual animal.

Two sections per animal were counted and averaged to obtain the mean for each animal. Phospho S^{133}-CREB-positive cells were counted in each animal at two different AP levels corresponding to bregma +1.8 mm (anterior striatum) and -0.80 mm (posterior striatum and cortices) (Paxinos and Watson, 2007). To control for variations in staining between sections, the background (i.e. an area containing no staining) was subtracted in each section. P-S^{133}CREB immunoreactivity was found to be exclusively nuclear (figure 2.12 B). No trace of nuclear labeling was detected in sections in which the primary antibody was omitted.

Mouse monoclonal antibody against p-CREB (S^{133}, diluted 1:500; Upstate Biotechnology USA; Cod. 05-667) was used for IHC. Antibody binding was detected using a secondary biotinylated goat anti-mouse antibody (diluted 1:300).

2.5 INTRACEREBRAL MICRODIALYSIS

General Principles Intracerebral microdialysis is a well-established technique which can be used in awake, freely moving animals. Since its introduction in routine experimental basic research it has been widely used in rodents to monitor extracellular levels of neurotransmitters and endogenous compounds, levels of drugs and their metabolites, to locally deliver drugs and toxins in the brain, to alter local ionic environment and to correlate behavioral changes to neurotransmission.

The procedure for the *in-vivo* microdialysis experiments in the rodent brain requires: the selection and construction of the microdialysis probe; implantation of the probe into the target brain region; collection of dialysate samples and quantification of the analytes (neurotransmitters, metabolites) contained. Attention to the details of each of these facets is critical for the achievement of accurate and reproducible results.

The key element in this technique is the microdialysis probe, a tiny tube made of a semi-permeable membrane which is inserted into the tissue (figure 2.13). A semi-permeable membrane has tiny "pores" in it through which low molecular weight solutes can pass. During the microdialysis experiment, the inside of the membrane is perfused with artificial cerebrospinal fluid (aCSF), and the outside of the membrane is in direct contact with the compartment to be sampled. Briefly, the idea of microdialysis is to mimic the passive function of a capillary blood vessel by perfusing a thin dialysis tube implanted into the tissue. The concentration of compounds in the perfusate thus reflects the composition of the extracellular fluid due to the diffusion of substances back and forth over the membrane (Ungerstedt, 1982).

The recovery of substances from the extracellular compartment depends on the length and molecular weight cut-off of the membrane as well as the composition and flow rate of the perfusate.
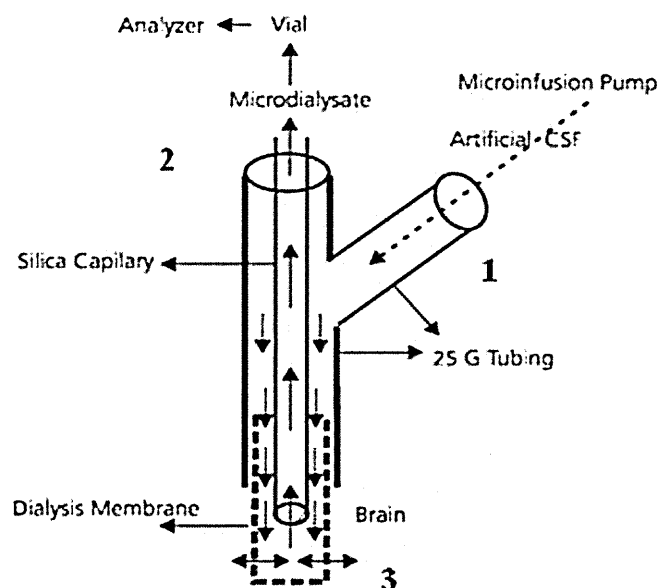
Fig. 2.13
The microdialysis probe Schematic representation of a concentric microdialysis probe.
The probe consists of an inflow (1) and outflow (2) tube separated by a tube made of dialysis membrane (3).

Microdialysis probes can be divided into four types on the basis of their geometry: concentric, side-by side, U-shaped, and horizontal. The concentric probes are more suitable for studies in rodents; can be used in combination with a guide cannula, which can be inserted into the brain sometime prior to the actual experiments (up to 2 months). The surgical procedure is straightforward and can be completed within 20 min. Although ready-to-use probes of varying dimensions can be obtained from commercial sources, our choice falls onto handmade concentric probes types, which are of course less expensive. Calibration of handmade probes is essential, thus pilot *in-vitro* recovery studies were conducted to determine the flow rate of the perfusate that yields optimal

quantification of the analyte under study. *In-vitro* recovery data also provide important information on probe efficiency.

Protocol   In these studies I used a concentric dialysis probes made of Cuprophan membrane (216 μm outer diameter, 3000 Da cutoff, Sorin Biomedica, Mirandola, Italy) prepared essentially as described elsewhere (Robinson and Whishaw 1988). The membrane exposed to the brain tissue was 4 mm long. The day before the experiment, rats were anesthetized by intraperitoneal (IP) injection of 3 mL/kg Equithesin and placed on a stereotaxic frame.

The dialysis probe was implanted in the mPFC at: AP +3.7 and L ±0.7 mm from bregma and DV -4.8 mm from dura surface (Paxinos and Watson 2007).

About 20 h after surgery, the probe was perfused with aCSF (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 0.6 M NaH_2PO_4) at 1 μL/min with a CMA/100 pump. After an equilibration period of 2 or 3 hr samples collection began. At least three stable baselines were collected before drug treatment.

A schematic diagram of the apparatus is shown in figure 2.14.
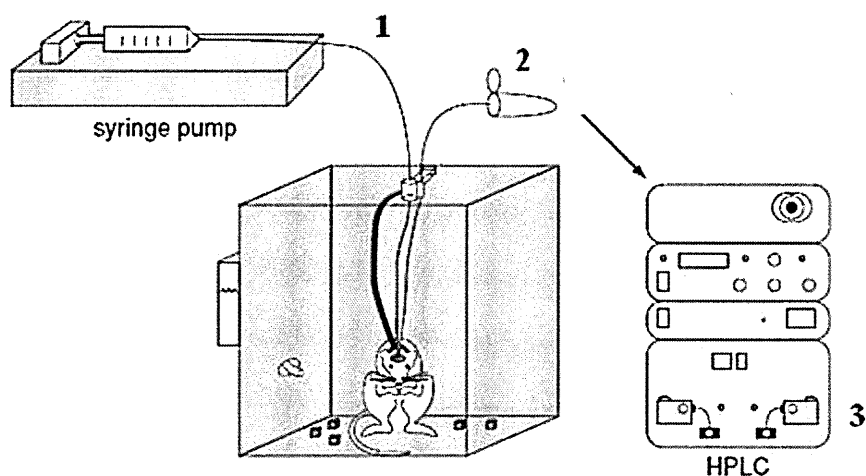
Fig. 2.14
Sequence of steps to perform a microdialysis experiment.
Artificial CSF, is pushed through the probe at a slow flow rate by a syringe pump (1) across the dialysis membrane. Analytes in the extracellular space are collected into the perfusate and then pushed through the outflow tubing into a collection vial (microcentrifuge tube) (2). The collected sample is then analyzed by HPLC-fluorometry (3).

GLU was separated through a 4.6 x 80 mm C18 reverse-phase column. The mobile phase contained 0.05 M Na_2HPO_4, 28% methanol, pH 6.4 with 85% phosphoric acid at a flow rate of 1 mL/min with a LC20-AD HPLC pump.

Since glutamate is not natively fluorescent, samples were derivatized with o-phthalaldehyde (OPA) in the presence of a thiol group such as β-mercaptoethanol which allowed for transformation of glutamate into fluorescent product (Donzanti and Yamamoto 1988). Five μL of derivatizing reagent were added to 5 μL sample, thoroughly mixed and immediately injected into the HPLC by a refrigerated autosampler set at 4°C.

GLU was measured by a fluorescence detector. Excitation and emission wavelengths were 335 and 450 nm. Detection limit was 0.1 pmol/5 μL (signal-to-noise ratio = 2). The peak of the glutamate was recognized by the retention time compared with the retention time of the standard. The concentration of GLU in the sample, not corrected for the *in-vitro* recovery, was calculated automatically by a data analysis system based on the calibration curve calculated on three different standard prepared daily. Intracerebral microdialysis has been used in these studies to measure GLU release in cortical samples from rats treated with (R)-CPP alone or in combination with LY379268. The protocol used has been extensively validated in our laboratory.

2.6 DRUGS, MICROINJECTION AND TREATMENT SCHEDULE

Drugs Drugs used in these studies were the following: (R)-CPP [3-(R)-2-carboxypiperazin-4-propyl-1-phosphonic acid] (Tocris Cookson Inc. MO); LY379268 [(-)-2-oxa-4-aminobicyclo (3.1.0) hexane-4, 6-dicarboxylate] (Eli Lilly, USA) and Sp-cAMP [Sp-(adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt hydrate)] (Sigma Aldrich, USA). All drugs were dissolved in a solution of phosphate buffered saline (PBS, pH=7.4).

(R)-CPP (50 ng/ µL) and Sp-cAMP (10.5 or 21 nmol/0.5 µL) were microinfused into the mPFC, LY379268 (0.1 mg/kg) was administered peripherally by a subcutaneous injection. In the microdialysis experiment (R)-CPP (100 µM) was dissolved in the aCSF and perfused through the probe implanted in the mPFC.

The dose of (R)-CPP was chosen based on previously published results from our laboratory (fig. 2.15).
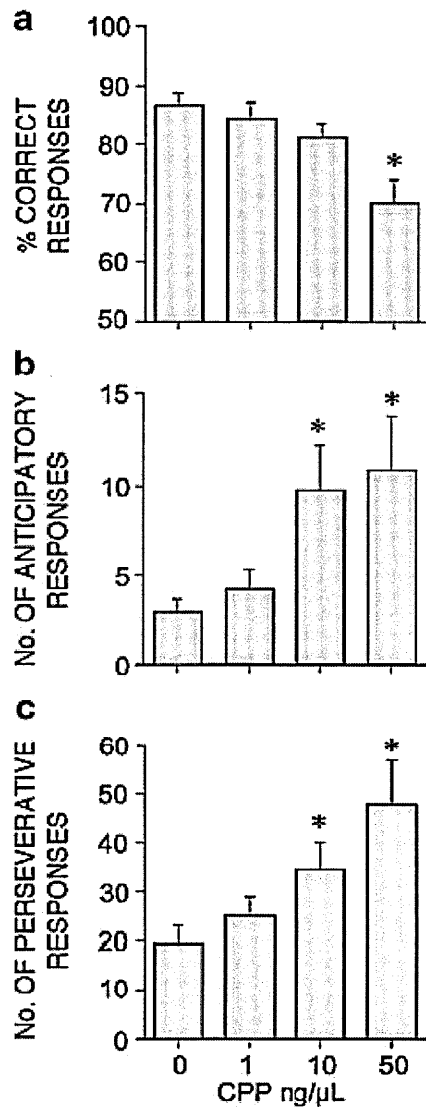
Fig. 2.15

Effects of intra mPFC (R)-CPP on behavioural performance

Effects of 1, 10, and 50 ng/μL of (R)-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). (R)-CPP and vehicle were administered at least 48 h apart, according to a Latin-square design.

Infusion of (R)-CPP, had profound effects on rats' attentional performance in the 5-CSRT task. Although the following data have been already published (Mirjana et al. 2004) they are reported as they represent the starting point of this thesis. (R)-CPP injected into the mPFC had profound effects on rats' attentional performance: it impaired attention as shown by a decrease in accuracy of visual discrimination (measured by % correct responses) and it caused a loss of inhibitory response control exemplified by enhanced anticipatory and perseverative responding.

The histograms show mean±SEM of eight rats. *P<0.05 vs 0 (Tukey's test).

Microinjection 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 (R)-CPP, Sp-cAMP or appropriate vehicles was delivered at a rate of 0.5 μL/min by a 10 μL Hamilton syringe, mounted in a CMA/100 infusion pump connected by PP10 tubing to the injection units. Injection units were left in place for 1 min after infusion to allow for drug diffusion.

Treatment Schedule Rats were given vehicle or LY379268 20 min before the bilateral injection of 1.0 μL side vehicle or 50 ng/μL side (R)-CPP into the mPFC. Rats were given vehicle or Sp-cAMP 2 min before the bilateral injection of (R)-CPP into the mPFC. The behavioural session started 10 minutes after (R)-CPP.

In the behavioural experiments 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.

With the exception of microdialysis study in which (R)-CPP was infused in the FC through the probe, in the rest of the experiments performed in this thesis, (R)-CPP was microinjected into the mPFC. This procedure is extensively employed and has many advantages. A major advantage is the reversibility of the effects that could not be found with other techniques such as neurotoxic or neurochemically selective lesions.

2.7 HISTOLOGY

Histology refers to the microscopic study of tissue. The visualization of the brain's cytoarchitecture is an important complement to the studies in neuroscience, which permits to identifying areas affected by a lesion and verifying the placement of an injection. At the end of experiments (except for western blotting experiments), rats were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and killed by decapitation. The brain was removed and frozen on dry ice. Frozen brains were sliced into 30 μm thick slices by a cryostat, and then mounted on polylysine-coated slides.

In this study I used cresyl violet, a Nissl stain that colours cell bodies a brilliant violet. Nissl is a term used by classical cytologists for the endoplasmic reticulum (ER). Since all cells contain ER, cresyl violet will stain both neurons and glia. Cresyl violet staining on adjacent cryostat cut sections was carried out according to published procedures (Kawamura and Niimi 1972).

The staining procedure consists of sequentially dipping the slides in about a dozen different solutions for specified amounts of time: 1 min H_2O; 5 min EtOH 70%, 5 min EtOH 100% and 5 min xylene. The steps were repeated in reverse order then slides were dipped for 3 min in 0.5% Cresyl-violet solution. Slides were washed ten times in H_2O and ten times in EtOH 70% followed by 1 min EtOH 95% plus 3% acetic acid, 3 min EtOH 100% and 5 min xylene.

The sequence of solutions is designed to accomplish the following: an initial alcohol soak removes lipids (fats) and fixation chemicals from the tissue; submersion of sections in stain; tissue dehydration by a series of alcohol baths. Finally the unstained parts of the tissue are made transparent by a clearing agent (xylene). Sections were then mounted with Permount and observed by light microscopy (Olympus BX51, Germany). Correct probe

and cannula placement was checked by visual inspection of the tracks on the stained coronal sections. Only rats with correct cannula and probe placements were considered in the results.

2.8 STATISTICAL ANALYSIS

Statistical analysis was performed using the StatView 5.0 software. One-way or two-way ANalysis Of VAriance (ANOVA) was used, followed by the appropriate *post-hoc* test. The significance limit was set at $p \leq 0.05$. The application of a specific *post-hoc* test (e.g. Tukey's test) is needed after the achievement of a significant main effect (one- and two-way ANOVA) or interaction, (two-way ANOVA) in order to determine differences between single means.

The main dependent behavioural variables selected for analysis were: choice accuracy (the proportion of correct responses / number of correct + incorrect responses), the proportion of omissions (the number of omissions / number of correct + incorrect + omissions), the number of anticipatory and perseverative responses, the correct response latency (the time from the stimulus onset to a correct response). All the behavioural data were analysed using SAS-V9 statistical software package.

Data from biochemical experiments were expressed as mean percentage of controls (± S.E.M.). The effects of (R)-CPP, LY379268 and Sp-cAMP were compared to vehicle controls by unpaired Student's t-test. The effects of (R)-CPP in combination with

LY379268 were analyzed by between-subjects two-way ANOVA with factors LY379268 and (R)-CPP.

Extracellular levels of GLU expressed as percentages of basal values were analyzed by ANOVA for repeated measures with treatments ((R)-CPP and LY379268) as the between-subject factors and time as the within-subject factor. The analysis was applied to the part of the curve from 20 to 80 min after systemic drug injection, which corresponded to the duration of (R)-CPP infusion.

On all occasions when the ANOVA indicated a significant effect of main factors or (R)-CPP x LY379268, or (R)-CPP x Sp-cAMP interactions (the significance level for F values was set at $P<0.05$), the treatments mean values were compared between them by Tukey's test.

CHAPTER 3

EFFECTS OF BLOCKADE

OF NMDAR IN THE mPFC

ON CREB PHOSPHORYLATION

CREB phosphorylation has been implicated in numerous cognitive functions: learning, various aspects of memory such as short and long term as well as working memory, memory formation for response and place strategy, appetitive and fear conditioning, cocaine self-administration and in the control of attention and impulsivity (see General Introduction).

In mice, acute systemic administration of NMDAR antagonist increase the phosphorylation of CREB on the active site S^{133} (p-CREB) both in the striatum and PFC as shown by Svenningsson et al. (2003) an effect that has been associated with impairments in pre-pulse inhibition and hyperactivity. Similarly, the NMDAR antagonist-induced attention performance deficit in the 5-CSRT task was associated with increased p-CREB level in the striatum of DBA mice (Pozzi et al. 2010), indicating a likely relationship between p-CREB and attention.

I set to further investigate this relationship by examining the effects of conditions that impair attention such as blockade of NMDAR in the mPFC on CREB phosphorylation at some nodes of cortico-striatal projection such as PFC, dorsal (CPu) and ventral (NAC) striatum which have been shown to importantly contribute to executive functions including attention (see General Introduction).

The competitive NMDAR antagonist (R)-CPP was injected into the mPFC of behaviourally naïve rats at the same dose and conditions employed in the behavioural experiments. In an attempt to examine the dynamics of the effects of (R)-CPP on p-CREB levels, two time points have been selected: the first corresponding to the beginning of the

behavioural test (10 min after (R)-CPP injection) and the second corresponding to the end of the behavioural test (40 min after (R)-CPP injection).

Immunohystochemical technique was used to detect the number of p-CREB-positive cells in coronal sections comprising cortical and striatal regions and western immunoblotting method to measure p-CREB levels in tissue homogenate from cortical and striatal samples of (R)-CPP-infused rats.

I also attempted to identify the likely signalling cascade through which (R)-CPP may have influenced CREB phosphorylation on serine 133 by examining its effects on some kinases such as c-AMP-dependent protein kinase A (PKA), calcium/calmodulin-dependent kinase II (CaMKII) and extracellular-regulated MAP kinase (ERK1/2) which are regulated by GLU and dependent on $Ca2^{+}$-permeable NMDA receptor activity (see General Introduction).

3.1 EFFECTS OF (R)-CPP ON p-CREB STUDIED BY IHC

The experimental timeline is reported below.
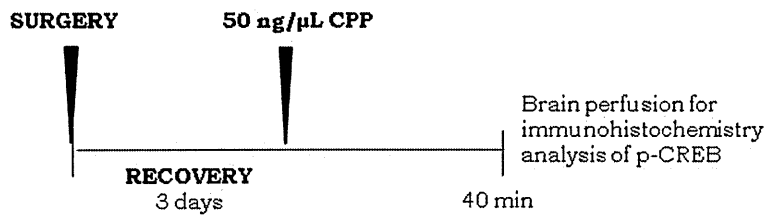
A comprehensive description of experimental procedures (animals, surgery, microinjections and western blot) is reported in General methods (Chapter 2). Briefly, rats were bilaterally implanted with stainless steel cannulae in the mPFC then they had 3 days to recover from surgery. On the day of the experiment 50 ng/μL (R)-CPP or 1 μL vehicle (PBS) were infused bilaterally in the mPFC. Forty min after (R)-CPP infusion rats were anaesthetized and intracardially perfused with a fixative solution for immunohistochemical analysis of the number of p-CREB-positive cells.

The number of p-CREB-positive cells was counted in cortical and some subcortical regions such as caudate-putament (CPu) and nucleus accumbens (NAC) that receive glutamatergic excitatory afferents from the FC. In particular p-CREB positive cells were counted in the motor and cingulate cortices, in two different aspects of the CPu (i.e. medial and lateral) and in the core and shell regions of the NAC (fig.3.1). Phospho-CREB immunoreactivity was not measured in slices containing the medial regions of the frontal cortex (which is the precise site of (R)-CPP infusion) because as a consequence of cannulae implantation and (R)-CPP injection, the area showed some sign of gliosis along the injection needle track that could prevent a precise quantification of cells.

The prelimbic (PL) and infralimbic (IL) cortices send their axons rostrally to the motor and the cingulate cortices thus changes in p-CREB in these areas may reflect at least in part the changes in PL and IL regions of PFC (fig.3.1).
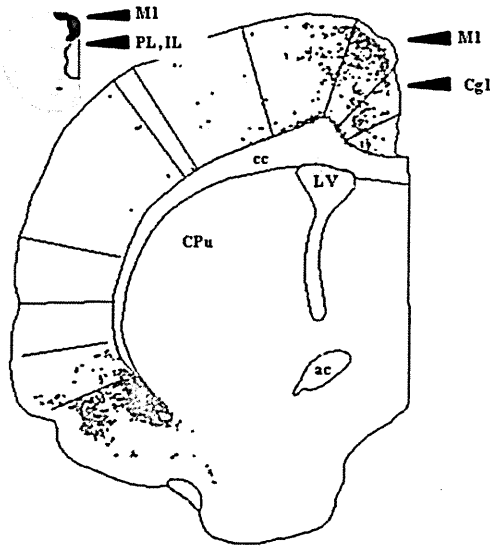
Fig.3.1
Coronal sections showing distribution of retrogradely labeled cells in posterior cortical areas
Coronal sections showing distribution of retrogradely labeled cells in posterior cortical areas following delivery of Fluoro-Gold (red) and Fast Blue (blue) in frontal cortical areas as indicated by the coloured insets.

Abbreviations: PL, prelimbic cortex; IL, infralimbic cortex; Cg1, cingulate cortex; M1, motor cortex; ac, anterior commissure; cc, corpus callosum; CPu, caudate putamen; LV, lateral ventricle.

(R)-CPP increased p-CREB in the FC and CPu but not in the NAC
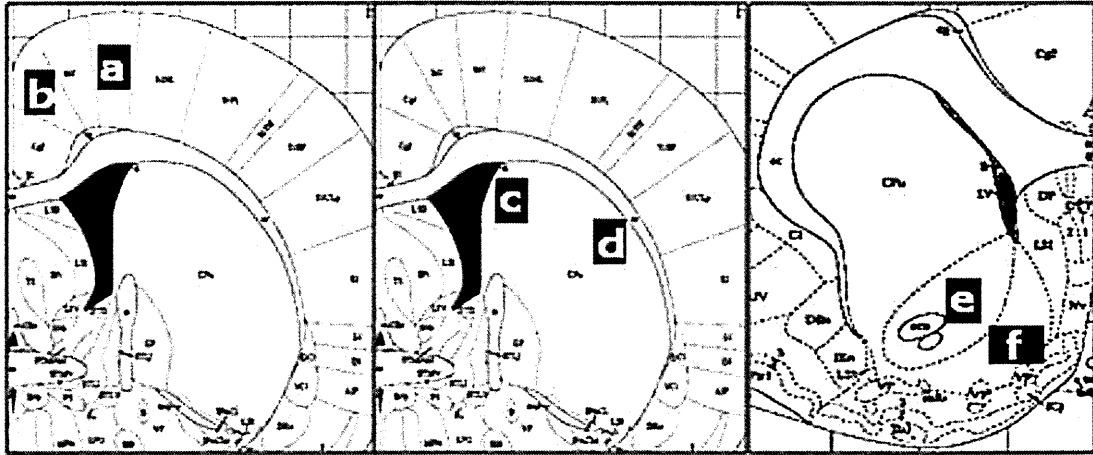
Fig.3.2
A schematic drawing that illustrate the areas where p-CREB-positive cells were counted.
Areas considered for cells counting were as follows: **a**, motor cortex 1; **b**, cingulate cortex 1; **c**, medial striatum; **d**, lateral striatum (at bregma -0.80); **e**, nucleus accumbens core and **f**, nucleus accumbens shell (at bregma +1.80).
The size of the counting frame was fixed at $0.96 \, mm^2$. See materials and methods for details.

Quantification of p-CREB-positive cells in the cortical regions, CPu and NAC after intracortical (R)-CPP is shown in fig.3.3.

Forty minutes after (R)-CPP infusion, p-CREB-positive cells were increased across primary motor and cingulate regions of FC (both $P<0.05$; Student's t-test). In contrast, the number of p-CREB-positive cells was decreased both in the medial and lateral region of the CPu (both $P<0.05$; Student's t-test). No changes in the number of p-CREB-positive cells were detected in the core or the shell regions of the NAC (both $P>0.05$; Student's t-test).
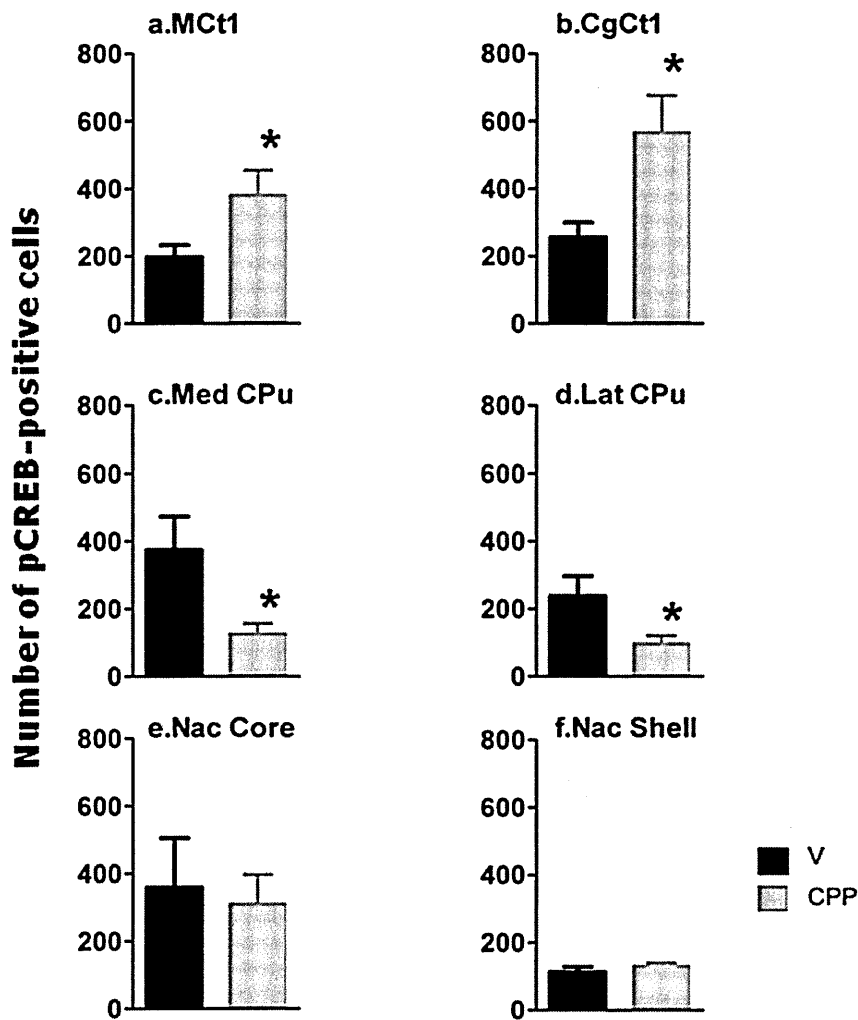
Fig.3.3

(R)-CPP-induced changes in the number of p-CREB-positive cells in cortical and striatal areas

Quantitative effects of (R)-CPP on p-CREB-positive cells in the cortical and striatal regions compared to saline (V). V or (R)-CPP were bilaterally infused in the mPFC and rats sacrificed 40 min later. Histograms represent the number of positive cells (mean±SEM). *P<0.05 vs saline (V) (Student's t-test).

The number of p-CREB–positive cells was as follows: **a,** MCtl V=197±35 and CPP=380±75; **b,** CgCtl V=256±43 and CPP=566±110; **c,** MedCPu V=375±99; CPP=124±32; **d,** LatCPu V=238±58; CPP=96±25; **e,** Nac core V=360±146; CPP=310±87; **f,** Nac shell V=113±15; CPP=130±10.

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p-CREB in the orbitofrontal cortex was increased by (R)-CPP

Recently, Sun et al. (2010) reported a positive association between impulsivity measured in the 5-CSRT task (ie. increased anticipatory responding) and S^{133}CREB phosphorylation in the orbitofrontal cortex (OFC). They showed that yohimbine (a noradrenergic $\alpha2$ antagonist) at the dose, which increased impulsive responding in rats, selectively increased p-CREB within the OFC but not mPFC or NAC.

They also reported that over-expression of the dominant negative antagonist mCREB within the OFC blocked yohimbine's effects on impulsivity, whereas viral-mediated gene transfer to regionally overexpress CREB in this region increased impulsive responding and potentiated the pro-impulsive actions of yohimbine.

Thus, I measured the effect of (R)-CPP, which also increased impulsivity as shown in fig. 3.1 on the number of p-CREB–positive cells in the rat OFC. I found that 40 min after (R)-CPP the number of p-CREB-positive cells was increased in the lateral and ventral OFC (Fig 3.4 C and C') compared to saline-infused rats (fig 3.4 B and B'). Only slices of 2 control and 2 treated rats were analyzed, making impossible a statistical analysis.

Therefore it could not be excluded that (R)-CPP-induced impulsivity may reflect at least in part changes in p-CREB in the OFC.
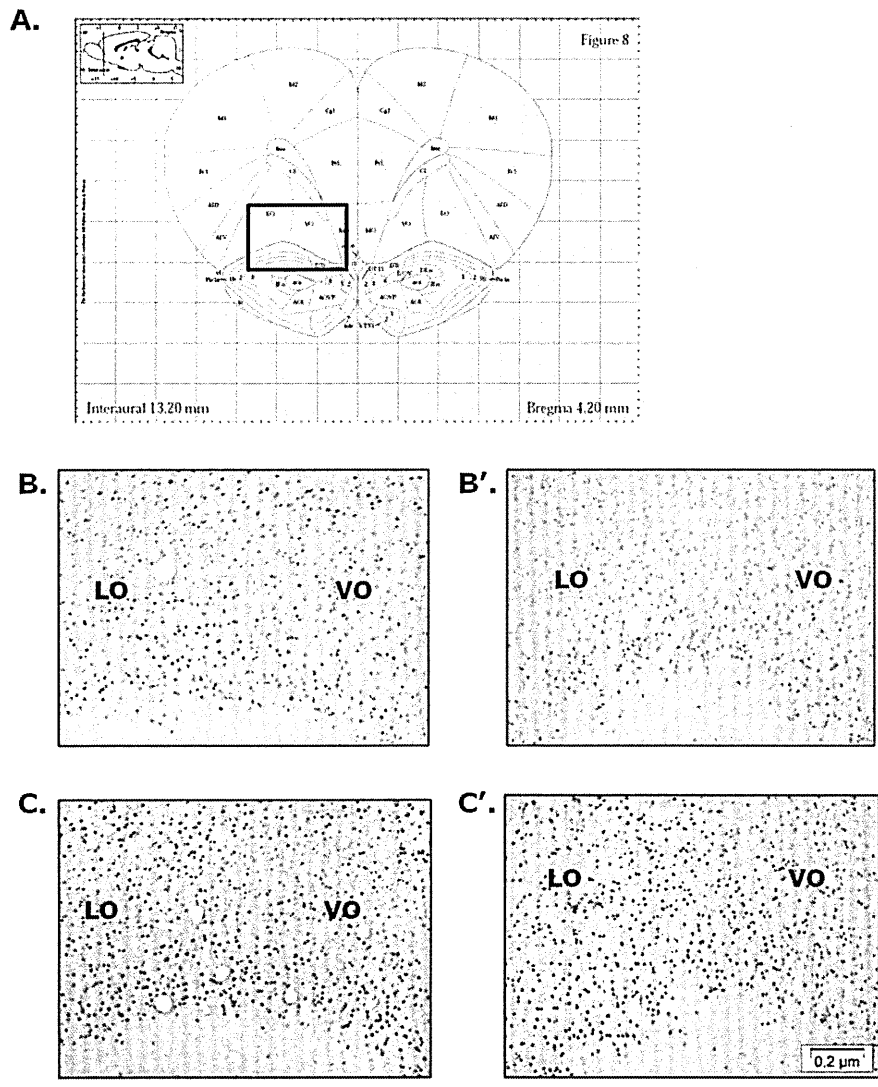
Fig. 3.4

(R)-CPP-induced p-CREB-positive cells in the rat orbitofrontal cortex.

(A) Schematic drawing of the coronal section showing orbital region of the frontal cortex (bregma 4.20; Paxinos and Watson 2007) and representative images illustrating immunostaining of p-CREB-positive cells 40 min after intracortical infusion of saline (**B, B'**) or CPP (**C, C'**). Areas in the inset represent the lateral (LO) and the ventral (VO) orbitofrontal cortex.

3.2 EFFECTS OF (R)-CPP ON p-CREB STUDIED BY WESTERN BLOT

In the following experiment western blot was used to measure levels of p-CREB as well as phosphorylation of various protein kinases in tissue samples at different time points after (R)-CPP infusion.

The experimental timeline is reported below.
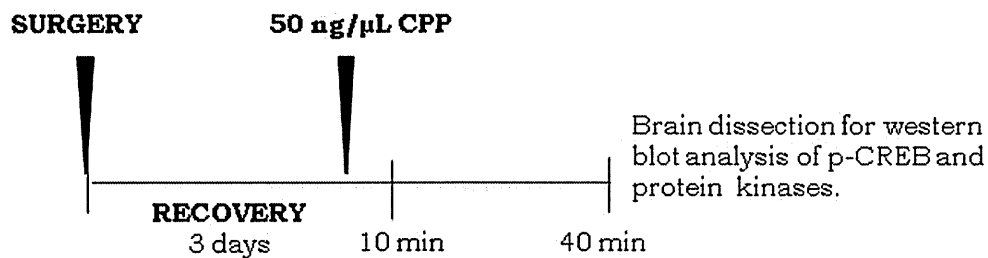
SURGERY   50 ng/µL CPP

Brain dissection for western
blot analysis of p-CREB and
protein kinases.

RECOVERY
3 days   10 min   40 min

A comprehensive description of experimental procedures (animals, surgery, microinjections and western blot) is reported in general methods (Chapter 2). Briefly, rats were bilaterally implanted with stainless steel cannulae in the mPFC then they had 3 days to recover from surgery. On the day of the experiment 50 ng/µl (R)-CPP or vehicle (PBS) 1 µl were infused bilaterally in the mPFC. Ten or 40 min after (R)-CPP infusion rats were sacrificed and their brain rapidly dissected out. FC, CPu and NAC samples were stored at -80°C until phosphorylation of CREB and of various protein kinases was measured by western blot.

(R)-CPP induced changes in p-CREB in the FC and CPu but not in the NAC

Figure 3.5 shows that blockade of NMDAR in the mPFC by (R)-CPP has differential effects on p-CREB levels in the FC, CPu and NAC.

The time-course analysis showed that (R)-CPP had biphasic effects on p-CREB in the FC. Ten minutes after intra-mPFC injection of (R)-CPP (fig. 3.5 A), p-CREB was decreased while 40 minutes after (R)-CPP (fig. 3.5 B) it was significantly increased (both $P<0.05$, comparison between V and CPP by Student's t-test).

CREB phosphorylation in the CPu was also affected by intra-mPFC (R)-CPP but in contrast to what reported for FC, levels of p-CREB were significantly and uniformly decreased at both time points (10 min shown in fig. 3.5 A and 40 min shown in fig. 3.5 B) (both $P<0.05$, comparison between V and CPP, Student's t-test).

No changes in p-CREB levels in the NAC were reported either at 10 min (fig. 3.5 A) or 40 min (fig. 3.5 B) after (R)-CPP injection in the mPFC (both $P>0.05$, comparison between V and CPP, Student's t-test).

CREB (T-CREB) levels in the FC, CPu and NAC were not affected by (R)-CPP (data not shown).

A. 10 min after CPP

p-S^{133}CREB
■ V
▨ CPP

← 43 kDa

B. 40 min after CPP

← 43 kDa

FC   CPu   NAC
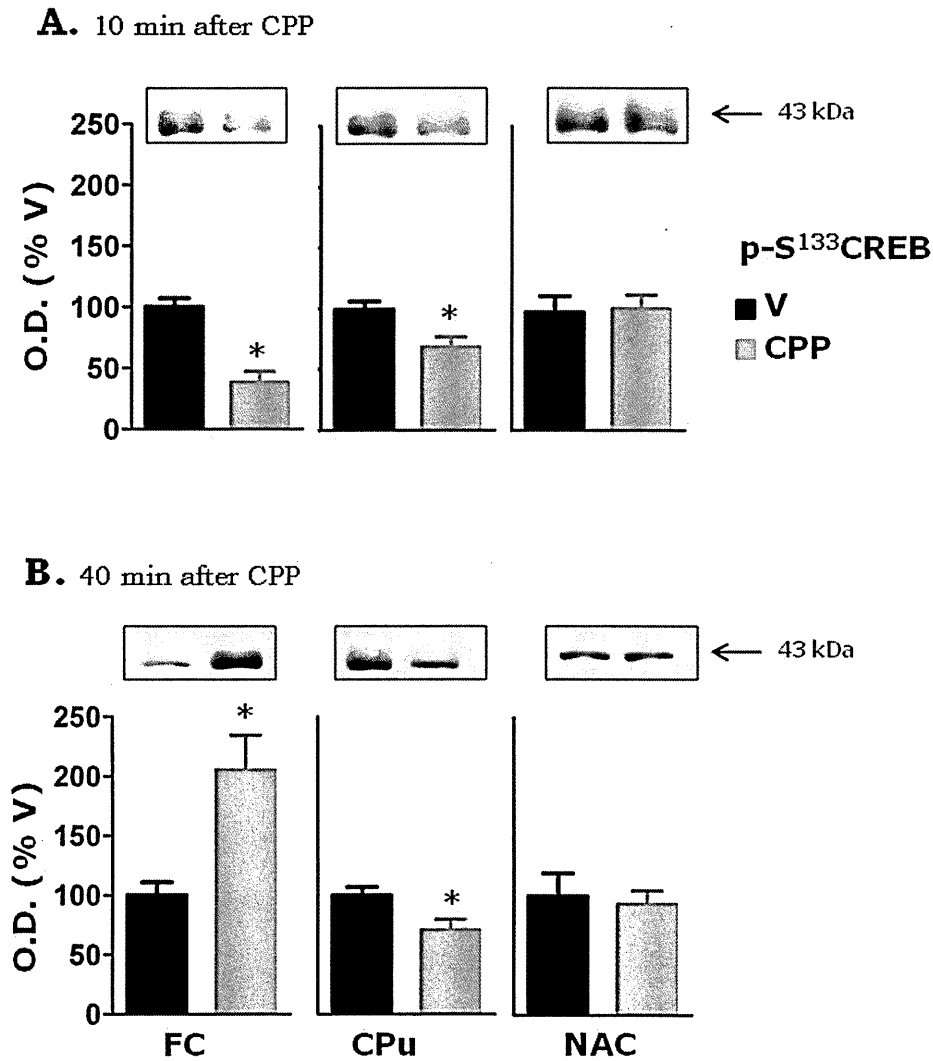
Fig 3.5

Effects of (R)-CPP on p-CREB levels.

Representative immunoblots and quantification of p-CREB levels in the FC, CPu and NAC of saline- (V) and (R)-CPP-infused rats (CPP). V or (R)-CPP were infused bilaterally in the mPFC and rats sacrificed 10 (A) or 40 (B) min later. Histograms represent the mean±SEM of 6 rats per group expressed as percentage of controls.

*$P<0.05$ vs V (Student's t-test).

The values for (R)-CPP expressed as the percentage of controls (V) are: A, FC 39±9%; CPu 68±8%; NAC 100±11%. B, FC 205±29%; CPu 71±9%; NAC 93±11%.

Total levels of CREB were not modified by (R)-CPP (not shown).

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3.3 EFFECTS OF (R)-CPP ON PROTEIN KINASES STUDIED BY WESTERN BLOT

It is well known that CREB phosphorylation on serine 133 can be regulated by several signalling proteins thus representing an important convergence element for different intracellular pathways. Among the activators of CREB, the cAMP-dependent protein kinase A (PKA), calcium/CaM-dependent kinases (CaMKs) and extracellular regulated kinase (ERK) pathways play an important role.

Therefore, the effects of (R)-CPP on the phosphorylation of PKA (T^{197} on the catalytic subunit), ERK1/2 ($T^{202/}Y^{204}$), and CaMKII (T^{286}), were examined in the same tissue homogenates where p-CREB levels were previously measured. Among the kinases measured in the FC, CPu and NAC after (R)-CPP only the catalytic subunit of PKA phosphorylated on T^{197} (p-PKAc) was significantly decreased by (R)-CPP 10 and 40 min after (R)-CPP.

(R)-CPP decreased p-PKAc in the FC and CPu but not NAC

I used an antibody against the phosphorylated form of the catalytic subunit of PKA (p-PKAc). PKA normally exists in the cytoplasm as an inactive tetrameric holoenzyme which is dissociated into two free catalytic and two regulatory subunits. The PKA catalytic subunit (c) once activated, moves into the cell nucleus and phosphorylates serine residue 133 on CREB (Skalhegg and Tasken 2000). Figure 3.6 shows that 10 min after (R)-CPP infusion levels of p-PKAc were decreased both in the FC (fig. 3.6 A) (compared to V; $P<0.05$; Student's t-test) and in the CPu (fig. 3.6 A) (compared to V; $P<0.05$; Student's t-test) and this decrease lasted for at least 40 min (fig. 3.6 B) in both FC and CPu (both compared to V; $P<0.05$; Student's t-test).

No changes in p-PKAc in the NAC were detected either 10 (fig. 3.6 A) (compared to V, P>0.05; Student's t-test) or 40 min after (R)-CPP injection in the mPFC (fig. 3.6 B) (compared to V, P>0.05; Student's t-test).

(R)-CPP had no effect on total PKA levels in the FC, CPu and NAC (data not shown).

A. 10 min after CPP
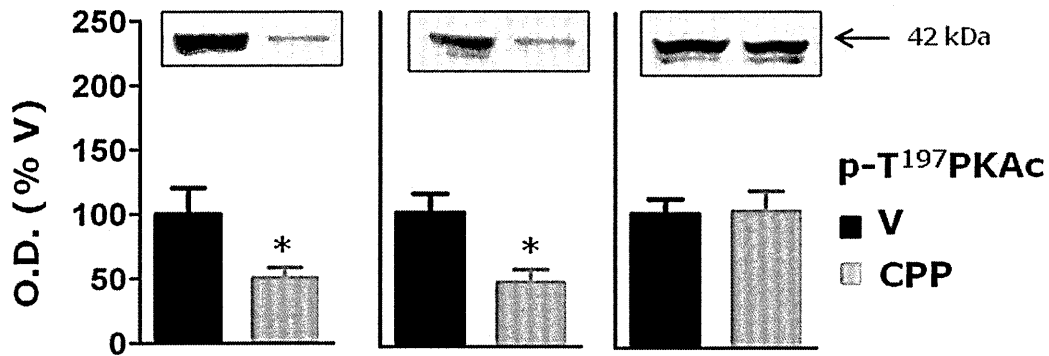
B. 40 min after CPP
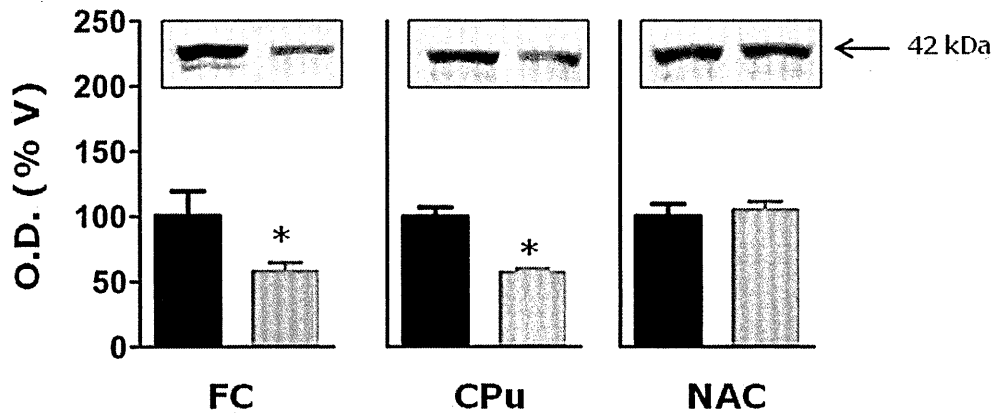
Fig. 3.6

Effects of (R)-CPP on p-PKAc levels

The effects of CPP injection in the mPFC on the levels of the catalytic subunit of PKA phosphorylated on p-PKAc in the FC, CPu and NAC

Representative immunoblots and quantification of p-PKAc levels in vehicle (V) and (R)-CPP-infused rats (CPP) 10 (A) or 40 (B) min later.

Histograms represent the mean±SEM of 5-6 rats per group expressed as percentage of controls. *$P < 0.05$ vs V (Student's t-test).

The values for (R)-CPP expressed as the percentage of controls (V) are: A, FC 51±8%; CPu 48±10%; NAC 102±15%. B, FC 57±19%; CPu 57±3%; NAC 105±6%.

Total levels of PKA were not modified by (R)-CPP (not shown).

(R)-CPP had no effect on p-ERK1/2 levels in the FC and CPu but increased p-ERK1 levels in the NAC

It is well known that activation of the extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), causes phosphorylation of CREB on S^{133}, and the subsequent transcription of CRE-driven genes (Impey et al. 1996; Impey et al. 1998; Davis et al. 2000). Thus I investigated whether this kinase might be involved in the CPP-induced changes in p-CREB.

Western blot data show that levels of p-ERK1 and p-ERK2 were unchanged in FC and CPu 10 min (fig 3.7 B and C) or 40 min (fig 3.8 B and C) after infusion of CPP.

Levels of p-ERK1 in the NAC were significantly increased at 40 (fig 3.8 B) but not 10 min (fig 3.8 C) after (R)-CPP, whereas levels of p-ERK2 in the same region were unchanged at both time points (fig 3.7 C and 3.8 C).

No changes in total ERK1/2 levels were detected in the FC, CPu and NAC (data not shown).

A.

44 kDa p-ERK1
42 kDa p-ERK2

FC   CPu   NAC

p-T^{202}/Y^{204} ERK1/2

B. p-ERK1 10 min after CPP

■ V
▨ CPP

C. p-ERK2 10 min after CPP
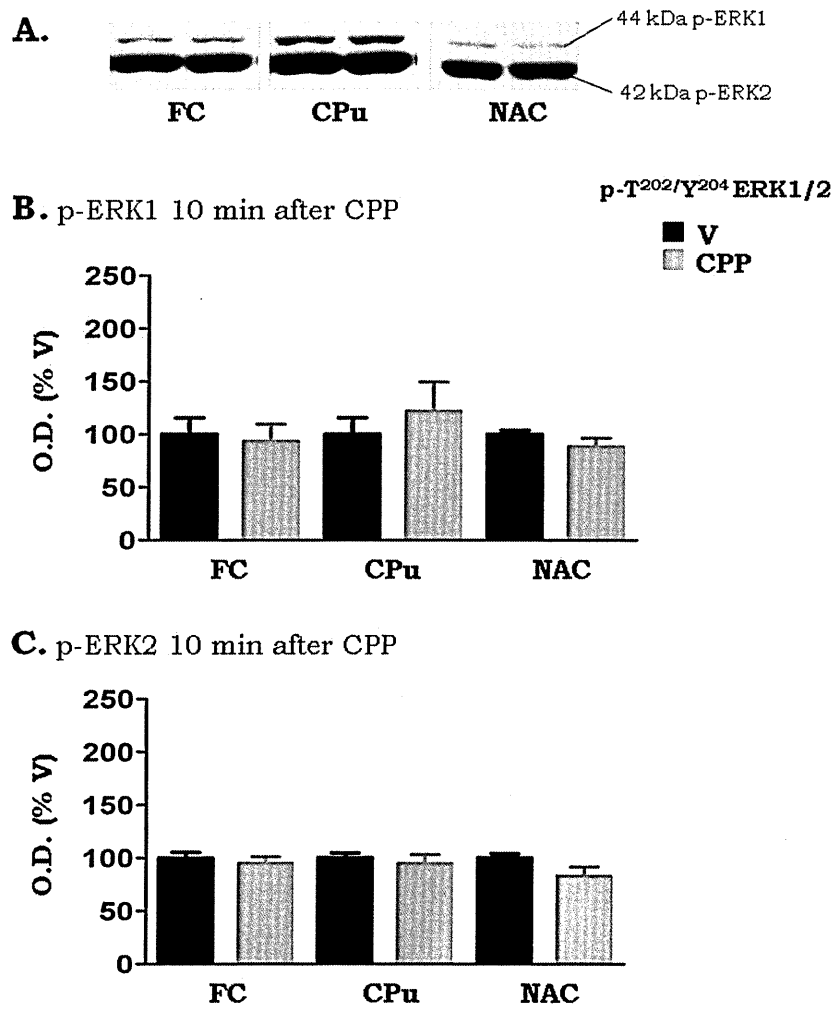
Fig. 3.7
Effects of (R)-CPP on p-ERK1 and p-ERK2 (10 min time point)
(R)-CPP had no effects on p-ERK1 and p-ERK2. Representative immunoblots (A) and quantification of p-ERK1 (B) and p-ERK2 (C) levels in vehicle (V) and (R)-CPP-infused rats (CPP). V or (R)-CPP were bilaterally infused in the mPFC and rats sacrificed 10 min later. Histograms represent the mean±SEM of 6-8 rats per group expressed as percentage of controls.
Total levels of ERK1/2 were not modified by (R)-CPP (not shown).

A.

44 kDa p-ERK1

42 kDa p-ERK2

FC CPu NAC

B. p-ERK1 40 min after CPP

p-T^{202}/Y^{204} ERK1/2

■ V
▨ CPP

C. p-ERK2 40 min after CPP
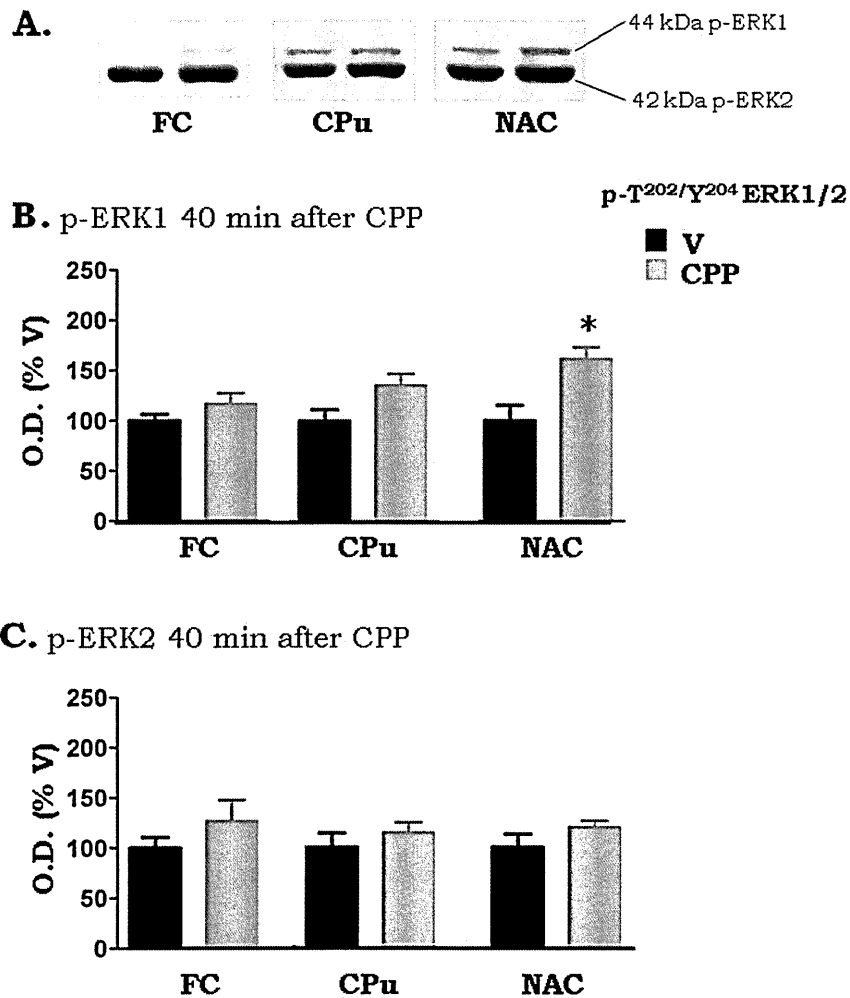
Fig.3.8

Effects of (R)-CPP on p-ERK1 and p-ERK2 (40 min time point)

Representative immunoblots (A) and quantification of p-ERK1 (B) and p-ERK2 (C) levels in vehicle (V) and (R)-CPP-infused rats (CPP). V or (R)-CPP were bilaterally infused in the mPFC and rats sacrificed 40 min later. Histograms represent the mean±SEM of 6-8 rats per group expressed as percentage of controls. *$P < 0.05$ vs V (Student's t-test).

Total levels of ERK1/2 were not modified by (R)-CPP (not shown).

(R)-CPP had no effect on p-CAMKII

I also investigated the effects of (R)-CPP on the phosphorylation of CaMKII on T^{286} (p-CaMKII) an enzyme associated with the calcium influx through the NMDA receptor and responsible for CREB phosphorylation on serine 133.

Figure 3.9, shows that injection of (R)-CPP in the mPFC 10 min (A), or 40 min (B) after had no effects on p-CaMKII levels in the FC, CPu or in the NAC.

Total CaMKII levels were not affected by (R)-CPP (data not shown).
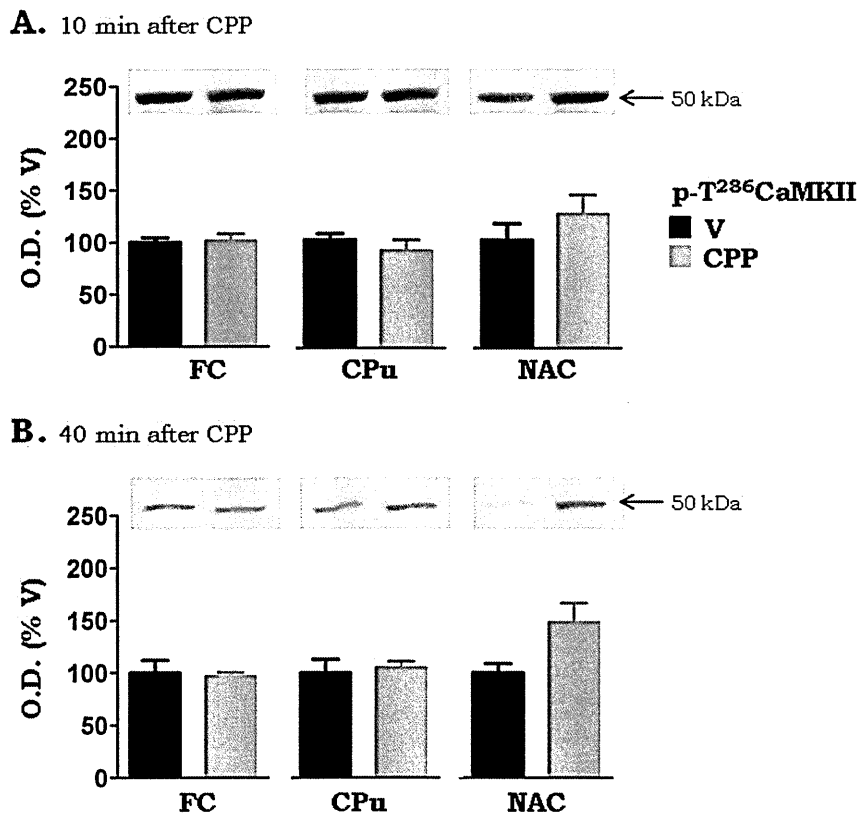
Fig. 3.9
(R)-CPP did not affect p-CaMKII
Representative immunoblots and quantification of p-CaMKII levels in vehicle (V) and (R)-CPP-infused rats (CPP). V or (R)-CPP were bilaterally infused in the mPFC and rats sacrificed 10 or 40 min later. Data are expressed as percentage of controls. Histograms represent mean±SEM of 5-7 rats per group. *P<0.05 vs V (Student's t-test).
Total levels of CaMKII were not modified by (R)-CPP (not shown).

3.4 DISCUSSION

The data provide evidence that the effects of the competitive NMDAR antagonist (R)-CPP injected in the mPFC on CREB phosphorylation were regionally specific; it was biphasic in the PFC, a decrease followed by an increase, it consistently decreased in the CPu and it was unaffected in the NAC. The analysis of p-CREB's upstream protein kinases show that in regions where p-CREB levels were affected by (R)-CPP such as FC and CPu, p-PKAc levels were significantly and consistently decreased. The data also show that intracellular pathways dependent on ERK1/2 or CaMKII kinases were not involved in the (R)-CPP-induced changes in p-CREB.

Biphasic effects of (R)-CPP in the mPFC on p-CREB levels in the FC.

Blockade of NMDAR in the mPFC by (R)-CPP had a biphasic effect on p-CREB levels in the FC: 10 min after intracortical infusion p-CREB levels decreased whereas 40 min later they increased. The decrease in p-CREB levels was surprising, as no other in vivo studies have ever reported a decrease after NMDAR antagonists.

The increase in p-CREB levels 40 min after (R)-CPP as measured by western blot technique was confirmed and extended by immunohistochemical determination. P-CREB-positive cells were increased across primary motor (MCt1) and cingulate (CgC1) regions of the FC. I also found that the number of p-CREB-positive cells was increased in lateral (LO) and ventral (VO) regions of the orbitofrontal cortex (OFC), which is consistent with the findings of Sun et al. (2010). Similar increases in p-CREB levels in the FC have been reported after non-competitive NMDAR antagonists such as PCP and

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MK-801 in mice (Pozzi et al. 2010; Svenningsson et al. 2003) but also in rats (Pozzi et al. unpublished results).

CREB is activated by phosphorylation on serine residue 133 (S^{133}) through many different signalling cascades among which is the cAMP / protein kinase (PKA) pathway (Mayr and Montminy 2001). Interestingly p-PKAc levels in the FC were also decreased 10 min after (R)-CPP injection. The phosphorylation of T^{197} is a necessary step in the catalytic activity of PKA (Jin 2007; Moore et al. 2002) thus its reduction may play a role in the (R)-CPP-induced decrease of p-CREB. Surprisingly, p-PKAc levels were reduced in the FC 40 min after (R)-CPP. Despite the decrease in p-PKAc p-CREB levels were increased suggesting that other non-PKA dependent mechanisms are involved in the (R)-CPP-induced increase in p-CREB.

Biphasic CREB phosphorylation has been observed in electrophysiological but also behavioural studies. For example, CREB phosphorylation shows a distinct temporal dynamics with fast increase, a return to baseline followed by a sustained and long lasting increase during hippocampal LTP tetanic stimulation in rats or long term facilitation (LTF) in Aplysia as well as during aversive learning in rats (Bernabeu et al. 1997; Liu et al. 2007; Schulz et al. 1999). In addition, single brief application of DA induced first a decrease and then an increase in depolarisation evoked firing of PFC neurons (Gulledge and Jaffe 1998 and 2001). This biphasic course may be a general feature of responding of neuronal tissue to DA as it has been observed in striatal (Williams and Millar 1990), hippocampal (Gribkoff and Ashe 1984) and PFC neurons (Gorelova and Yang 2000; Gulledge and Jaffe 1998 and 2001; Seamans et al. 2001).

Interestingly, administration of NMDAR antagonists causes first a rapid decrease in the firing of fast spiking putative GABA interneurons and subsequently an increase in the

firing of glutamatergic pyramidal neurons (Homayoun and Moghaddam 2007). Although the effects of decreased and increased firing of cortical interneurons and pyramidal cells respectively on p-CREB has yet to be determined, bursts of synaptic activity has been shown to increase p-CREB in hippocampal neurons (Bito et al. 1996). Over-expression of CREB in slice-cultures of the NAC and locus coeruleus (LC) neuronal cell types increased membrane excitability (Dong et al. 2006; Han et al. 2006). Sustained activation of cAMP-PKA pathway by forskoline or 8-Br-cAMP has also been shown to increase LC neurons firing (Cao et al. 2010). On the other hand, knockdown of CREB in slice cultures from floxed CREB mice decreased the firing of LC neurons and prevented the effects of cAMP stimulation (Cao et al. 2010). Thus it could not be excluded that the biphasic course of action of (R)-CPP on p-CREB may reflect the decrease followed by the increase in neuronal firing in the PFC and that different neuronal populations (GABA vs GLU) may be involved.

(R)-CPP infused in the mPFC has been shown to strongly increase neuronal GLU efflux in this cortical region (Ceglia et al. 2004) (see also Chapter 4). There is evidence that behavioural deficit and neurochemical changes such as increased DA release in the PFC induced by NMDAR antagonists are reversed by blockade of (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) or stimulation of type II mGlu receptors (Greco et al. 2005; Homayoun et al. 2005; Moghaddam and Adams 1998; Moghaddam et al. 1997) (see also data presented in Chapter 4). Thus it could be hypothesised that GLU released from presynaptic terminals can enhance the intracellular Ca^{2+} levels by activating AMPA and mGlu receptors positively coupled to CREB phosphorylation (Choe and

Wang 2002; Ginty et al. 1993; Lindemeyer et al. 2006; Perkinton et al. 1999; Uslaner et al. 2009; Zirpel et al. 2000).

In addition to enhancing GLU efflux NMDAR antagonists increase the release of DA and 5-HT in the PFC (Martin et al. 1998; Moghaddam et al. 1997). Cortical DA and 5-HT modulate GLU and GABA transmission in the PFC through diverse signalling cascades that target numerous intracellular substrates to regulate cellular excitability (Greengard et al. 1999; Svenningsson et al. 2002). A key actor in the integration of DA and GLU is DARPP32 (Fernandez et al. 2006), the DA and cAMP-regulated phosphoprotein of 32 kDa. DARPP32 is a phosphatase inhibitor and a major target for GLU signalling (Nishi et al. 2005). Although DARPP32 impacts cortical inhibition through D2-dependent regulation of GABA current in pyramidal cells, the D1-PKA modulation of GABA fast spiking interneurons in the PFC occurs independently of DARPP32 (Trantham-Davidson et al. 2008). The ability of NMDA receptor antagonists to increase p-CREB has been linked to an interaction with DARPP32, as $T^{34} \rightarrow A\_DARPP32$ point mutation abolished the effects of PCP on p-CREB (Svenningsson et al. 2003). In the same study Svenningsson showed that the effects of amphetamine (but not those of PCP) on p-DARPP32 was abolished in brain slices made from D_1 KO mice suggesting that the effects of PCP on p-DARPP32 were independent of increased DA tone on D1 receptor.

In our previous study we showed that the ability of PCP to increase p-CREB might be linked to an interaction with $5-HT_{2A}$ receptors as a consequence of PCP increasing endogenous 5-HT tone (Pozzi et al. 2010). Consistently, the $5-HT_{2A}$ receptor agonists DOI and LSD increased p-CREB (Chalecka-Franaszek et al. 1999; Svenningsson et al. 2003). Moreover, PCP was not able to increase p-CREB in animals depleted of 5-HT by

the 5-HT synthesis inhibitor PCPA (Pozzi et al. 2010). DARPP32 appears essential also for 5-HT transmission as behavioural and gene transcription effects induced by compounds that selectively release 5-HT were greatly reduced in DARPP32 KO mice (Svenningsson et al. 2002). Furthermore stimulation of 5-HT$_{2A}$ receptors by DOI increases the phosphorylation of Ser^{137} on DARPP32 which in turn controls the phosphorylation state of DARPP32 at Thr^{34} site, while ketanserine a 5-HT$_{2A}$ antagonist blocks the effects of 5-HT on Ser^{137}-DARPP32 (Svenningsson et al. 2002). However, the PCP-induced increase in p-CREB but not p-DARPP32 was completely abolished by blockade of 5-HT$_{2A}$ receptors (Pozzi et al. 2010). Together, these data indicate that 5-HT mechanisms modulate p-CREB independently of changes in DARPP32 phosphorylation.

Effects of (R)-CPP on p-CREB levels in the dorsal (CPu) and ventral (NAC) striatum

I report that local blockade of NMDAR in the PFC by (R)-CPP decreased p-CREB in the CPu at both 10 min and 40 min time points whereas it had no effects in the NAC.

The decrease in striatal p-CREB levels contrast with previous studies showing that systemic PCP increased p-CREB in the striatum of mice (Pozzi et al. 2010; Svenningsson et al. 2003). The reason for this discrepancy are most likely due to local versus systemic administration of NMDAR antagonists although I cannot disregard altogether, the competitive versus non-competitive nature of the NMDAR antagonists and species differences (mice versus rats) employed in these studies. It should be noted that the effects of intracortical (R)-CPP on p-CREB, p-PKA, p-ERK1/2 and p-CaMKII in dorsal and ventral striatal regions were indirect and most likely due to changes in the corticostriatal neurotransmission.

It is conceivable that (R)-CPP-induced GLU release in the mPFC could be reflected in changes in neuronal activity in the CPu. Interestingly, intracortical infusion of (R)-CPP (50 ng/μL) increased GLU, DA and GABA release in the CPu as measured by intracerebral microdialysis in rats (Agnoli and Carli unpublished) indicating a disinhibition of corticostriatal neurotransmission. Local application of GLU in the CPu, increases CREB phosphorylation via CaMK (Mao and Wang 2002; Vanhoutte et al. 1999) that acts through L-type Ca2+ channels (Rajadhyaksha et al. 1999) and MAPK (Choe and Wang 2001b; Mao et al. 2004; Wang et al. 2004). However, as I found a decrease in p-CREB it is unlikely that the increased GLU efflux contributed to it.

There is evidence that descending GLU pathways from the FC modulate the release of DA in subcortical areas such as the CPu but also in NAC (Karreman and Moghaddam 1996; Murase et al. 1993; Taber and Fibiger 1993 and 1995). In rats, this occur both directly, via GLU-ergic corticostriatal projections (Cheramy et al. 1986; Taber and Fibiger 1995), and indirectly through a direct cortical GLU projection to mesostriatal DA neurons in the substantia nigra (SN) and ventral tegmental area (VTA) (Karreman and Moghaddam 1996; Murase et al. 1993). Notably, intracerebral microdialysis experiments showed that intracortical infusion of 50 ng/μL (R)-CPP increased DA release in the CPu of rats (Agnoli and Carli unpublished).

A possible explanation for this decrease in p-CREB levels implicates the involvement of DA receptors in the CPu. The integration of corticostriatal information in the CPu by DA-ergic mechanisms depends critically on the class of DA receptor. In particular in the CPu, stimulation of D1 receptors (a receptor family positively coupled to the cAMP) or blockade of D2 receptors (negatively coupled to the cAMP) increased p-CREB (Chartoff et al. 2003; Konradi et al. 1994; Pozzi et al. 2003; Schmidt et al. 1998; Stoof and

Kebabian 1981). In the medium spiny neurons of the CPu, the PKA-dependent phosphorylation of DARPP32 on threonine 34 (T^{34}) site has been shown to induce p-CREB (Fernandez et al. 2006; Nishi et al. 2005; Valjent et al. 2005). Interestingly, a recent report has shown that activation of D2 receptor triggers divergent signalling cascades that inhibit multiple synaptic Ca2+ sources; it attenuates Ca2+ influx through NMDAR via a reduction of PKA activity but also through a PKA-independent pathway (voltage-gated calcium channels) (Higley and Sabatini 2010).

Both immunohystochemistry and western blot data show that p-CREB levels in the NAC were not affected by (R)-CPP. Accordingly, I found that in rats the acute systemic administration of PCP or MK-801 had no effect on p-CREB levels (see Table 1). This lack of effects on p-CREB levels in the NAC is surprising in view of cortical GLU afferents to NAC and to DA neurons of the VTA that in turn project to the NAC (Vertes 2004). In addition, intra-PFC (R)-CPP (albeit at a dose 20 times higher) increased DA efflux in the NAC (Del Arco et al. 2008), which is not substantially different to what has been reported for CPu (Agnoli and Carli, unpublished results).

Modulation of protein kinases by (R)-CPP

In an attempt to identify the intracellular pathways that may have lead to changes in p-CREB I examined some kinases that are regulated by GLU and depend on Ca^{2+}-permeable NMDA receptor activity such as the protein kinase A (PKA), the calcium/calmodulin-dependent kinase II (CaMKII) and the extracellular-regulated MAP kinase (ERK1/2), (Dash et al. 1991; Sheng et al. 1991; Vanhoutte et al. 1999).

I used an antibody against the phosphorylated form of the catalytic subunit of PKA (PKAc). PKA normally exists in the cytoplasm as an inactive tetrameric holoenzyme

which is dissociated into two free catalytic and two regulatory subunits. The PKA catalytic subunit (c) once activated, moves into the cell nucleus and phosphorylates residue S133 on CREB (Skalhegg and Tasken 2000). Among the kinases measured in the FC, CPu and NAC after (R)-CPP only the catalytic subunit of PKA phosphorylated on T^{197} (p-PKAc) was significantly decreased at both time points (10 and 40 min after CPP). The effects of (R)-CPP on p-PKA were discussed in the previous paragraphs of this chapter.

Despite the fact that the MAPK/ERK pathway can be activated in response to increased Ca^{2+} levels (Fiore et al. 1993; Vanhoutte et al. 1999) the present results show that there were no changes in phosphorylation of T^{202}/Y^{204}-ERK1/2 in the FC and CPu in response to (R)-CPP infusion thus ruling out the involvement of these kinases in the (R)-CPP-induced changes in p-CREB levels. Accordingly, PCP had no effect on p-ERK1/2 in mice' FC and CPu (Pozzi et al. 2010; Svenningsson et al. 2003).

However, the ability of PCP to affect ERK1/2 phosphorylation in other brain regions has been reported. Administration of PCP dose-dependently increased p-ERK1/2 in the rat cerebellum without affecting those in the hippocampus, brainstem, and FC (Kyosseva et al. 2001), whereas Enomoto et al. (2005) found elevated level of p-ERK1/2 in the hippocampus of PCP-treated mice. The p-ERK1 was increased in the NAC 40 min after (R)-CPP. This effect appears to be independent of p-CREB as no changes in p-CREB levels in this region were detected. However, it could not be excluded that p-ERK1 in the NAC might participate in the (R)-CPP-induced behavioural impairments.

Another protein strictly associated with the calcium influx through NMDA receptor is CaMKII, which is a crucial mediator of synaptic plasticity (Colbran and Brown 2004). Consistent with my previous data with systemic PCP (Pozzi et al. 2010), (R)-CPP had no effect on phospho-T^{286}-CaMKII in FC, CPu and NAC thus ruling out the involvement of this kinase in (R)-CPP-induced changes in p-CREB levels. However, it cannot be excluded that other CaMK isoforms such as CaMKIV and VI might be involved.

Table 3.1.

5-CSRTT	CPP			PCP			MK-801		
ACCURACY	↓			↓			↓		
IMPULSIVITY	↑			↑			↑		
COMPULSIVITY	↑			↑			↑		
p-CREB	FC	CPu	NAC	FC	CPu	NAC	FC	CPu	NAC
10 min	↓	↓	N.E.	N.E.	↑	_	↑	↑	_
40 min	↑	↓	N.E.	↑	N.E.	N.E.	↑	N.E.	N.E.

Summary of behavioural and biochemical effects of NMDAR antagonists
NMDAR antagonists-induced behavioural responses in the 5-CSRT task and CREB phosphorylation
This table summarized the effects of (R)-CPP (50 ng/μL intra mPFC), PCP (2.5 mg/kg i.p.) and MK-801(0.15 mg/kg i.p.) on 5-CSRT task performance and p-CREB levels in the rat FC, CPu and NAC.
↓ reduction; ↑ increase; **N.E.** no effect;

Phosphorylation of CREB was affected by (R)-CPP and by non-competitive NMDAR antagonists PCP and MK-801, which share the ability to impair attention and executive functions in the 5-CSRT task. As can be appreciated in summary Table 3.1, there are discrepancies between the behavioural effects of NMDAR antagonists and the dynamics of qualitative changes in the levels of p-CREB in the various regions of rat brain. Indeed, 10 min after intracortical (R)-CPP p-CREB was significantly decreased in the FC, by contrast it was increased or not changed after MK-801 and PCP, respectively. On the other hand, all compounds increased p-CREB levels in the FC 40 min after their administration (Table 3.1). In the CPu p-CREB was decreased 10 and 40 min after (R)-CPP, whereas it was increased at 10 but not at 40 min after PCP or MK801 (Table 3.1). Likewise, discrepancies in the effect of NMDA receptor antagonists on p-CREB levels in cortical and subcortical regions of the brain have been observed also in mice. Acute systemic administration of PCP significantly increased p-CREB levels in the FC and CPu of CD1 mice (Svenningsson et al. 2003). These results suggest that although p-CREB might be involved in the mechanisms by which NMDAR antagonists induced behavioural impairments in the 5-CSRT task, its role is still undefined and somewhat controversial. Indeed, it is not clear whether the genetic make-up of animals (rats versus mice), the competitive or uncompetitive nature of the NMDAR antagonists or the route of administration (i.e intra mPFC versus systemic) play a major role in determining the direction and time course of changes in p-CREB levels across different brain areas.

Recently, it has been shown that preventing CREB phosphorylation by viral-vector gene transfer in the FC, accuracy measured in the 5-CSRT task was impaired (Paine et al. 2009). This finding partially contrasts with what is suggested by the present data. However, in the study by Paine et al. (Paine et al. 2009) inhibition of CREB

phosphorylation was made in rats performing the task while I examined the effects of (R)-CPP on p-CREB in rats that were behaviourally naïve. Thus it could not be excluded that (R)-CPP may have different effect on p-CREB depending on whether or not rats are performing the task. This aspect has been addressed in Chapter 5.

In conclusion, it is suggested that changes in CREB phosphorylation in the cortex and dorsal striatum induced by blockade of NMDAR in the mPFC may be not directly linked to attention performance deficits but might likely reflect changes in the activity of corticostriatal circuitry involving many diverse actors among which are PKA signalling and neurotransmitters.

CHAPTER 4

EFFECTS OF LY379268 ON (R)-CPP-INDUCED COGNITIVE DEFICITS, p-CREB CHANGES AND GLUTAMATE RELEASE

In the previous chapter (Chapter 3) I have reported that NMDAR antagonists at doses that affected attention and response control in the 5-CSRT task affect phosphorylation of CREB on S^{133} in the FC and CPu. In particular I showed that in rats, (R)-CPP infused in the mPFC both increased and decreased p-CREB in the FC (10 and 40 min after infusion, respectively) and reduced it in the CPu.

Interestingly, I recently reported that in mice the attentional performance deficit induced by PCP in the 5-CSRT task and the PCP-induced p-CREB changes in the CPu were prevented by the same dose of the serotonin $5HT_{2A}$ receptor antagonist, M100907, suggesting that up-regulation of p-CREB may be associated with impairment in attentional performance in the 5-CSRT task (Pozzi et al. 2010).

As mentioned in the General Introduction (Chapter 1), administration of NMDAR antagonists increased GLU release in the FC. Accordingly, recent work in my laboratory has shown that an increased GLU efflux in the PFC was associated with (R)-CPP-induced impairments in accuracy of visual discrimination, but not impulsivity or perseveration in the 5-CSRT task (Ceglia et al. 2004; Calcagno et al. 2009; Calcagno et al. 2006; Carli et al. 2006; Mirjana et al. 2004) (see Table 4.1).

Table 4.1

Summary of the effects of different agents on (R)-CPP-induced deficits on various aspects of attentional performance in the 5-CSRTT and on GLU efflux in the rat PFC.

	5-CSRTT			GLU
	ACCURACY	IMPULSIVITY	COMPULSIVITY	Efflux (PFC)
Intra mPFC (R)-CPP	↓	↑	↑	↑
Haloperidol [a] (D2 antagonist)	NO EFFECT	R	R	NO EFFECT
M100907 [b,c] (5HT$_{2A}$ antagonist)	R	R	NO EFFECT	R
8-OH-DPAT [d,e] (5HT$_{1A}$ agonist)	R	NO EFFECT	R	R
Ro60-0175 [f] (5HT$_{2C}$ agonist)	R	R	NO EFFECT	R
SB242084 [g] (5HT$_{2C}$ antagonist)	NO EFFECT	NO EFFECT	NO EFFECT	NO EFFECT

↓ reduction; ↑ increase; R reversal.

[a] Baviera et al, 2008; [b] Mirjana et al, 2004; [c] Ceglia et al, 2004; [d] Carli et al, 2006; [e] Calcagno et al, 2006; [f] Calcagno et al, 2009; [g] Higgins et al, 2003 (in this study MK801 was administered systemically)

The metabotropic GLU receptors (mGluRs) are classified into three groups (I, II and III) based on structural homology, signal transduction mechanisms and pharmacological properties (Nakanishi 1992). These receptors modulate GLU neurotransmission in an anatomically and functionally distinct manner (Conn and Pin 1997; Schoepp and Conn 2002). The group II family of mGluRs consist of mGluR2 and mGluR3 highly expressed in cortical neurons (Ohishi et al. 1994; Petralia et al. 1996) and functions as a presynaptic negative regulatory mechanism, modulating excitatory GLU and inhibitory GABA transmission (Anwyl 1999; Schoepp 2001). The mGluR2/3 mediated inhibition of evoked GLU release in PFC is thought to be one possible mechanism through which the agonists at these

mGluRs prevent NMDAR antagonists-induced rise in cortical GLU efflux. Indeed, in pharmacological studies, the systemically active mGlu2/3 receptor agonists LY354740 and LY379268 attenuated the disruptive effects of PCP on cognitive functions, stereotypy, locomotion and cortical GLU efflux (Moghaddam and Adams 1998; Schoepp 2001). Interestingly the selective mGlu2/3 receptor agonist LY379268 prevented PCP-induced impairment in attention and response control in mice performing the 5-CSRT task (Greco et al. 2005).

In this chapter I present the results of the experiments investigating the hypothesis that activation of mGlu2/3 receptors using LY379268, might be sufficient to prevent (R)-CPP-induced attention deficits via a modulation of CREB phosphorylation and cortical GLU release.

Specifically, I examined the effect of LY379268 given systemically on (R)-CPP-induced attention performance deficit and executive control over performance (such as anticipatory "impulsive" and perseverative responses) in the 5-CSRT task. In addition, by western blot technique and intracerebral microdialysis in awake animals I examined whether and how the behaviourally active dose of LY379268 interfered with the (R)-CPP-induced p-CREB changes in the FC and CPu and the (R)-CPP-induced rise in GLU efflux in the FC.

4.1 LY379268 PREVENTED (R)-CPP-INDUCED COGNITIVE DEFICITS

The 5-CSRT task was used to investigate whether (R)-CPP-induced cognitive deficits were prevented by LY379268. The timeline of this experiment is reported below.
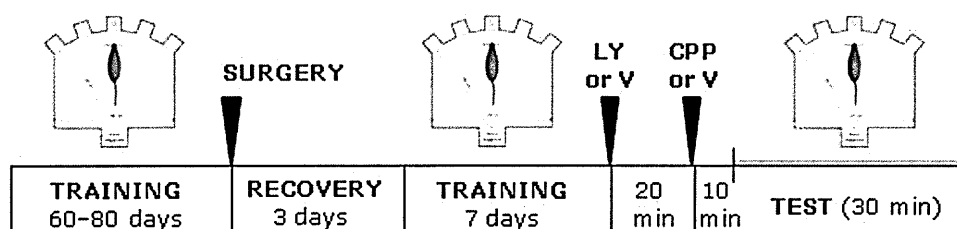
A comprehensive description of experimental procedures is reported in General Methods (Chapter 2). Briefly, rats were trained on the 5-CSRT task for several days until they reached a baseline performance of 80% correct responses. Then they were bilaterally implanted with stainless steel cannulae in the mPFC and had 3 days to recover from surgery. Rats were then re-trained for a week to re-establish their pre-surgery baseline performance.

On the experimental days rats received the combinations of subcutaneous injections of PBS (2 mL/kg) or LY379268 (0.1 mg/kg) 20 min before bilateral injection of PBS (1 µL/side) or (R)-CPP (50 ng/µL/side) into the mPFC. Ten minutes after intra-mPFC injection rats were put into the box and the test session started. Each rat received all drugs combinations according to a Latin-square design. At least two days were left between experimental days. Rats were always tested on these "free" days to re-establish the baseline and check for the lasting effects of drugs.

RESULTS

LY379268 prevented (R)-CPP-induced impairments in accuracy and anticipatory but not perseverative responding.

Figure 4.1 shows that 0.1 mg/kg LY379268 alone administered subcutaneously 20 min before bilateral injections of vehicle (V) into the mPFC had no effect on any measure of rats' performance. When LY379268 was administered in combination with intra mPFC 50 ng/µL (R)-CPP, it reversed the (R)-CPP-induced impairments in accuracy (% correct) (**A**) and anticipatory responding (**B**) but was without effects on (R)-CPP-induced increase in perseverative responses (**C**). 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 (R)-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 (R)-CPP significantly decreased % correct responses compared to vehicle controls (V+CPP compared to V+V; P<0.05) and that (R)-CPP-injected rats pre-treated with 0.1 mg/kg LY379268 but not vehicle made significantly more correct responses than (R)-CPP-injected rats (LY+CPP compared to V+CPP; P<0.05) (Fig.4.1**A**).

Similarly, ANOVA performed on the anticipatory response data showed a significant interaction between LY379268 and (R)-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 (R)-CPP (V+CPP compared to V+V, P<0.05) and LY379268 decreased it (LY+CPP compared to V+CPP, P<0.05) (Fig.4.1**B**).

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Statistical analysis of perseverative responses data by ANOVA indicated that LY379268 had no effect on (R)-CPP-induced perseverative over-responding (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) (Fig.4.1C). Post-hoc comparison of treatment means showed a significant increase in perseverative responses due to (R)-CPP (V+CPP compared to V+V, P<0.05) injection but no effect of LY379268 on its own (LY+V compared to V+V, P>0.05) or on (R)-CPP-induced increase (LY+CPP compared to V+CPP; P>0.05).
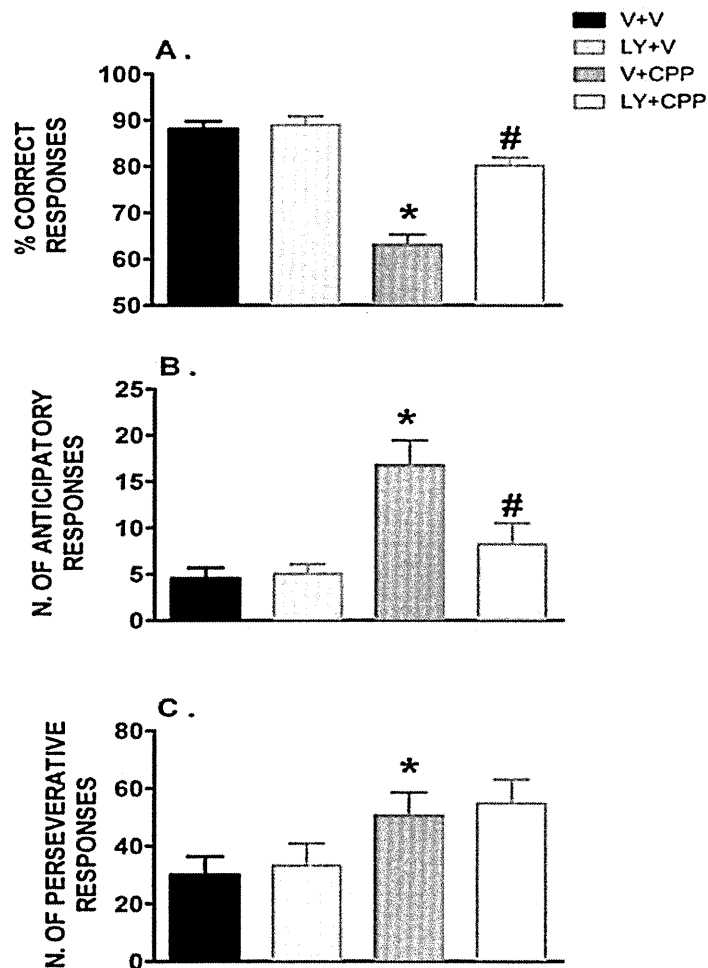
Fig. 4.1
LY379268 prevented (R)-CPP-induced cognitive deficits
Histograms represent the effects of LY379268 alone or in combination with (R)-CPP on correct responses (**A**), anticipatory (**B**) and perseverative responses (**C**).
One µL of saline (V+V) or (R)-CPP (50 ng/µL; V+CPP) was bilaterally infused in the mPFC. Rats received LY379268 alone (LY+V) or in combination with (R)-CPP (LY+CPP). LY379268 0.1 mg/kg was injected subcutaneously twenty min before (R)-CPP.
Ten min after (R)-CPP rats started the test session. (R)-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+V; # P<0.05 vs. V+CPP; (Tukey's test).

Omissions showed in Table 4.2, were increased by (R)-CPP ($F_{1,24}$=116.0; P<0.0001) but not 0.1 mg/kg LY379268. However, the proportion of omissions made by rats receiving (R)-CPP was not further affected by LY379268 (LY379268 x CPP, $F_{1,24}$=2.2; P=0.15). Table 4.2 also shows that rats receiving (R)-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).

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

Table 4.2
(R)-CPP-increased omissions and correct response latency
Each value is the mean ± SEM of 9 rats. LY379268 at a dose of 0.1 mg/kg (LY 0.1) or vehicle (V) were injected subcutaneously 20 min before bilateral injections of vehicle (V, 1µL) or (R)-CPP (CPP, 50 ng/µL) 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. V+V; Tukey's test.

The effects of 0.3 mg/kg LY379268 on (R)-CPP-induced deficits were also tested. At this dose LY379268 by itself had no effect on accuracy and on anticipatory or perseverative responses but greatly increased the percentage of omissions (from 10.0±1.4 to 22.1±4.4; $P<0.05$) and correct response latencies (from 0.54±0.03 to 0.70±0.08 sec; $P<0.05$). When given in combination with (R)-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. The mean proportion of omissions and correct response latencies after 0.3 mg/kg LY379268 plus (R)-CPP reached 60.3±5.3 % and 1.47±0.11 sec respectively. However, 0.3 mg/kg LY379268 greatly reduced anticipatory (from 14.0±3.4 to 3.7±1.7; $P<0.05$) but not perseverative over-responding (from 49.3±7.1 to 41.7±7.5; $P>0.05$) of (R)-CPP-injected rats. Accuracy data were not reliable due to a very low number of correct and incorrect trials and are not reported.

Rats' baseline performance before starting intra-cortical (vehicle or (R)-CPP; n=9) and subcutaneous (vehicle or LY379268; n=9) injections was stable across days (Fig. 4.2). They performed the task with very high accuracy (between 78-96 % correct responses; 5-17 % omissions, respectively) and a low number of anticipatory (between 1-17) and perseverative (between 10-40) responses. Rats were fast to make a correct response (0.46-0.7 sec) and to collect the earned food pellet (0.44-2.0 sec).

During the "free between-treatment" days rats' performance was similar to that during baseline, suggesting that there were no carry-over effects of various treatments and their combinations.
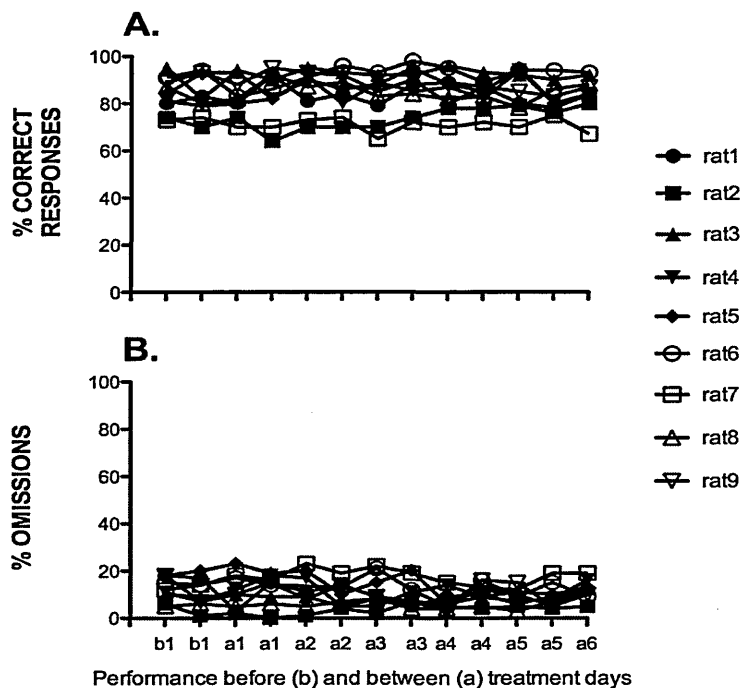
Fig. 4.2

Baseline behavioural performance

Accuracy (**A**) (% CORRECT RESPONSES) and omissions (**B**) (% OMISSIONS) of individual rats (n=9) for the 2 days before the start of injections in the mPFC (b1 and b2) and during the 2 days after each treatment day (a1, after the first treatment day – a6 after the last treatment day).

4.2 LY379268 PREVENTED (R)-CPP-INDUCED p-CREB CHANGES IN THE FC

I next investigated the effect of LY379268 at the dose that prevented (R)-CPP-induced attention deficits in the 5-CSRT task on (R)-CPP-induced p-CREB changes in the rat FC and CPu. The timeline of this experiment is reported below.
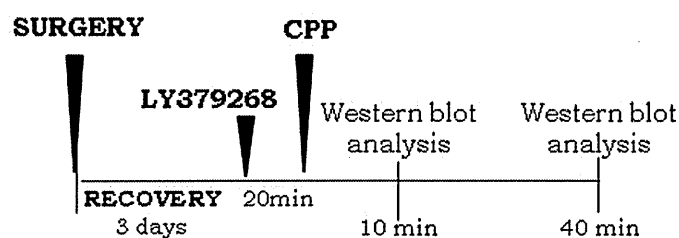
SURGERY   CPP

LY379268   Western blot   Western blot
            analysis   analysis

RECOVERY   20min
3 days   10 min   40 min

A comprehensive description of experimental procedures is reported in General Methods (Chapter 2). Briefly, rats were bilaterally implanted with stainless steel cannulae in the mPFC and had 3 days to recover from surgery. A group rats was pretreated subcutaneously with PBS (2 mL/kg) or LY379268 (0.1 mg/kg) 20 min before intra-PFC injections of PBS (1 µL side) or (R)-CPP (50 ng/µL side). These rats were sacrificed 10 or 40 min after (R)-CPP (i.e. 30 min and 1 h after LY379268, respectively).

RESULTS

LY379268 prevented (R)-CPP-induced p-CREB changes in the FC

As shown in figure 4.3, p-CREB levels in the FC were decreased 10 min (**A**) and increased 40 min (**B**) after intracortical (R)-CPP infusion thus replicating the data presented in Chapter 3. Both effects were abolished by pre-treatment with a subcutaneous dose of 0.1 mg/kg LY379268 as indicated by the significant interaction between LY379268 and (R)-CPP at both 10 (Fig.4.3A) ($F_{1,18}$=4.4; P<0.05) and 40 (Fig.4.3B) min ($F_{1,21}$=10.4; P<0.05) after (R)-CPP. Furthermore, comparison between treatment means by Tukey's test indicated that p-CREB levels of (R)-CPP-injected rats decreased compared to those after saline-injection at 10 but increased at 40 min (comparison between V+CPP with V+V; P<0.05). Comparing treatment means of rats that had LY+CPP with those that received V+CPP showed that at 10 min time point p-CREB levels were increased (P<0.05) whereas at 40 min time point the p-CREB levels were decreased (P<0.05). However, on its own LY379268 significantly increased the levels of p-CREB but only 30 min after injection (Fig. 4.3A) (comparison between V+LY and V+V; P<0.05, Tukey's test). One hour after LY379268 injection (Fig.4.3B) levels of p-CREB were similar to those measured in saline injected rats (comparison between LY+V and V+V; P>0.05, Tukey's test).

Levels of T-CREB were not modified by any treatments (not shown).
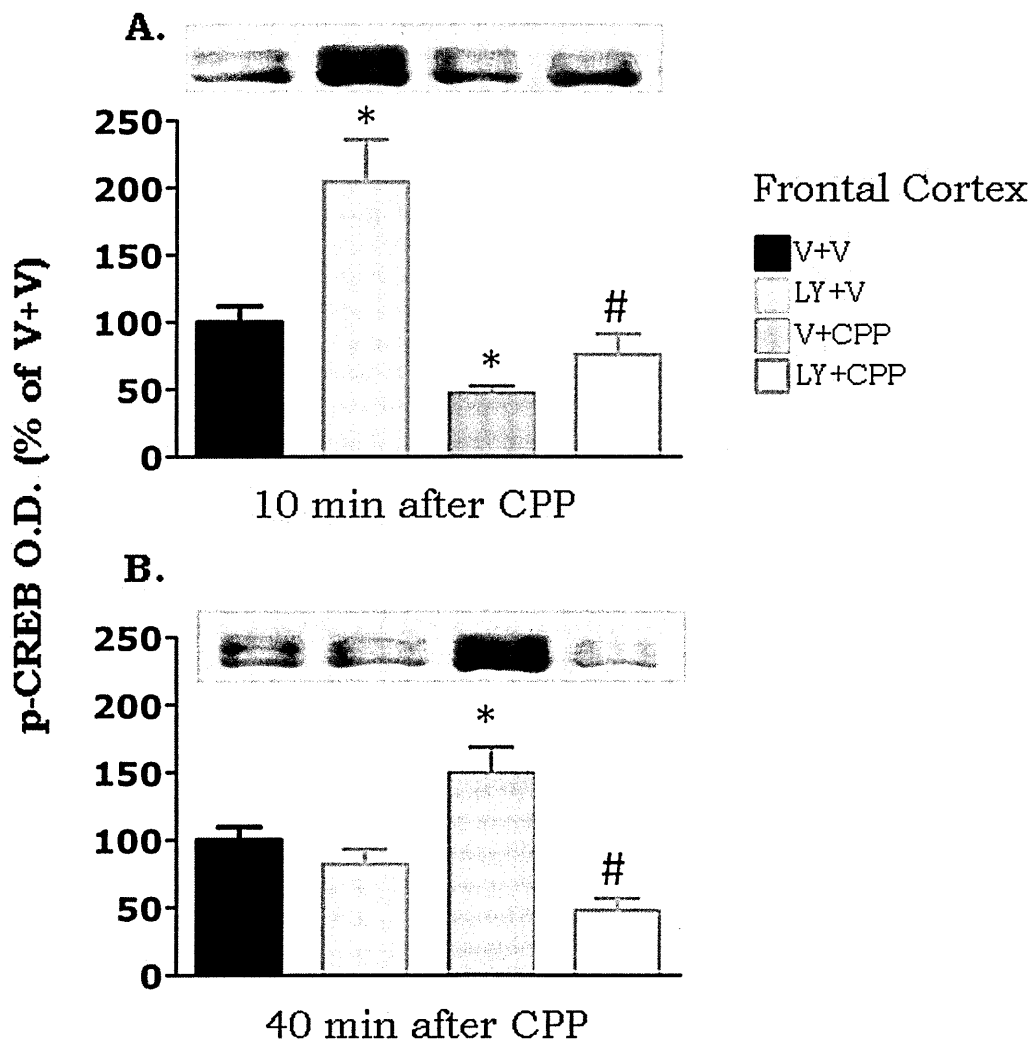
Fig. 4.3
LY379268 prevented (R)-CPP-induced pCREB changes in the FC
Representative immunoblots and quantification of (R)-CPP-induced p-CREB changes in the FC of rats pre-treated with 0.1 mg/kg LY379268.
LY379268 was given 20 min before (R)-CPP and rats were sacrificed 10 (**A**) or 40 min (**B**) after (R)-CPP. Data are expressed as percentage of values in saline-treated rats (V+V). Histograms represent mean±SEM of 6 rats per group.
*$P<0.05$, vs V+V; # $p<0.05$, vs V+CPP (Tukey's test).

LY379268 did not prevent (R)-CPP-induced p-CREB decrease in the CPu

LY379268 was unable to reverse the effects of (R)-CPP on p-CREB levels in the CPu. Figure 4.4 shows that (R)-CPP-induced decrease in p-CREB levels in the CPu were not prevented by pretreatment with 0.1 mg/kg LY379268 both at 10 (**A**) and 40 min (**B**) as indicated by the two-way ANOVA (LY379268xCPP, $F_{1,19}$=2.2; P>0.05 and LY379268xCPP, $F_{1,20}$=0.08; P>0.05, respectively).

However, the F values for the main effect of (R)-CPP were significant at both time points and further analysis by Tukey's test indicated that (R)-CPP decreased the levels of p-CREB 10 and 40 min after injection (comparison between V+V and V+CPP, P<0.05). Although at 10 min time-point (Fig.4.4A) levels of p-CREB in rats receiving 0.1 mg/kg LY379268 plus (R)-CPP were higher than those receiving saline plus (R)-CPP this difference was not statistically significant (P>0.05; Tukey's test).

Figure 4.4**B** shows that 40 min after (R)-CPP p-CREB levels in rats receiving the combination of LY379268 plus (R)-CPP were not different from those receiving V plus (R)-CPP (P>0.05; Tukey's test).

LY379268 had no effect on p-CREB levels in rats injected with intra-cortical saline at either 10 (**A**) or 40 (**B**) min time points (comparison between LY379268+V and V+V; P>0.05, Tukey's test). Levels of T-CREB were not modified by any treatment (not shown).
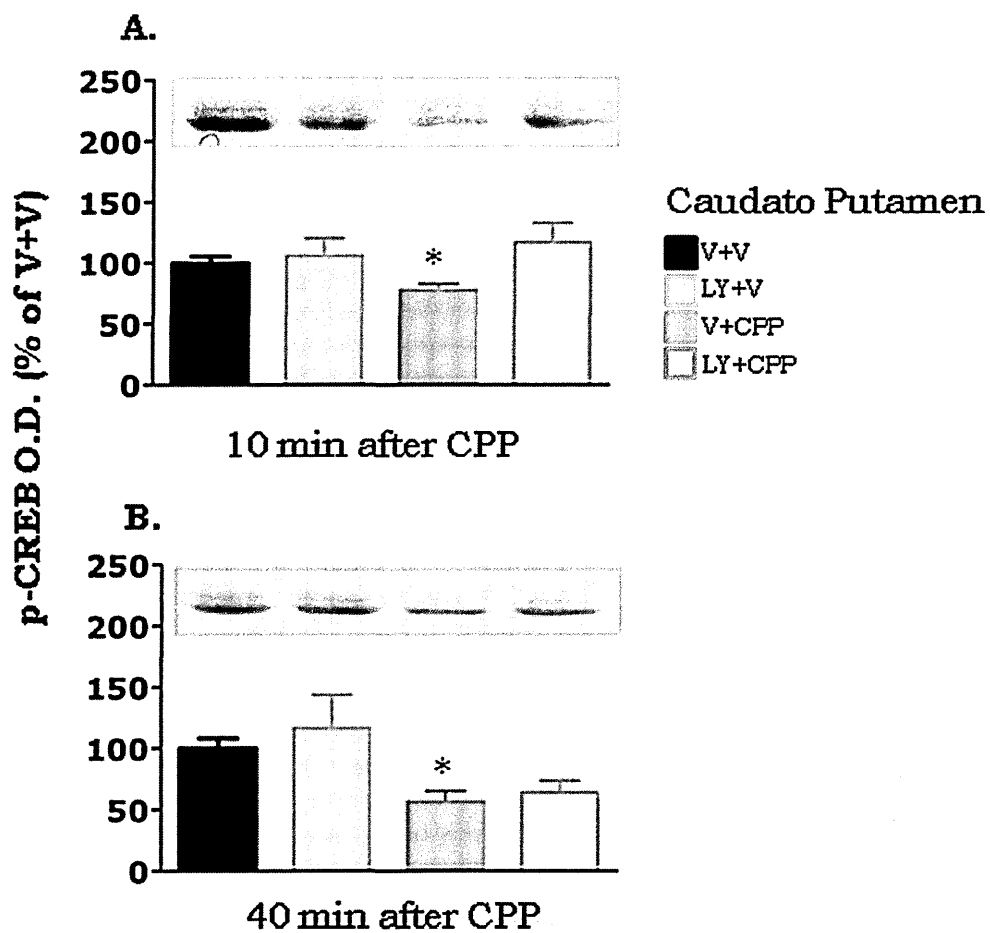
Fig. 4.4
LY379268 did not prevent (R)-CPP-induced p-CREB decrease in the CPu.
Representative immunoblots and quantification of (R)-CPP-induced p-CREB changes in the CPu of rats pre-treated with 0.1 mg/kg LY379268.
LY379268 was given 20 min before (R)-CPP and rats were sacrificed 10 (**A**) or 40 min (**B**) after (R)-CPP. Data are expressed as percentage of values in saline-treated rats (V+V). Histograms represent mean±SEM
of 6 rats per group.
* $P<0.05$, vs V+V; (Tukey's test).

4.3 LY379268 SELECTIVELY INCREASED p-CaMKII IN THE FC

To unveil the signal pathways recruited by LY379268 in its ability to prevent (R)-CPP-induced changes in p-CREB, I tested whether LY379268 might interfere with the upstream kinases that regulate phosphorylation of CREB on serine 133, such as PKA, ERK1/2, and CaMKII.

Levels of phosphorylated kinases in the FC and CPu were measured 1 hour after LY379268 (the time point corresponding at 40 min after (R)-CPP in the previous experiment). Dilutions of antibodies for protein kinases were as follows: p-T^{286}-CaMKII (1:10000), p-T^{197}-PKAc (1:4000) and p-T$^{202/}$Y^{204}-ERK1/2 (1:10000). Levels of phosphorylated kinases and CREB were measured in the same samples.

RESULTS

The effects of LY379268 on p-CREB and protein kinases are reported in Fig.4.5.

I found that levels of p-CREB were not changed 1h after LY379268 administration both in the FC (**A**) and in the CPu (**B**) thus confirming my previous results (see Figs 4.3B and 4.4B).

Administration of LY379268 increased the levels of p-CaMKII in the FC (Fig.4.5**A**) ($P<0.05$, Student's t-test; comparison V versus LY379268) while it was without effect on p-CaMKII levels in the CPu (Fig.4.5**B**) ($P>0.05$, Student's t-test; comparison V versus LY379268).

One hour after LY379268, levels of p-PKAc were increased both in the FC (Fig.4.5**A**) and in the CPu (Fig.4.5**B**) compared to vehicle-injected rats ($P<0.05$, comparison between V and LY379268, Student's t-test).

LY379268 did not affect levels of p-ERK1/2 either in the FC (Fig.4.5**A**) or in the CPu (Fig.4.5**B**) ($P>0.05$, Student's t-test).

The effects of LY379268 on these kinases in the NAC were not examined.

A. FC

B. CPu
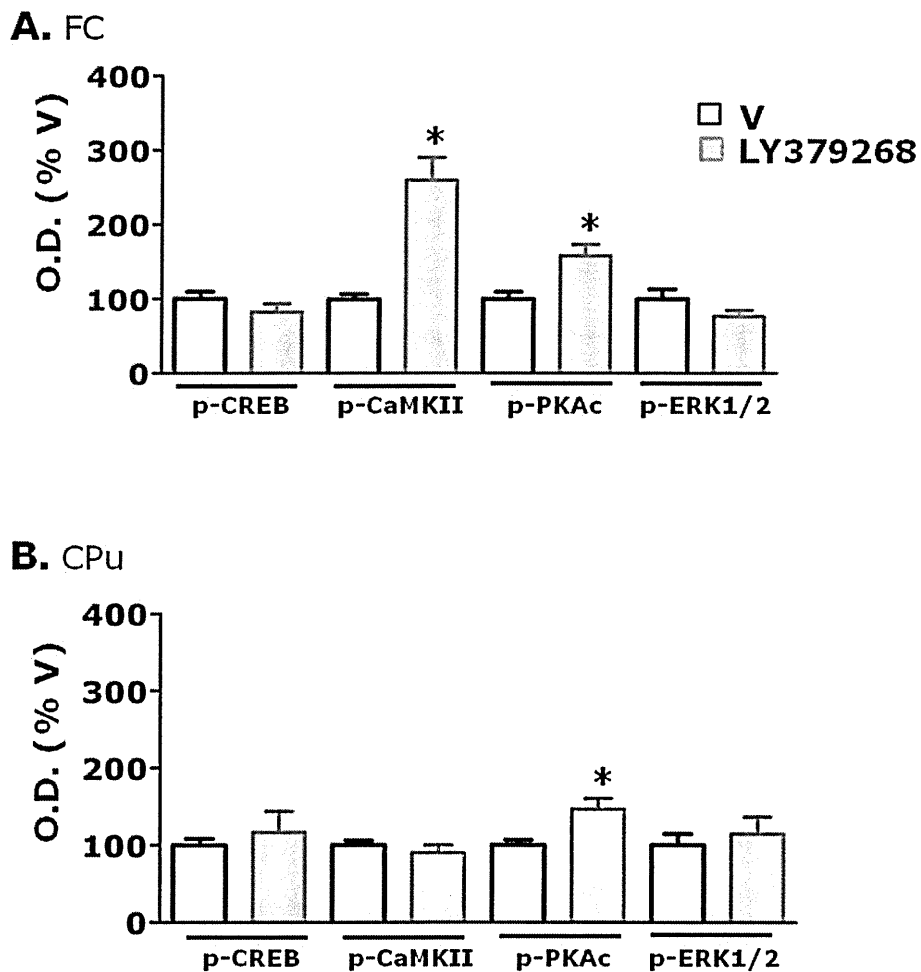
Fig. 4.5

Effects of LY379268 on phosphorylated CREB and protein kinases

Histograms depict quantitative effects of LY379268 on p-CREB, p-CaMKII, p-PKAc and p-ERK1/2 levels in the FC (**A**) and CPu (**B**), compared to vehicle (V). Vehicle or LY379268 (0.1 mg/kg) were given s.c. and rats were sacrificed 1 hour later.

Data are expressed as percentage of values (mean±SEM) in vehicle-injected rats (V). N=6/8 replications each bar.

*P<0.05 vs V (Student's t-test).

4.4 LY379268 PREVENTED (R)-CPP-INDUCED GLUTAMATE RELEASE

In the following experiment I tested the hypothesis that LY379268, at the dose that prevented (R)-CPP-induced attention deficits and (R)-CPP-induced p-CREB changes in the FC might be effective in preventing (R)-CPP-induced cortical GLU release. The timeline of the experiment is reported below.

SURGERY   LY379268   HISTOLOGY

Start
Sampling

100 μm CPP
infusion

24 hours recovery 20 min 1h 1h

Intracerebral Microdialysis
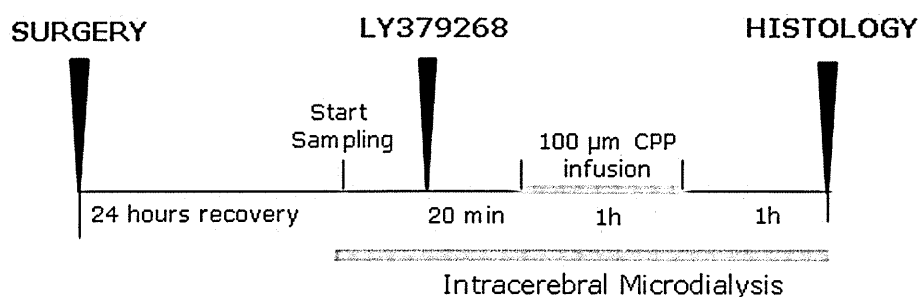
A detailed description of experimental procedures is reported in General Methods (Chapter 2). Briefly, rats were implanted with a microdialisys probe in the mPFC (Fig.4.6).

Microdialysis
probe
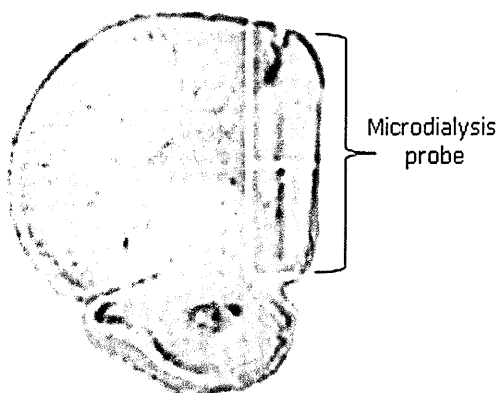
Fig. 4.6
Localization of the microdialysis probe in the mPFC.
The picture shows the exact localization of the concentric microdialysis probe in the rat mPFC. Coordinates are as follows: AP+3.7, L±0.7 mm from bregma and DV-4.8 mm from dura surface (Paxinos and Watson 2007).

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On the experimental day (24 h after surgery) (R)-CPP was dissolved in aCSF and infused for 60 min through the probe into the mPFC at the concentrations of 100 μM. Control rats were perfused for the same time with aCSF alone.

LY379268 (0.1 mg/kg) was dissolved in PBS and injected S.C. 20 min before (R)-CPP, during the phase of stable GLU output (defined as three consecutive baseline samples not differing by more than 20%). Control rats were given PBS s.c.

RESULTS

LY379268 prevented (R)-CPP-induced rise of extracellular GLU in the mPFC

Basal levels of extracellular GLU in the rats' mPFC are reported in Table 4.3.

mPFC	Basal GLU pmol/20μL
aCSF+PBS	17±3 (N=6)
LY+aCSF	19±2 (N=6)
PBS+CPP	22±4 (N=5)
LY+CPP	25±3 (N=6)

Table 4.3
Basal levels of extracellular GLU in the mPFC in each group of rats implanted with the microdialysis probe. Data are expressed as pmol/20 μL (5-6 rats).
The mean basal level of GLU was 21±3 pmol/20 μL (not corrected for recovery) (N=23). The number of animals in each group is shown in brackets.

Figure 4.7 shows that infusion of artificial CSF trough the probe and systemic administration of PBS had no effects on extracellular GLU in the rat mPFC (**A**). The infusion of 100 μM (R)-CPP trough the probe increased extracellular GLU in the rat mPFC reaching 250% of basal values at 40 min (**B**).

LY379268 (0.1 mg/kg), which had no significant effect by itself (**A**) ($F_{1,19}$=1.8, p=0.2), abolished the (R)-CPP-induced rise of extracellular GLU (**B**). ANOVA showed highly significant effects of (R)-CPP ($F_{1,19}$=6.2, P=0.02), LY379268 x (R)-CPP ($F_{1,19}$=17.4, P=0.0005) and LY379268 x (R)-CPP x time ($F_{7,133}$=7.2, P<0.0001).

A.

B.
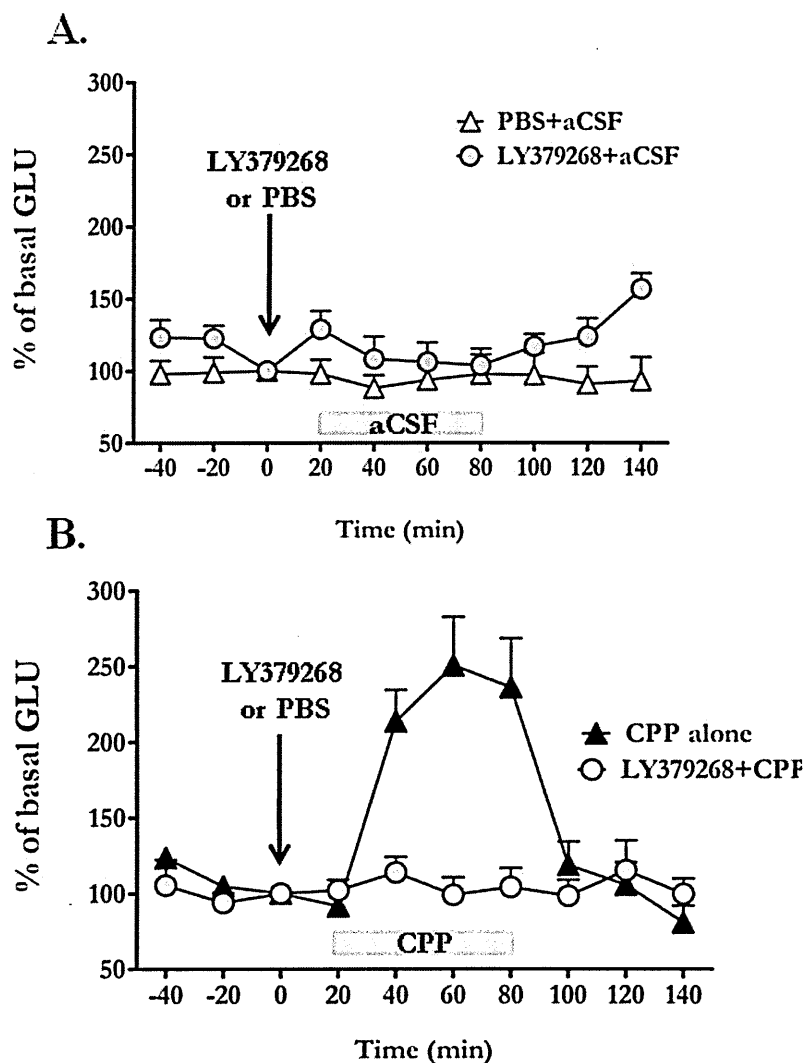
Fig 4.7
Effect of (R)-CPP alone and in combination with LY379268 on GLU efflux in the mPFC.
(A) LY379268 alone has no effect on basal GLU in the mPFC. LY379268 or PBS was injected S.C. 20 min before aCSF perfusion (arrow). a-CSF was perfused through the probe for 60 min, starting 20 min after LY379268 or PBS, as indicated by the horizontal bar.
(B) LY379268 prevented (R)-CPP-induced rise of extracellular GLU.
LY379268 was injected S.C. 20 min before (R)-CPP (arrow). (R)-CPP was perfused through the probe for 60 min, starting 20 min after LY379268 or PBS, as indicated by the horizontal bar.
Extracellular levels of GLU, not corrected for in vitro recovery of the probe, were expressed as percentages of basal values. Basal values (time 0) are the mean of three stable consecutive samples.

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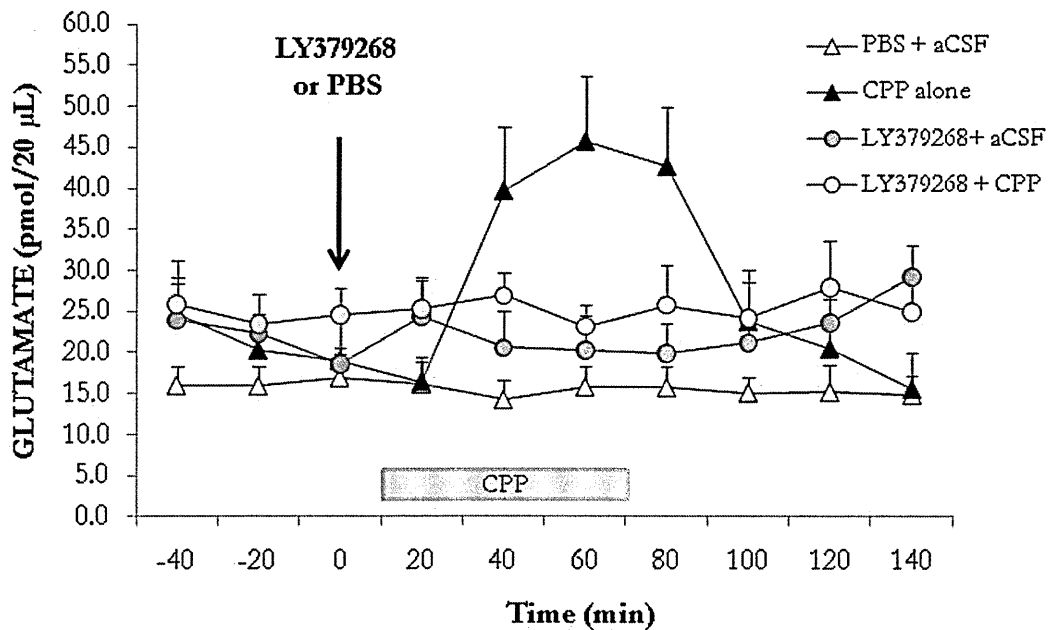
Fig 4.8
Graph showing the results reported in fig.4.7 expressed as raw data.
LY379268 or PBS was injected S.C. 20 min before (R)-CPP (arrow). (R)-CPP was perfused through the probe for 60 min, starting 20 min after LY379268 or PBS, as indicated by the horizontal bar. Basal levels of GLU (time 0), not corrected for in vitro recovery of the probe, were calculated as the average of three stable consecutive samples.

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4.5 DISCUSSION

The findings from these experiments show that (R)-CPP-induced cognitive dysfunctions in the 5-CSRT task are associated with changes in the phosphorylation of CREB on residue S^{133} and excessive GLU release in the FC. Indeed, activation of mGluR2/3 by the group II selective agonist LY379268 ameliorated accuracy and some aspects of response control such as impulsivity disrupted by the blockade of NMDAR in the mPFC and prevented (R)-CPP-induced p-CREB changes and the increased GLU release in FC.

LY379268 and cognitive deficits

The results of the behavioural experiment show that LY379268 reversed accuracy impairments and anticipatory over-responding induced by intra-mPFC (R)-CPP but had no effect on perseverative responses. This pattern of effects contrast with those reported by Amitai and Markou (2010) using Wistar rats. They found that 1.0 mg/kg LY379268 administered concomitantly with repeated PCP treatment exacerbated accuracy impairment. LY379268 has been reported to exacerbate also PCP-induced ataxia (Cartmell et al. 2000a). Intriguingly, 1.0 mg/kg LY379268 had additive effects on PCP-induced increase in omissions, and correct response latency (Amitai and Markou, 2010), which is similar to what reported in the present study with 0.3 mg/kg. In addition, the dose of 0.3 mg/kg (present study) and that of 3.0 mg/kg in Amitai and Markou (2010) administered on their own increased the percentage of omissions and correct response latency to a similar degree. Thus it could be argued that compared to Wistar rats, Lister hooded rats, may be more sensitive to the effects of LY379268.

We previously reported that in mice performing the same behavioural task LY379268 at doses 10 time higher than those used in the present experiment had no effect on PCP-induced accuracy deficit but completely abolished the increase in anticipatory and perseverative responses (Greco et al. 2005). Furthermore, in mice the 5-HT$_{2A}$ receptor antagonist, M100907 abolished PCP-induced accuracy impairment and the increase in anticipatory and perseverative responses whereas in rats M100907 abolished accuracy impairment and anticipatory responding but was not able to decrease perseverative over-responding induced by systemic MK-801 (Higgins et al. 2003) or intra-mPFC (R)-CPP (Carli et al. 2006; Mirjana et al. 2004; Pozzi et al. 2010). The contrasting findings are not surprising as differences in effective drugs doses and qualitative effects across species and strains have been reported. The route of administration, the schedule of treatment (acute or repeated) and the competitive/non-competitive nature of NMDAR antagonists may have contributed to the reported inconsistencies.

Although LY379268 have been used at higher doses in some previous studies (Amitai and Markou, 2010) the doses used in the present study are close to those significantly reducing DOI-induced head-twitches (Klodzinska et al. 2002) and supposed to yield brain concentrations close to the IC$_{50}$ (10 nM) for the displacement of [^{3}H]341495 binding to mGlu2/3 receptors and the EC$_{50}$ (3-6 nM) for the inhibition of forskolin-stimulated cAMP production (Imre 2007). Non-selective effects of LY379268 can be ruled out as doses of LY379268 close to those used in the present study or even higher were prevented by the selective mGluR2/3 antagonist LY341495 (Di Liberto 2010; Molinaro et al. 2009).

Injected in the mPFC (R)-CPP has been shown to increase motor activity (Del Arco et al. 2008; Mirjana et al. 2004) suggesting that LY379268 could have prevented accuracy deficit by blocking (R)-CPP-induced hyperactivity. However, it is difficult to explain the accuracy deficit as a simple motor effect as correct and incorrect responses in this task have the same motor requirements. In addition we have shown that accuracy deficit and increased omissions induced by 50 ng/side (R)-CPP were completely abolished when the attentional load on performance was decreased, by increasing stimulus duration from 0.5 to 1.0 sec (Mirjana et al. 2004). Furthermore LY379268 prevents hyperactivity at doses at least 10 times higher than those used in the present study (Cartmell et al. 1999 and 2000b; Imre et al. 2006; Lorrain et al. 2003a). The (R)-CPP-induced increase in omissions and latency to make a correct response might reflect motivational factors. However, decreasing the motivation for food by pre-feeding the animals increases the proportion of omissions and response latency but had no effect on accuracy and response control (Carli and Samanin 1992). Therefore, it is unlikely that (R)-CPP-induced impairments were a consequence of hyperactivity, poor motivation or a failure to make associations or remember the general rules of the task as well as that 0.1 mg/kg LY379268 prevented attention deficit by decreasing hyperactivity or interfering with motivation. At 0.3 mg/kg LY379268 greatly increased omissions and latencies to make a correct response and to collect the earned food pellet suggesting that at high doses LY379268 may interfere with motivational factors.

Despite differences in behavioural processes under study, the present findings are largely compatible with the behavioural studies showing that mGluR2/3 agonists such as LY354740 and LY379268 reduced hyperlocomotion, stereotypy and

deficits in working memory, induced by non-competitive NMDAR antagonists given systemically (Moghaddam and Adams 1998; Swanson and Schoepp 2002). However, no effects of mGluR2/3 agonists on NMDAR antagonists-induced pre-pulse inhibition deficit and hyperlocomotion have also been reported (Imre et al. 2006; Henry et al. 2002).

LY379268 prevented (R)-CPP-induced p-CREB changes in FC: possible role of CaMKII

Using western blot analysis I found that the dose of LY379268 that prevented (R)-CPP-induced accuracy impairments in the 5-CSRT task also prevented (R)-CPP-induced changes of p-CREB in the FC but not in the CPu. This indicates that p-CREB in the rat FC, but not in the CPu, might be specifically associated to aspects of accuracy.

I found that 30 min, but not 1h, after LY379268, p-CREB levels were significantly increased in the FC. Although this increase was significant only at the time point corresponding to the start of the behavioural task (i.e. 30 min after LY379268), this result shows that p-CREB can be increased without there being a behavioural deficit.

Although mGluR2/3 belongs to a class of inhibitory G protein-coupled receptors that are negatively linked to adenylyl cyclase (Conn and Pin 1997; Pin and Acher 2002; Schoepp et al. 1996a; Schoepp et al. 1996b), the ability of LY379268 to increase p-CREB was not surprising. Indeed, some studies have shown that activation of mGluRII can increase cAMP levels in cat visual cortex (Reid et al.

1996), in slices of the neonatal rat hippocampus (Schoepp et al. 1996c) and in cellular population of hypothalamic origin (Sortino et al. 1996).

Moreover, stimulation of mGluR2/3 by agonists such as DCG IV and 1-CCG-I, produced an approximately 70% decrease of basal cAMP levels with a concomitant increase in the p-CREB/CREB ratio in neuronal cell cultures, an effect similar to that elicited by a group I mGluR agonist (Harris et al. 2004). Cultured cortical neurons express both group I and II mGlu receptors and evidences for synergism between them have been found (Cartmell et al. 1993). Thus it is possible that the LY379268-induced increase in p-CREB, might be due to a synergistic interaction between mGluR group I and II receptors. However, the activation of group II mGluR can result in a variety of others physiological effects that can influence p-CREB, including the modulation of voltage-dependent conductance (Charpak et al. 1990) and, at higher doses, neurotransmitter release (Imre et al. 2006).

Western blot analysis was performed to identify the protein kinases recruited by LY379268 to prevent (R)-CPP-induced changes in p-CREB levels. In the FC I found that both p-PKA and p-CaMKII protein levels were significantly increased by LY379268. Yet only p-CaMKII was selectively increased in the FC after LY379268 suggesting that the ability of LY379268 to prevent (R)-CPP-induced p-CREB changes in the FC, but not in the CPu, might originate from a specific modulation of the Ca^{2+}-dependent CaMK signaling in that region. Interestingly, recent findings indicate that, among the various mGlu receptors, the group II mGluR subtypes mGluR2 and mGluR3 play an important role in the modulation of spontaneous Ca^{2+} spikes (Koga et al. 2010).

Although speculative, the hypothesis that LY379268-induced changes in CaMKII signalling in the FC may be responsible of its ability to alleviate (R)-CPP-induced p-CREB changes, is supported by the findings that antipsychotic drugs such as haloperidol, clozapine and risperidone significantly impact the calcium second messenger system as shown by in-situ hybridization studies where these drugs selectively increased mRNA isoform II (but not isoforms I and III) of CaMK (Lee et al. 2007; Rushlow et al. 2009).

Interestingly, CaMKII is known to regulate GLU receptors as it induces the phosphorylation of the channel domain of the NMDA receptor/channel in the postsynaptic densities thus causing an enhancement of Ca^{2+} influx through the channel (Kitamura et al, 1993).

Moreover, CaMK can regulate PKA and others signaling pathways, such as PKB/Akt and MAPK, which are also affected by haloperidol and clozapine (Dwivedi and Pandey 1999; Emamian et al 2004; Pozzi et al 2003; Soderling 1999).

In conclusion, although, the exact mechanism by which LY379268 reversed the effects of (R)-CPP on cortical p-CREB remains to be identified, these data shown that is sufficient to restore levels of p-CREB in the FC to reduce the attention deficits induced by (R)-CPP as the effects of LY379268 were restricted to the FC.

LY379268 prevent (R)-CPP-induced GLU efflux in the FC

Intracortical perfusion of (R)-CPP significantly increased extracellular GLU in the mPFC consistently to previous experiments in my laboratory (Ceglia et al. 2004). The intracortical concentration of (R)-CPP that significantly increased extracellular

GLU (i.e. 100 μM) fit well with those used in vitro and in vivo to selectively block

NMDAR (Lehmann et al. 1987; Del Arco and Mora 2002). The effect of (R)-CPP is

mimicked by intracortical infusion of the competitive NMDAR antagonist AP7

(Ceglia et al. 2004; Perkins et al. 1982) and (RS)-CPP (Abekawa et al. 2006).

However, systemic but not intracortical non-competitive NMDAR antagonists such

as ketamine (Lorrain et al. 2003b) and MK-801 (Lopez-Gil et al. 2007) increased

cortical GLU efflux. Although it is not known whether the different mode of

blocking NMDAR or other factors may account for these discrepancies, these

results indicate that selective blockade of NMDAR in the mPFC is sufficient to

increase extracellular GLU in this brain region.

As discussed in the General Introduction (Chapter 1) it has been suggested that the

apparently paradoxical effect of NMDAR antagonist on GLU release depends on

the inhibition of NMDAR predominantly on the fast spiking putative GABA

interneurons that in turn decrease the inhibitory drive on PFC pyramidal output

neurons (Homayoun and Moghaddam 2007). Indeed, NMDAR antagonists,

including (R)-CPP, reduced extracellular GABA in the mPFC (Calcagno et al.

2009; Yonezawa et al. 1998) and induced cortical excitation in humans (Breier et al.

1997; Holcomb et al. 2005; Vollenweider et al. 1997) and in behaving animals

(Gozzi et al. 2008b). Accordingly, the $GABA_A$ receptor antagonist bicuculline

injected in the PFC of monkey has been shown to disrupt the performance of a

delayed response task (Sawaguchi et al. 1988) and loss of spatial tuning in neurons

engaged by working memory (Rao et al. 2000).

The mGluR2/3 are primarily distributed in forebrain regions (Petralia et al. 1996) and autoradiographic studies using H^3-LY354740 have shown a substantial binding within layer V of the mPFC (Marek et al. 2001; Marek et al. 2000) where they are associated with presynaptic membranes (Ohishi et al. 1994; Petralia et al. 1996). Their activation mediates presynaptic depression and inhibits electrically evoked GLU release both in vivo and in vitro (Cartmell and Schoepp 2000; Marek et al. 2000). LY379268 which by itself had no effect on GLU efflux prevented the (R)-CPP-evoked GLU release in the PFC. The data are consistent with previous reports showing that activation of cortical pre-synaptic mGluR2/3 by LY354740 or LY379268 reduces PCP- or ketamine-induced GLU efflux (Lorrain et al. 2003b; Moghaddam and Adams 1998) and MK801-induced changes in firing rate of pyramidal mPFC neurons (Homayoun et al. 2005).

A possible limitation is the difference in the (R)-CPP administration between behavioural and microdialysis experiments. As in behavioural experiments (R)-CPP was injected into the mPFC for 1 min while in microdialysis experiment (R)-CPP was continuously infused through the probe for 60 min. Thus it may be argued that the two conditions are not comparable. However, we found that the injection of (R)-CPP at the same dose (i.e. 50 ng/μL) and route of administration (i.e. intra mPFC) used in the 5-CSRT task, induced a rapid increase of extracellular GLU which was similar in magnitude to that observed after its infusion through the probe (Calcagno et al. 2009).

LY379268 mimics the effects of the 5-HT$_{2A}$ receptor antagonist M100907 which prevented the NMDAR antagonists-induced impairments in attention performance, the increase in GLU efflux in the PFC and the changes in p-CREB (Ceglia et al. 2004; Mirjana et al. 2004; Pozzi et al. 2010). This is consistent with previous findings showing that the 5-HT-evoked excitatory post-synaptic currents were enhanced by the mGluR2/3 antagonist LY341495 and inhibited by agonists (1S,3S)-ACPD and LY354740 and by 5-HT$_{2A}$ receptor antagonist M100907 (Aghajanian and Marek 2000; Marek et al. 2000). Additionally, activation of 5-HT$_{2A}$ receptors by DOI increased excitatory postsynaptic currents and potentials, GLU release (Scruggs et al. 2003), activated c-*fos* in PFC and induced a behavioural syndrome characterized by head twitches. All these effects were blocked by LY354740 and LY379268 (Aghajanian and Marek 2000; Gewirtz and Marek 2000; Klodzinska et al. 2002; Zhai et al. 2003).

The interaction between 5-HT$_{2A}$ and mGlu$_{2/3}$ receptors might reside in their co-localization on the apical dendrites of neocortical layer V pyramidal cells (Aghajanian and Marek 1999) rich in 5-HT terminals and 5-HT$_{2A}$ receptors (Aghajanian and Marek 1997; Blue et al. 1988). This functional analogy may also derive from the mGluR2 interaction through the specific transmembrane helix domain with the 5-HT$_{2A}$ receptor (Gonzalez-Maeso et al. 2008). This neuronal location is opposed to that found in other cortical regions such as fronto-parietal where mGlu2/3 and 5-HT$_{2A}$ binding appears to localize to different laminas (II-IV and Va, respectively) (Marek et al., 2000). Thus, mGluR2/3 receptors may play an important role in the integration of synaptic activity. These findings are of special

significance in light of a recent phase II clinical trial showing some efficacy of an mGlu2/3 agonist in treatment of schizophrenia (Patil et al. 2007).

In conclusion, by behavioural, biochemical and neurochemical studies I showed that (R)-CPP-induced cognitive deficits in the 5-CSRT task were associated with changes in p-CREB levels and increased GLU release in the FC. It can be hypothesised that (R)-CPP-induced increase in p-CREB might be secondary to the rise of extracellular GLU, which in turn activate non-NMDA GLU receptors such as AMPA or mGlu positively coupled to CREB phosphorylation (Choe and Wang, 2002; Perkinton et al., 1999; Uslaner et al., 2009). Thus, LY379268 by suppressing GLU release may have prevented the changes in p-CREB levels with the possible involvement of CaMKII and PKA cascades (see scheme in Fig. 4.9).

Thus, the evidences that LY379268 has attention enhancing effect and modulates p-CREB and GLU efflux in the FC may contribute to an emerging understanding of the molecular mechanisms that regulate different aspects of the executive functions such as attention and response control but also in cognitive deficits dependent on NMDA receptor hypofunction in the PFC. They also point to the potential that novel approaches aiming at molecular mechanisms to modify CREB phosphorylation in the FC may lead to a new generation of treatments for cognitive disorders of schizophrenia.
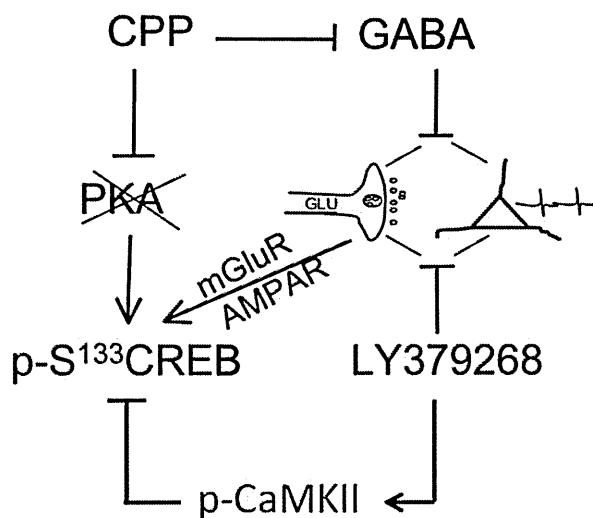
Fig 4.9
Putative mechanisms involved in (R)-CPP-induced rise of p-CREB levels in the FC and its attenuation by the activation of mGlu2/3 receptor with LY379268.

The effect of (R)-CPP could reflect the increase of glutamate (GLU) release and cortical pyramidal cells firing which in turn stimuluates CREB phosphorylation presumably through the activation of glutamatergic non-NMDAR such as mGlu and AMPA.

By suppressing the effect of (R)-CPP on extracellular GLU and cellular activity or by phosphorylating CaMKII, LY379268 may prevent CREB phosphorylation on S^{133}.

A role of PKA in (R)-CPP effects on frontocortical p-S^{133}CREB is unlikely as the reduction in p-T^{197}PKA levels is not compatible with enhanced p-S^{133}CREB. However, PKA may play a major role in (R)-CPP-induced reduction of p-S^{133}CREB in the CPu. ($\rightarrow$) Activation; ($\top$) Inhibition; (X) Unlikely pathway.

CHAPTER 5

EFFECTS OF Sp-cAMP ON

(R)-CPP-INDUCED ACCURACY DEFICITS

IN THE 5-CSRT TASK

There is substantial evidence that phosphorylation of CREB on S133 might have a role in the GLU NMDAR antagonist-induced inattention and executive function deficits. In the previous chapters (Chapters 3 and 4) I have reported that local blockade of cortical NMDAR by intra-mPFC administration of the competitive antagonist (R)-CPP, at the dose that impair attentional functioning and response control in the 5-CSRT task (Mirjana et al. 2004), increased and decreased p-CREB levels in the FC and decreased it in the CPu of behaviorally naïve rats compared to controls.

Moreover, in behaviourally naïve mice, acute systemic administration of PCP and MK-801, increased levels of p-CREB both in the FC and CPu, an effect that has been associated with impairments in pre-pulse inhibition and hyperactivity (Svenningsson et al. 2003). Similarly, I found that PCP- and MK-801-induced attention performance deficits in the 5-CSRT task were associated with increased p-CREB level in the CPu of mice (Pozzi et al. 2010), indicating a likely role of p-CREB in the control of attention and executive functions.

Recently, it has been shown that preventing CREB phosphorylation on S133 by viral-vector gene transfer in the PFC, accuracy in the 5-CSRT task was impaired (Paine et al. 2009). This latter finding contrasts in part with the evidences that behavioural deficits induced in the same task by (R)-CPP, PCP and MK-801 were associated to increased p-CREB levels in the FC (this thesis; Pozzi et al. 2010; Svenningsson et al. 2003). However, in the study by Paine et al. (2009) inhibition of CREB phosphorylation was made in rats performing the behavioural task while the effects of (R)-CPP, PCP and MK-801 on p-CREB were examined in rats that were behaviorally naïve (Pozzi et al. 2010;

Svenningsson et al. 2003). Thus it could not be excluded that the effects of NMDAR

antagonists on p-CREB levels depend on whether or not rats are performing the task.

5.1 EFFECTS OF (R)-CPP ON p-CREB IN RATS PERFORMING THE TASK

To address the possibility that (R)-CPP-induced p-CREB changes might depend on the behavioral status of the rats, the next experiment was settled out to evaluate p-CREB levels in the FC, CPu and NAC of trained rats (rats infused with (R)-CPP and performing the 5-CSRT task) compared to (R)-CPP-induced p-CREB changes in untrained rats (rats habituated to the testing chambers but not performing the task). Two experimental manipulations (i.e. behavioural status and drug treatment) lead to four experimental groups: untrained+vehicle; untrained+CPP; trained+vehicle and trained+CPP. The timeline of this experiment is represented below.

A. TRAINED
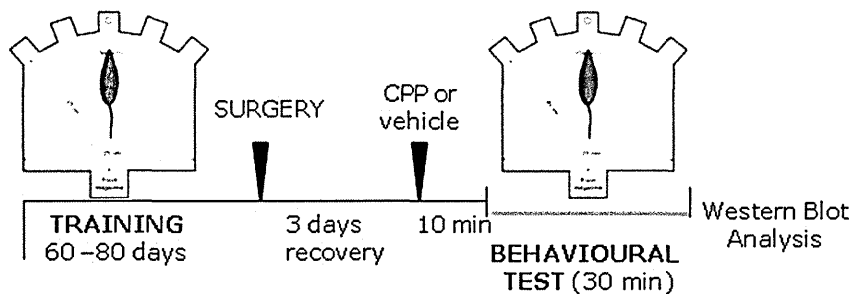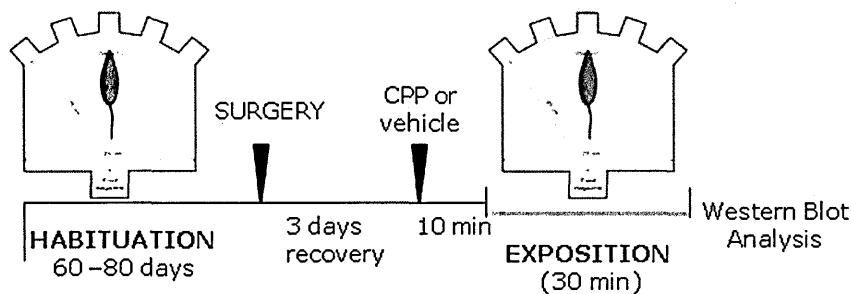
| TRAINING | SURGERY | CPP or vehicle | BEHAVIOURAL | Western Blot |
| 60–80 days | 3 days recovery | 10 min | TEST (30 min) | Analysis |

B. UNTRAINED

| HABITUATION | SURGERY | CPP or vehicle | EXPOSITION | Western Blot |
| 60–80 days | 3 days recovery | 10 min | (30 min) | Analysis |

A comprehensive description of experimental procedures is reported in General Methods (Chapter 2). Briefly, after two weeks of food deprivation (when the body weight had stabilized to about 85-90% of their initial weight) rats were divided in two groups. One group of rats was trained in the 5-CSRT task until they reached a baseline performance of 80% correct responses (**A**), while the other group was kept food-deprived and habituated to the testing chamber (**B**). Then they were bilaterally implanted with stainless steel cannulae in the mPFC and had 3 days to recover from surgery. On the days of the experiment (R)-CPP or vehicle was infused in the mPFC as described previously. Ten min after (R)-CPP infusion, trained rats started the behavioural session that last for 30 min while untrained rats were exposed to the behavioural box for 30 min. Forty minutes after (R)-CPP infusion both groups of rats were sacrificed and their brains dissected for western blot analysis of p-CREB and T-CREB levels in the FC, CPu and NAC.

RESULTS

Confirming my previous findings with behaviourally naïve rats, figure 5.1 (A) shows that intra-mPFC infusion of 50 ng/µL (R)-CPP increased p-CREB in the FC in untrained rats ($P<0.05$, versus V, Student's t-test) but decreased p-CREB in trained rats ($P<0.05$, versus V; Student's t-test). As shown in figure. 5.1 (B), levels of p-CREB were increased in the CPu of untrained rats but this increase was not statistically significant ($P>0.05$, versus V; Student's t-test). p-CREB levels in the CPu of trained rats were not affected by R-CPP ($P>0.05$, versus V; Student's t-test). Interestingly, p-CREB levels in the NAC of trained rats were strongly increased ($P<0.05$, versus V; Student's t-test) compared to no effect in untrained rats (Fig. 5.1; C).

Figure 5.2 shows that (R)-CPP had no effect on T-CREB levels in the FC (**A**), CPu (**B**) and NAC (**C**) of untrained rats (all $P>0.05$, versus V; Student's t-test). In rats performing the task (trained) (R)-CPP decreased T-CREB levels in the FC (Fig. 5.2 **A**), but increased it in the NAC (Fig. 5.2 **C**) (both $P<0.05$, versus V ; Student's t-test). No effects were detected in the CPu (Fig. 5.2 **B**) of trained rats ($P>0.05$, versus V ; Student's t-test).

A. FC

B. CPu

p-CREB
- ■ V
- ▨ CPP

C. NAC
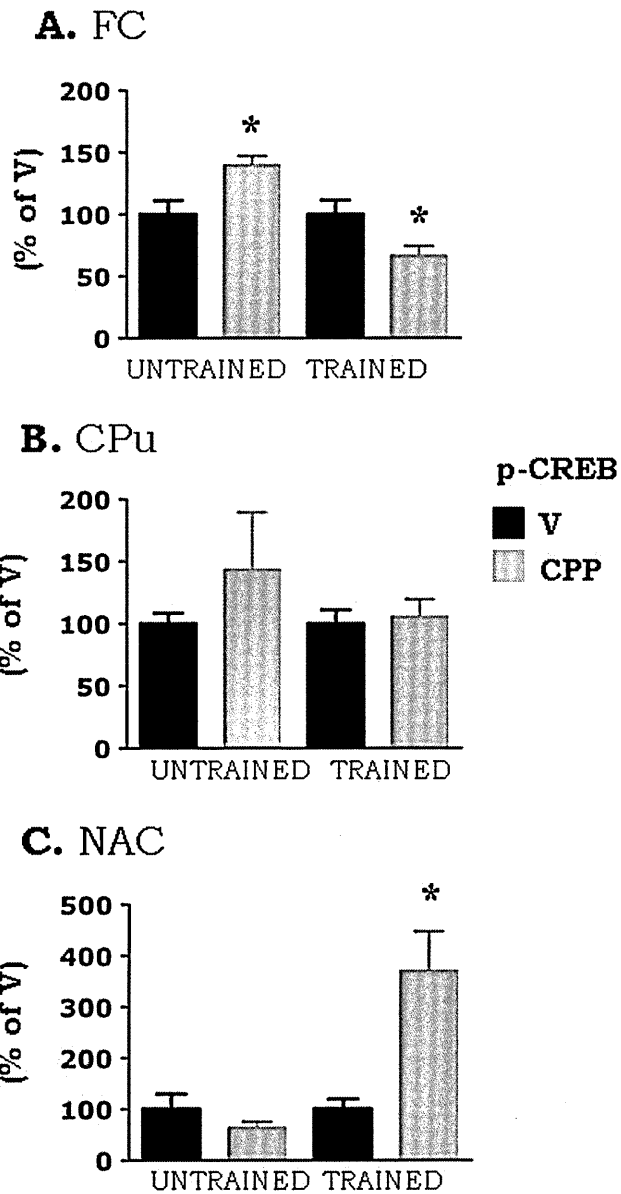
Fig 5.1

Quantification of p-CREB levels in untrained and trained rats

Histograms depict quantitative effects of saline (V) or (R)-CPP (CPP) on p-CREB levels in the FC, CPu and NAC in untrained and trained rats. Saline or CPP were bilaterally infused in the mPFC and rats sacrificed 40 min later. Data are expressed as the percentage of controls (V). The histograms show mean ± S.E.M. of 5-7 rats.

*P<0.05 vs V (Student's t-test).

A. FC

B. CPu

C. NAC
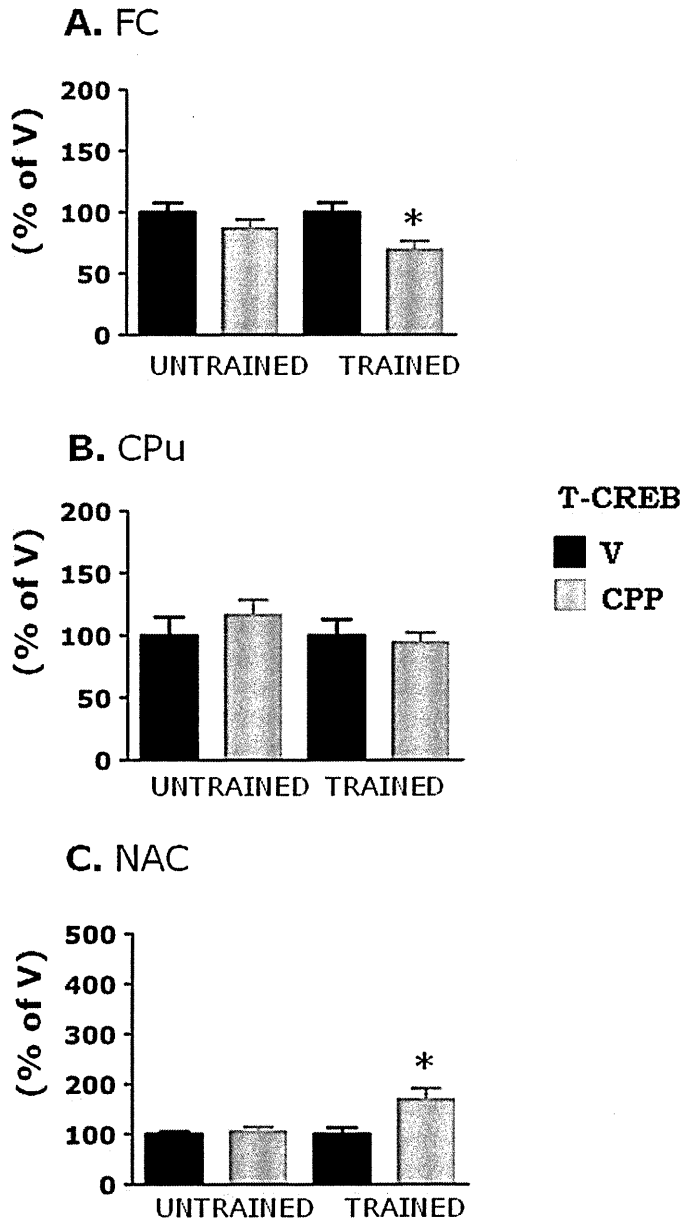
T-CREB
■ V
▨ CPP

5.2
Quantification of T-CREB levels in untrained and trained rats
Histograms depict quantitative effects of saline (V) or (R)-CPP (CPP) on T-CREB levels in the FC, CPu and NAC in untrained and trained rats. Saline or CPP were bilaterally infused in the mPFC and rats sacrificed 40 min later. Data are expressed as the percentage of controls (V). The histograms show mean ± S.E.M. of 5-7 rats.
*P<0.05 vs V (Student's t-test).

5.2 EFFECT OF INTRACORTICAL INFUSION OF Sp-cAMP ON p-CREB LEVELS

In the previous experiment I found that in contrast to increasing p-CREB levels in untrained or behaviourally naïve rats (Chapters 3 and 4) (R)-CPP decreased levels of p-CREB in the FC of rats performing the 5-CSRT task. This finding led to the hypothesis that increasing p-CREB levels in this region might be sufficient to prevent (R)-CPP-induced attention deficits.

As discussed in the General Introduction (Chapter 1), phosphorylation of residue S133 on CREB protein can be achieved by the intervention of various protein kinases. One prominent and well-characterized mechanism is activation of adenylate cyclase, which increases production of cyclic adenosine 3',5'-monophosphate (cAMP).

cAMP can have intracellular actions activating various proteins like EPAC (Exchange Protein Activated by cAMP; Seino and Shibasaki 2005), MAP kinases and the PKB pathway (Pearson and Cobb 2002; Hansen et al. 1999; Frodin et al. 1994) that might influence CREB activity. Moreover, cAMP induces the release of Ca^{2+} that in turn may stimulate CaMK and PKC (Sheng et al. 1990) but also it activates cAMP-dependent protein kinase (PKA) (Greengard et al. 1999). PKA can in turn phosphorylate many intracellular proteins and translocate into the nucleus where it phosphorylates CREB.

Many compound have been developed that interact with this signaling pathway. Rolipram increases cAMP and PKA activity by inhibiting the phosphodiesterase type IV enzyme (PDE4), preventing the breackdown of cAMP. A different drug, Sp-adenosine-3',5'-cyclic monophosphorothioate (Sp-cAMP), acts as a competitive agonist of the cyclic nucleotide–binding domains on PKA. It mimics the effects of cAMP, including activation of PKA and PKA-dependent intracellular cascades (Van Haastert et al. 1984; Punch et al.

1997; Huang and Kandel 1998; Self et al. 1998). Downstream consequences of PKA activation by Sp-cAMP include phosphorylation of CREB on S133 (Carlezon et al. 2005). Its enatiomer, Rp-cAMP, inhibits cAMP actions and PKA activity (Fig. 5.3).

Compared to other cAMP analogues (i.e. dibutyryl-cAMP or 8-Br-cAMP), Sp-cAMPS is a cell permeable, potent and specific activator of PKA and has a higher resistance against cyclic nucleotide-dependent phosphodiesterases (PDEs) (Fig 5.3).

Fig. 5.3
Schematic drawn showing the compounds frequently used to modulate cAMP–PKA activity
(Arnsten et al. 2005)
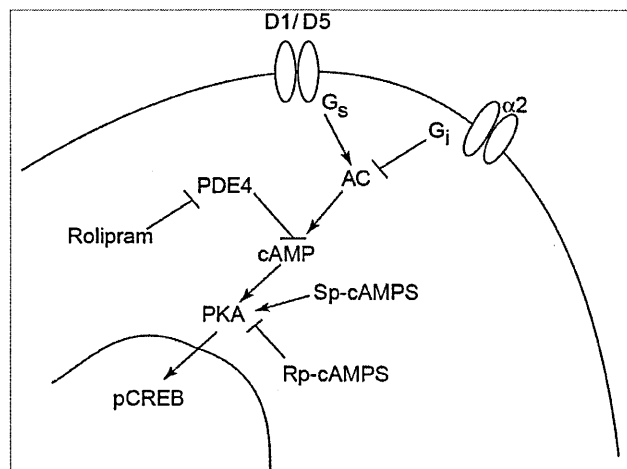
Sp-cAMP acts downstream of cAMP and its activity is independent from levels of cAMP in the cell as it binds directly to the PKA regulatory subunits ("R" in Fig 5.4). This avoids non-specific effects of cAMP on other protein kinases and ion channels. Thus, by specifically increasing the activity of PKA, Sp-cAMP represents a unique tool for the activation of S^{133}-CREB phosphorylation *in vivo*.

Fig.5.4
Mechanisms of actions of Sp-cAMP
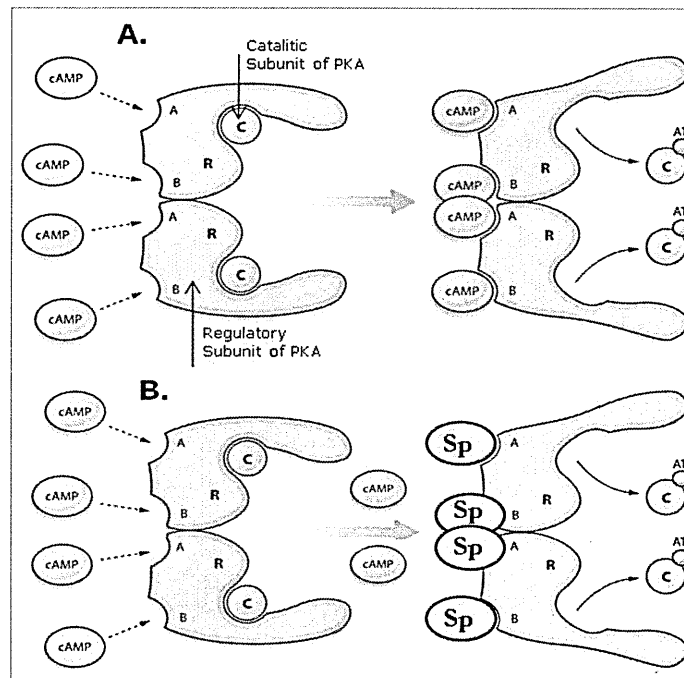
A. In its inactive state, PKA consists of a tetramer of two regulatory (R) and two catalytic (C) subunits. Each regulatory subunit has two cAMP-binding sites; cAMP binding releases the catalytic subunits, which become bound to ATP and go on to phosphorylate serine and threonine residues on the substrate sequence.
B. Binding of Sp-cAMP (Sp) to PKA release the two catalytic subunits (C) which become bound to ATP and go on to phosphorylate serine and threonine residues on the substrate sequence.

Consistent literature indicates that the intracerebral administration of Sp-cAMP impaired some types of cognitive performance. For example, post-training infusions of Sp-cAMP into the mPFC markedly impaired delayed alternation performance in rats (Taylor et al. 1999); impaired cortical function in both rats and monkeys (Ramos et al. 2003), and exacerbated working-memory deficits in aged rats (Arnsten et al. 2005). Moreover, the study of Paine et al, (2009) showed that the post-training infusion of Sp-cAMP into the

mPFC had no effect on accuracy but it did increase omissions in rats performing the 5-CSRT task (Paine et al. 2009), while Rp-cAMP had the opposite effect.

As Sp-cAMP do not penetrate the blood-brain barrier when given systemically, the compound was injected directly into the mPFC. Therefore, I determined the dose response effects and the time course of intracortically infused Sp-cAMP on p-CREB levels in the FC, CPu and NAC of rats. The timeline of this experiment is reported below.
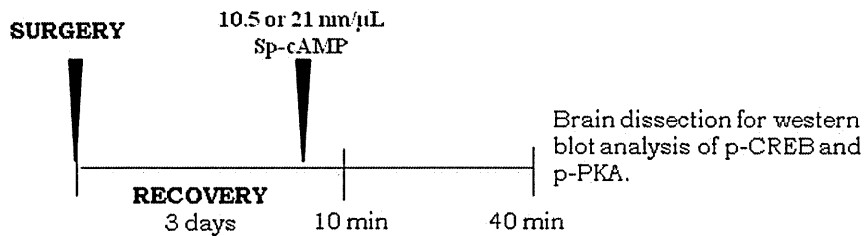
Briefly, rats were implanted monolaterally with stainless steel cannulae in the mPFC and had 3 days to recover from surgery. On the experimental day rats were gently held and the infusion needle was inserted into the cannula after removing the stylet.

Rats received saline (0.5 µL) or Sp-cAMP at 10.5 or 21 nmol dissolved in 0.5 µL saline in the mPFC. Sp-cAMP and vehicle were delivered at a rate of 0.5 µL/min. Injection unit was left in place for 1 min after infusion to allow for drug diffusion. Ten or 40 min after Sp-cAMP or saline rats were killed by decapitation. The FC, CPu and the NAC from both hemispheres were rapidly dissected out, frozen on dry ice and stored at −70 °C for western blot analysis. The non-injected hemisphere was used as the control.

RESULTS

Effect of Sp-cAMP on p-CREB levels

Figure 5.5 shows the effects of intracortical infusion of Sp-cAMP on p-CREB levels 10 (*left*) and 40 (*right*) min after infusion as measured by western blot. I found that 21 nmol/0.5 µL Sp-cAMP increased p-CREB levels in the FC (**A**) and CPu (**B**) (both $P < 0.05$ compared to V; Dunnett's test). P-CREB levels were decreased in the NAC 10 min after 21 nmol Sp-cAMP (**C**) ($P > 0.05$ compared to V; Dunnett's test). At 10.5 nmol Sp-cAMP had no effect on p-CREB in the FC (**A**), CPu (**B**) and NAC (**C**) 10 min after injection (all $P > 0.05$ compared to V; Dunnett's test).

Forty min (*right*) after the infusion of 21 nmol of Sp-cAMP, p-CREB levels were still increased in the FC (**A**) ($P < 0.05$ compared to V; Dunnett's test), while in the CPu (**B**) and in NAC (**C**) p-CREB returned to basal levels (both $P > 0.05$ compared to V; Dunnett's test). Lower doses of Sp-cAMP were not tested.

The effects of Sp-cAMP on cortical p-CREB levels were restricted to the infused hemisphere as in the FC taken from the contralateral hemisphere p-CREB levels remained unchanged (not shown).

A. p-CREB FC

B. p-CREB CPu

C. p-CREB NAC

10 min   **40 min POST-INFUSION**
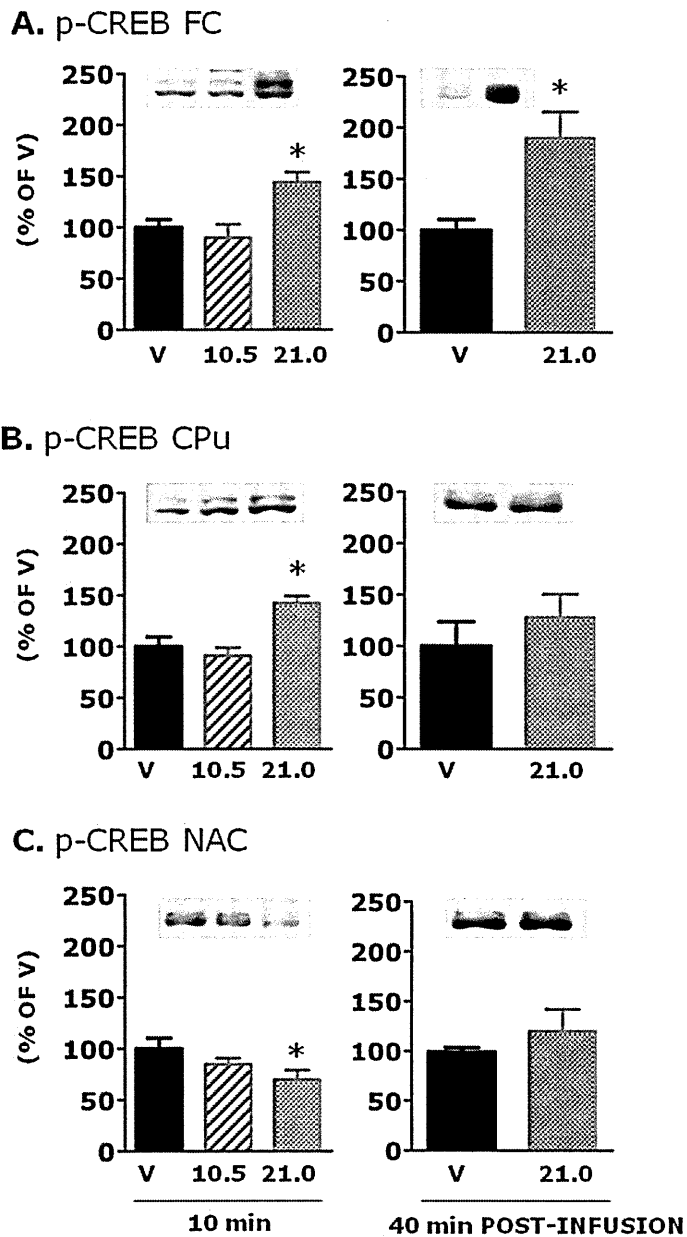
Fig. 5.5
Effects of Sp-cAMP on p-CREB levels.
Representative immunoblots and quantification of p-CREB levels in the FC (**A**), CPu (**B**) and NAC (**C**) of rats infused with vehicle (V), 10.5 or 21 nmol/0.5 μL Sp-cAMP in the mPFC. Histograms represent mean±S.E.M. of 5-6 rats per group expressed as percentage of controls (V). *P<0.05 vs V (Dunnett's test and Student's t-test for 10 and 40 min, respectively).

Effect of Sp-cAMP on p-PKAc levels in the FC

As Sp-cAMP is supposed to increase the phosphorylation of CREB on S133 by increasing the availability of the catalytic (c) subunits of PKA, I measured levels of PKAc phosphorylated on threonine T^{197} in the same samples of the FC used to assess the effects of Sp-cAMP on p-CREB.

Forty minutes after infusion of 21 nmol Sp-cAMP in the mPFC, levels of p-PKAc were significantly increased compared to controls (V=100±2.5 and Sp-cAMP=150±26, P<0.05; Student's t-test) (Fig.5.6).
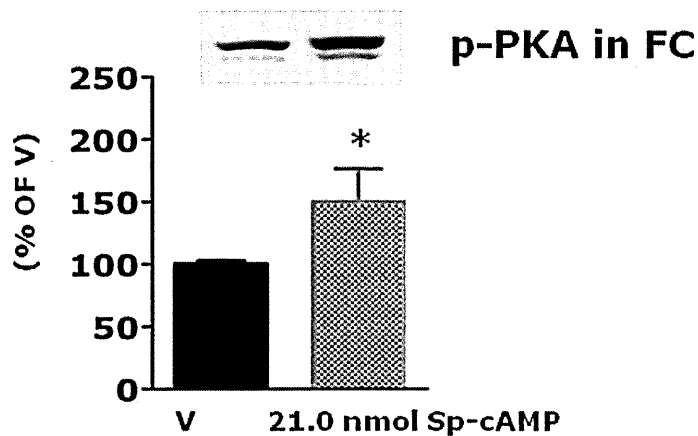
Fig 5.6
Effects of Sp-cAMP on p-PKAc levels.
Quantification of p-T^{197}PKAc levels in the FC of rats infused with vehicle (V) or 21 nmol/0.5 µL Sp-cAMP in the mPFC. Rats were sacrificed 40 min later for western blot analysis.
Data are expressed as percentage of controls (V). Histograms represent mean ± S.E.M. of 4 rats. *P<0.05 vs V (Student's t-test).

The results of this experiment clearly indicated that 21 nmol Sp-cAMP significantly increased p-CREB and p-PKAc levels in the FC. Therefore, this dose was selected for the behavioural experiment.

5.3 EFFECTS OF Sp-cAMP ON (R)-CPP-INDUCED ATTENTION DEFICITS

I showed that in the FC of rats performing the task, (R)-CPP decreased p-CREB levels as opposed to the increase found in the FC of behaviourally naïve rats at the same time point (i.e. 40 min after (R)-CPP) indicating that the (R)-CPP-induced behavioural impairment in rats performing the 5-CSRT task might be associated to low levels of p-CREB in this region. Thus it might be hypothesized that increasing the levels of cortical p-CREB by Sp-cAMP might prevent the behavioural deficit induced by (R)-CPP.

The timeline of this experiment is reported below.
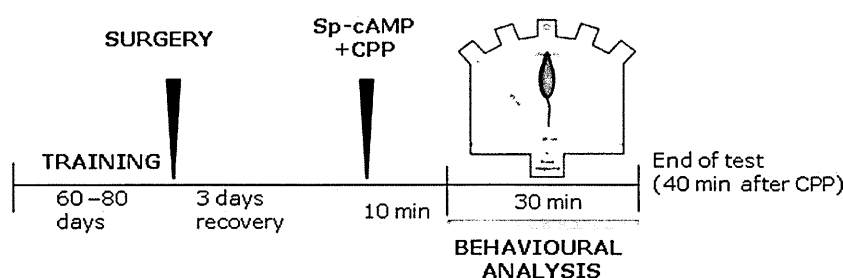
Briefly, on each experimental day rats trained to perform the task received a bilateral infusion of 0.5 µL vehicle or 21 nmol/0.5 µL side Sp-cAMP into the mPFC and after 2 min they received an additional infusion of 1 µL PBS or 50 ng/µL side (R)-CPP. Ten minutes after (R)-CPP infusion rats were put into the boxes and the test session started. Each rat received all drugs combinations 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 the lasting effects of drugs.

RESULTS

Sp-cAMP prevents (R)-CPP-induced impairments in the 5-CSRT task

Fig. 5.7 shows that (R)-CPP (V+C) injected in the mPFC decreased the percentage of correct responses **(A)** and increased anticipatory responding **(B)**. In contrast to the previously published reports (but see also Chapter 4), in this experiment (R)-CPP only marginally increased perseveratives responses **(C)** and the percentage of omissions **(D)**. Sp-cAMP reversed the (R)-CPP-induced impairment in accuracy **(A)** and the increase in anticipatory responding **(B)**.

A two-way within-subject ANOVA performed on accuracy (% correct response) showed a significant interaction between Sp-cAMP and (R)-CPP ($F_{1,21}=13.9$, P<0.01) **(A)**. Post-hoc comparisons of treatments means showed that (R)-CPP decreased accuracy (V+C versus V+V; P<0.05) and that rats receiving Sp-cAMP+CPP had higher accuracy than rats injected with (R)-CPP alone (Sp+C versus V+C). Sp-cAMP alone had no effect on accuracy (comparison Sp+V versus V+V, P>0.05) **(A)**.

A two-way ANOVA showed a significant effect of (R)-CPP on the number of anticipatory responses (V+C versus V+V; P<0.05). This increase was prevented by Sp-cAMP as indicated by the significant interaction between Sp-cAMP and (R)-CPP ($F_{1,21}=7.13$, P<0.05) **(B)**. On its own Sp-cAMP had no effect on anticipatory responses (Sp+V versus V+V; P>0.05) **(B)**.

There was no significant main effect of Sp-cAMP ($F_{1,21}$=0.07, P>0.05) or (R)-CPP ($F_{1,21}$=0.02, P>0.05) or interaction between Sp-cAMP and R-CPP ($F_{1,21}$=3.25, P>0.05) for perseverative responses (**C**) indicating that Sp-cAMP and (R)-CPP alone or in combination had no effect on perseverative responding (both P>0.05; V+C and Sp+V versus V+V) (**C**).

Statistical analysis on the percentage of omissions (**D**) showed a significant interaction between Sp-cAMP and (R)-CPP ($F_{1,21}$=13.3, P<0.01) and main effects of Sp-cAMP ($F_{1,21}$=0.25, P>0.05) and (R)-CPP ($F_{1,21}$=0.25, P>0.05). The proportion of omissions was increased by (R)-CPP (even if it did not reach statistical significance) (V+CPP versus V+V; P>0.05) and Sp-cAMP (Sp+V versus V+V; P<0.05), when they were administered together their effect on omissions was not more apparent (**D**).
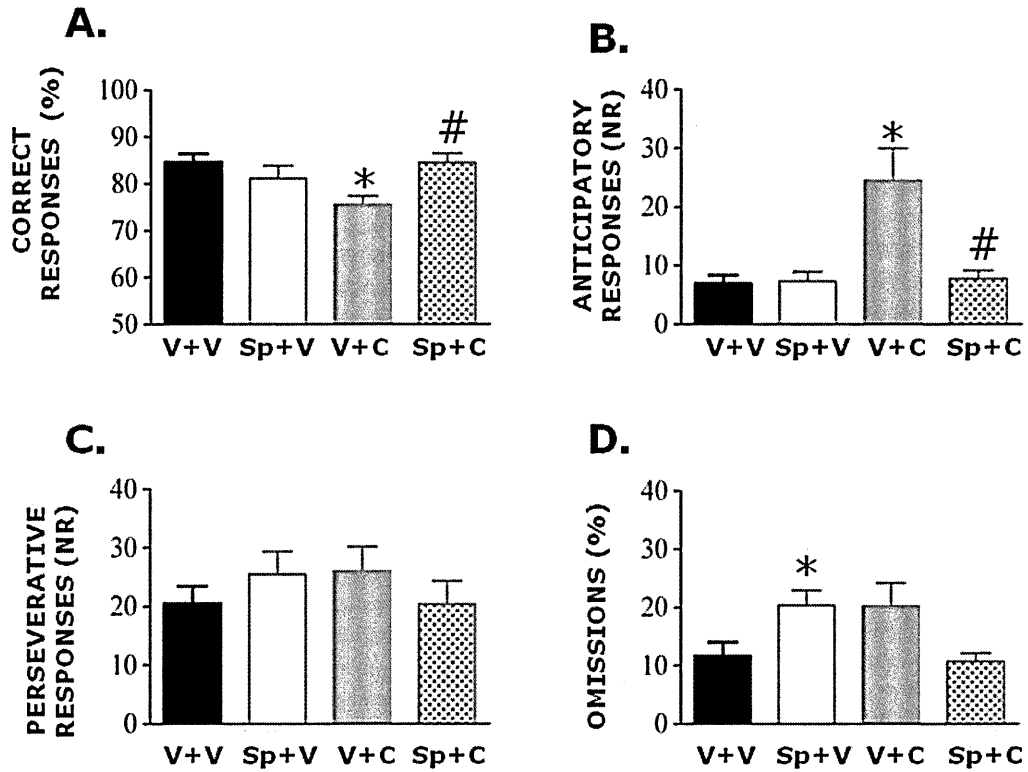
Fig 5.7
Effects of Sp-cAMP on (R)-CPP-induced performance deficit in the 5-CSRT task.
Histograms represent the mean±S.E.M (n=8 rats per group) of correct responses (A), the number of anticipatory (B), perseverative (C) and omissions responses (D).
Rats were intracortical infused with vehicle (V) or 21 nmol Sp-cAMP (Sp) 2 min before bilateral infusion of 1 µL of V or 50 ng/µL side (R)-CPP (C). Ten min later rats started the test session. Vehicle, (R)-CPP and Sp-cAMP in combination were administered according to a Latin-square design. Test sessions were at least 48 h apart. * P<0.05 vs. V+V; # P<0.05 vs. V+C; (Tukey's test).

5.4 DISCUSSION

The main findings of these series of experiments were that (R)-CPP had different effects on p-CREB levels in the FC and NAC depending whether or not the rats were performing the 5-CSRT task. In the FC of untrained rats p-CREB levels were increased whereas in rats performing the task levels of p-CREB in the FC were decreased. Remarkably only in rats performing the 5-CSRT task (R)-CPP increased the levels of p-CREB in the NAC.

The fact that intracortical infusion of the cAMP analogue Sp-cAMP, which increased p-CREB and p-PKAc levels in the FC, prevented (R)-CPP-induced deficits in accuracy and the increase in anticipatory responses in rats performing the 5-CSRT task, gives support to the hypothesis that p-CREB mechanisms within the FC may contribute to selective attention.

Effect of (R)-CPP in rats performing the 5-CSRT task

In the FC of rats performing the task (R)-CPP decreased p-CREB as opposed to the increase found in the FC of behaviourally naïve rats at the same time point (i.e. 40 min after (R)-CPP) indicating that the impaired attention performance on the 5-CSRT task might be associated to low levels of p-CREB in this region. These results are in accordance with those of Paine et al. (2009) who showed that reducing p-CREB levels in the FC cause attention deficits in the 5-CSRT task and with the evidences that regions of the FC such as prefrontal (PFC) and orbitofrontal (OFC) are critical for attention but also for aspects of executive control over performance such as impulsivity and compulsivity (Birrell and Brown 2000; Chudasama et al. 2003; Muir et al. 1996a; Passetti et al. 2002; Sun et al. 2010).

That low levels of p-CREB might contribute to impaired attention and aspects of executive control over performance is supported by data showing that Spontaneously Hypertensive Rats (SHR) have lower levels of p-CREB in the FC and lower accuracy in the 5-CSRT task compared to normotensive Wistar Kyoto Rats (WKY; Pozzi unpublished).

Impulsivity induced by (R)-CPP was also decreased by Sp-cAMP suggesting that in addition to attention, decreased p-CREB in the FC may contribute to impulsivity. However, these findings are in contrast with those reported recently by Sun et al. (2010) who found that over-expressing CREB protein within the OFC increased impulsivity. However, it should be noted that Sun et al. (2010) used a simplified version of the 5-CSRT, one-choice task, which places differential emphasis on attention and response control compared to the 5-CSRT task used presently. It could be argued that differences in the emphasis on response control or the cognitive processes engaged by the task may explain the observed differences in p-CREB.

Interestingly level of T-CREB were also decreased after (R)-CPP in the FC of rats performing the task. This finding was surprising since in behaviourally naïve rats we never found any change in total levels of CREB (this thesis). However, it could not be excluded that increased T-CREB in the FC might be due to extensive training itself or the actual performance on the 5-CSRT task when compared to untrained controls.

I found that (R)-CPP significantly decreased p-CREB in the CPu of naïve rats (Chapters 3 and 4). However, in the present experiment the same dose of (R)-CPP (50 ng/µL) had no effect on p-CREB in the CPu of rats that were either performing or not performing the

task. This result suggest that p-CREB in this region might have a minor role in the attentional deficits induced by (R)-CPP.

Whether this lack of effect might depend on the experimental procedures employed in the two different experiments is at present unknow. Indeed, compared to behaviourally naive rats used in the previous experiments the untrained control used here were food-deprived, manipulated and exposed to the testing chamber for few days.

While (R)-CPP had no effects on p-CREB in the NAC of untrained rats (see Chapters 3 and 4), it strongly increased p-CREB levels in the NAC of rats performing the task. These data are in accordance with findings showing that cortico-striatal projections to NAC importantly contribute to affective modulation of attentional performance (Christakou et al. 2004).

It has been reported that food restriction increased NMDAR-dependent CREB phosphorylation mediated by CaMKII and ERK1/2 in the NAC (Haberny and Carr, 2005). However, it's unlikely that the food restriction regimen used in this experiment might have induced specific changes in (R)-CPP-induced CREB phosphorylation in the NAC because both untrained and trained rats were food-deprived for the same period. Table 5.1 summarized the results.

	Untrained+CPP			5-CSRT+ CPP		
	FC	CPu	NAC	FC	CPu	NAC
T-CREB	N.E.	N.E.	N.E.	↓	N.E.	↑
p-CREB	↑	N.E.	N.E.	↓	N.E.	↑

Table 5.1.
Effects of (R)-CPP on CREB levels in rats not performing or performing the 5-CSRT task.
Effects of intra mPFC infusion of (R)-CPP (50 ng/µL) on T-CREB and p-CREB levels in the FC, CPu and NAC of rats not performing (Untrained+CPP) or performing (5-CSRT+CPP) the behavioural task.
↓ reduction; ↑ increase; **N.E.** no effect.

Effects of Sp-cAMP on p-CREB levels in the FC, CPu and NAC

I found that the infusion of 10.5 nmol Sp-cAMP into the rat mPFC was without effects on p-CREB levels in the FC, CPu and NAC, while the higher dose of 21 nmol significantly increased p-CREB levels in the FC and CPu but reduced it in the NAC.

To my knowledge this is the first evidence that infusion of Sp-cAMP into the mPFC increased S^{133}-CREB phosphorylation in these regions. Despite the fact that Sp-cAMP has been widely used to activate S^{133}-CREB in various behavioural studies (Aujla and Beninger 2001; Paine et al. 2009; Taylor et al. 1999) no attempt has been made in these studies to document that phosphorylation of CREB on S^{133} in the target region was effectively increased by Sp-cAMP.

Opposed to the increase in p-CREB levels found in the FC and CPu, p-CREB levels in the NAC were decreased after Sp-cAMP. Sp-cAMP infused in the NAC at a high dose of 80 nmol had no effect (Misra and Pandey 2006) or increased (Self et al. 1998) p-CREB and PKAc in this area (Misra and Pandey 2006). Reciprocal regulation of cortical versus subcortical regions is well established, for example, for levels of neurotransmitters such as dopamine; therefore it is unsurprising to find evidence of reciprocal changes in molecular signals in these brain regions.

Effect of Sp-cAMP on 5-CSRT task performance

I found that Sp-cAMP by itself had no effect on accuracy but increased the number of omissions in rats performing the 5-CSRT task. These results are consistent with a recent report showing that activation of cortical PKA by Sp-cAMP (0.021, 0.21 and 2.1 nmol) did not affect accuracy but increased omission errors (Paine et al. 2009).

In the behavioural study I used 21 nmol of Sp-cAMP a dose which was ten times higher to that used by Paine et al. (2009). This dose of Sp-cAMP was selected based on previous work in rats, which had shown that 21 nmol/0.5 µL Sp-cAMP significantly impaired rat's performance in a delayed alternation performance, whereas 0.21 and 2.1 nmol/0.5 µL had no effect (Taylor et al. 1999). In a preliminary study done in my laboratory we found that intra-mPFC infusion of 2.1 and 21 nmol/0.5 µL Sp-cAMP had no effect on accuracy in the 5-CSRT task (Carli unpublished). Importantly, 21 nmol of Sp-cAMP but not a lower dose of 10.5 nmol increased both p-PKAc and p-CREB of in the FC.

Sp-cAMP prevented (R)-CPP-induced behavioural deficits

The data from the behavioural experiment showed that Sp-cAMP, at a dose that increased p-CREB and p-PKAc in the FC, prevented (R)-CPP-induced accuracy deficit and anticipatory responding in the 5-CSRT task (see summary table 5.2).

These results suggest that a decreased activity of the PKA/CREB signalling pathway might have a negative influence on attention and impulsivity. This interpretation is supported by findings showing that the PKA inhibitor Rp-cAMP disrupted accuracy in the same task (Paine et al. 2009).

5-CSRTT	Sp	CPP	Sp+CPP
ACCURACY	N.E.	↓	R
IMPULSIVITY	N.E.	↑	R
COMPULSIVITY	N.E.	N.E.	N.E.
OMISSIONS	↑	N.E.	N.E.

Table 5.2.
Effects of (R)-CPP and Sp-cAMP (Sp) alone or in combination on
5-CSRT task performance.
↓ reduction; ↑ increase; **N.E.** no effect; **R** reverted.

It is not clear whether the beneficial effects of Sp-cAMP on (R)-CPP-induced behavioural impairments are the results of an exclusive action of Sp-cAMP on CREB phosphorylation. Indeed, the functional effects of increased PKA activity is an increased neuronal excitability (Dong et al. 2006; Lopez de Armentia et al. 2007; Trantham-Davidson et al. 2004 and 2008). As a consequence, activation of PKA might enhance synaptic transmission by increasing neurotransmitter release at synapses (Chavez-Noriega and Stevens 1992). Interestingly, NMDAR-mediated excitatory postsynaptic currents

(EPSCs) are increased by activation of PKA through stimulating G-protein coupled receptors (GPCRs), by forskolin or cAMP analogs or direct intracellular administration of PKA (Cerne et al. 1993; Tong et al. 1995).

PKA phosphorylates NR_1 and NR_2 NMDAR subunits (Leonard and Hell 1997) thus, activation of PKA with Sp-cAMP may transiently increase the activity of NMDAR resulting in increased Ca^{2+} influx into the cell. This aspect is particularly interestingly in view of the ability of LY379268 (that also prevented (R)-CPP-induced behavioural deficits) to increase the phosphorylation of CaMKII in the FC (see results chapter 4).

Sp-cAMP has been also shown to potentiate GLU release from nerve terminals (Herrero and Sanchez-Prieto 1996; Wang 2002). These results suggested the presence of a PKA-dependent facilitatory pathway in the nerve terminals from the cerebral cortex that might affect GLU release. However, as (R)-CPP increased GLU release in the mPFC, a facilitatory role of Sp-cAMP on GLU release should impair rather than improve the behavioural responses induced by (R)-CPP.

The relationship between Sp-cAMP-induced increase in CREB and PKAc phosphorylation and improvement of (R)-CPP-induced behavioural deficits deserve more attention. However, the results of these experiments suggest that reduced signalling in the PKA/CREB pathway in the FC has deleterious effects on attention and aspects of control over performance.

In conclusion these data suggest that alterations in CREB signalling pathway impaired the 5-CSRT task performance in rats and that modulation of cortical PKA activities that favour phosphorylation of CREB on S133 could be beneficial.

CHAPTER 6

SUMMARY AND CONCLUDING REMARKS

6.1 Summary of Findings

Hypofunction of NMDAR has been implicated in schizophrenia (Javitt and Zukin 1991; Tsai and Coyle 2002) and in cognitive deficit associated with this disease (Krystal et al. 2003). Administration of NMDAR antagonists has become a widely used pharmacological model of schizophrenia-like cognitive deficits in experimental animals. Microinjections of the competitive NMDAR antagonist (R)-CPP into the mPFC is sufficient to induce attentional impairment and increase impulsivity and perseverative over-responding in the five choice serial reaction time (5-CSRT) task, an analogue of the continuous performance test used to study attention in humans (Mirjana et al. 2004; Murphy et al. 2005). In this thesis I report an association between the transcription factors CREB, NMDAR antagonists and performance on the 5-CSRT task.

The present thesis was built with the aim of understanding to what extent the impairments in attention and inhibitory responses control induced by blockade of glutamate NMDAR in the rat PFC may actually depend on changes in CREB phosphorylation occurring in the cortico-striatal circuitry.

In order to achieve a better comprehension of the phenomena the approach has been characterized by experiments performed both *in vivo* and *ex-vivo*, in behaviourally naive rats as well as in rats trained to perform the behavioural task.

In Chapter 3 I report that in behaviourally naive rats, (R)-CPP at the dose that impairs attentional functioning and inhibitory response control (Mirjana et al.

2004), affected p-CREB in the FC and CPu but not in the NAC (summarized in Table 6.1). These results are compatible with previous findings showing that systemic PCP and MK-801 increase p-CREB in the FC and CPu (Pozzi et al. 2010; Svenningsson et al. 2003).

I also report (Chapter 3) that (R)-CPP infused in the mPFC increased p-CREB in the orbitofrontal cortex, a result compatible with the study of Sun et al. (Sun et al. 2010) showing a positive association between impulsivity measured in the 5-CSRT task (increased anticipatory responding) and p-CREB levels in the same region.

Amongst the three major CREB's upstream kinases that were measured (i.e. p-PKA, p-ERK1/2 and p-CaMKII), I found that only p-PKA was significantly decreased by (R)-CPP within the FC and CPu (Table 6.1). However, the precise role of PKA in the (R)-CPP-induced CREB phosphorylation in the FC was not clearly established. Indeed, while the decreased levels of p-PKA correlate with decreased levels of p-CREB in the CPu, in the FC a decrease in p-PKAc was associated with increased p-CREB levels suggesting that other mechanisms participate to (R)-CPP-induced CREB phosphorylation.

The fact that phosphorylation of ERK1/2 and CaMKII was not modified by (R)-CPP was not surprising as also the PCP- and MK-801-induced p-CREB changes were independent from these kinases (Pozzi et al. 2010; Svenningsson et al. 2003).

Table 6.1.

Effect of (R)-CPP on p-CREB and p-PKA levels.

p-CREB	FC	CPu	NAC
10 min	↓	↓	N.E.
40 min	↑	↓	N.E.
p-PKAc	**FC**	**CPu**	**NAC**
10 min	↓	↓	N.E.
40 min	↓	↓	N.E.

↓ reduction; ↑ increase; **N.E.** no effect.

In Chapter 4, I report that LY379268 ameliorated accuracy and some aspects of response control such as impulsivity disrupted by (R)-CPP (results summarized in Table 6.2). These results confirm and extend our previous findings in mice (Greco et al. 2005). I also report that LY379268 significantly prevents (R)-CPP-induced changes in p-CREB in the FC, but not in the CPu (Table 6.2). Moreover, the microdialysis experiments presented in Chapter 4, confirm that disinhibition of GLU transmission in the FC could account for the behavioural impairments induced by blockade of NMDAR in the FC (Baviera et al. 2008; (Calcagno et al. 2009; Calcagno et al. 2006; Carli et al. 2006; Ceglia et al. 2004; Mirjana et al. 2004), as pretreatment with LY379268, which prevented (R)-CPP-induced behavioural impairments in the 5-CSRT task, also prevented GLU release in the same region (Table 6.2).

Table 6.2.
Effects of (R)-CPP alone or in combination with LY379268 on 5-CSRT task performance, p-CREB levels and GLU release.

5-CSRT task	CPP	LY379268+CPP
ACCURACY	↓	R
IMPULSIVITY	↑	R
COMPULSIVITY	↑	↑
OMISSIONS	↑	↑

p-CREB	FC	CPu	FC	CPu
	↑	↓	R	↓

GLU	FC	FC
	↑	R

↓ reduction; ↑ increase; **R** reversal.

Interestingly, by studiyng the p-CREB's upstream protein kinases, I found that LY379268 increased p-PKA levels in FC and CPu while levels of p-CaMKII where selectively increased in the FC. These results suggest that the ability of LY379268 to prevent the (R)-CPP-induced p-CREB changes in the FC, might involve alterations in Ca^{2+}/ CaM signals in that region (Table 6.3).

Table 6.3.
Effects of LY379268 on protein kinases levels.

LY379268	FC	CPu
p-PKAc	↑	↑
p-CaMKII	↑	N.E.

↑ increase; N.E. no effect.

The results report in Chapters 3 and 4 were obtained in behaviourally naïve rats and, together with some published evidences (Pozzi et al. 2010; Svenningsson

et al. 2003), suggested that the NMDAR antagonists induced deficits in the 5-CSRT task may be associated to increased levels of p-CREB in the FC.

However, it has been shown that accuracy measured in rats performing the 5-CSRT task was impaired when levels of p-CREB in the FC were reduced by viral vector gene transfer (Paine et al. 2009).

Thus, to address the possibility that the effect of (R)-CPP on p-CREB might depend on the behavioral performance of the rats, p-CREB levels were measured in rats doing the 5-CSRT task (Chapter 5). Interestingly (R)-CPP-induced p-CREB levels were decreased in the PFC in rats performing the task as opposed to those that were food deprived, exposed to the box but were not trained on the task. There was also a significant increase in p-CREB levels in the NAC of (R)-CPP-treated rats performing the task, which I never found in the same region in behaviourally naïve rats.

Thus as summarized in Table 6.4, the data show that the direction of changes in p-CREB and the regions involved after blockade of NMDAR in the PFC may critically depend on the behavioural state of animals.

Table 6.4.
(R)-CPP-induced CREB changes in rats performing the task
Effects of intra mPFC infusion of (R)-CPP on T-CREB and p-CREB levels rats not performing (CPP alone) or performing (5-CSRT+CPP) the behavioural task.

	Untrained +CPP			5-CSRTT+CPP		
	FC	CPu	NAC	FC	CPu	NAC
T-CREB	N.E.	N.E.	N.E.	↓	N.E.	↑
p-CREB	↑	N.E.	N.E.	↓	N.E.	↑

↓ reduction; ↑ increase; **N.E.** no effect.

The c-AMP analogue Sp-cAMP was then used as a pharmacological tool to specifically increase p-CREB levels in the FC to see whether this was sufficient to prevent the deficits induced by (R)-CPP in rats performing the behavioural task.

I show in Chapter 5 that Sp-cAMP, at the dose that increased p-CREB in the FC (Table 6.5), prevented (R)-CPP-induced attention deficits in rats performing the task (Table 6.6), thus supporting a role for decreased cortical p-CREB in controlling attention and impulsivity.

Table 6.5.
Time course effects of intra mPFC infusion of Sp-cAMP on p-CREB levels.

Sp-cAMP	FC	CPu	NAC
p-CREB (10 min)	↑	↑	↓
p-CREB (40 min)	↑	N.E.	N.E.

↓ reduction; ↑ increase; **N.E.** no effect.

Table 6.6.

Effects of (R)-CPP on behavioural performance in the presence of Sp-cAMP

5-CSRTT	Sp	CPP	Sp+CPP
ACCURACY	N.E.	↓	R
IMPULSIVITY	N.E.	↑	R
COMPULSIVITY	N.E.	N.E.	N.E.
OMISSIONS	↑	N.E.	N.E.

↓ reduction; ↑ increase; **N.E.** no effect; R reversal.

6.2 Concluding remarks and clinical implications

This study has analyzed the pattern of (R)-CPP-induced CREB phosphorylation in naive, untrained and in rats trained to perform the 5-CSRT task, indicating that CREB, which is involved in modulating attentional processes, is differentially regulated in a region- and time-dependent manner by exposure to NMDAR antagonists. The present results point out that the stautus of the animal (trained vs. untrained) determines the effect of (R)-CPP on CREB phosphorylation indicating that studying the effects of drugs affecting attention in naïve animals might not be appropriate to investigate the underlying molecular mechanisms. At present, changes in CREB phosphorylation on S133 induced by NMDAR antagonists may be considered a sensitive index of NMDAR-induced changes in neuronal activity. Although no direct links can be established between changes in CREB phosphorylation and attention performance, findings in trained rats suggest that reduced CREB phosphorylation in the FC is associated with attention deficits.

There is little doubt that patients with schizophrenia have attentional impairment deficits in executive functions and working memory (Egan et al. 2001) but it is quite difficult to specify the underlying neuronal and neurochemical basis of these deficits. In humans, some lines of evidences implicate CREB cascade in the pathophysiology of schizophrenia. In particular, two novel variants have been found in the promoter region of the CREB gene in schizophrenic patients but replication studies are pending (Kawanishi et al. 1999). Binding of forskolin to adenylate cyclase was found

to be significantly elevated in the hippocampal regions in post mortem schizophrenic brains as measured by quantitative autoradiography (Kerwin and Beats 1990). Moreover, increased cyclic AMP response to forskolin was also found in human B-lymphocytes derived from schizophrenics (Natsukari et al. 1997).

Indeed, the regions which displayed altered CREB activity in response to NMDAR blockade are also key regions that are dysfunctional in schizophrenia. Thus, CREB may represent a molecular target for the development of novel drugs that may result effective on cognitive deficits of schizophrenia.

In summary, the results of this study have identified phospho-S133-CREB as a new downstream target modulated by NMDAR antagonists, which may be important in attention control.

CHAPTER 7

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