



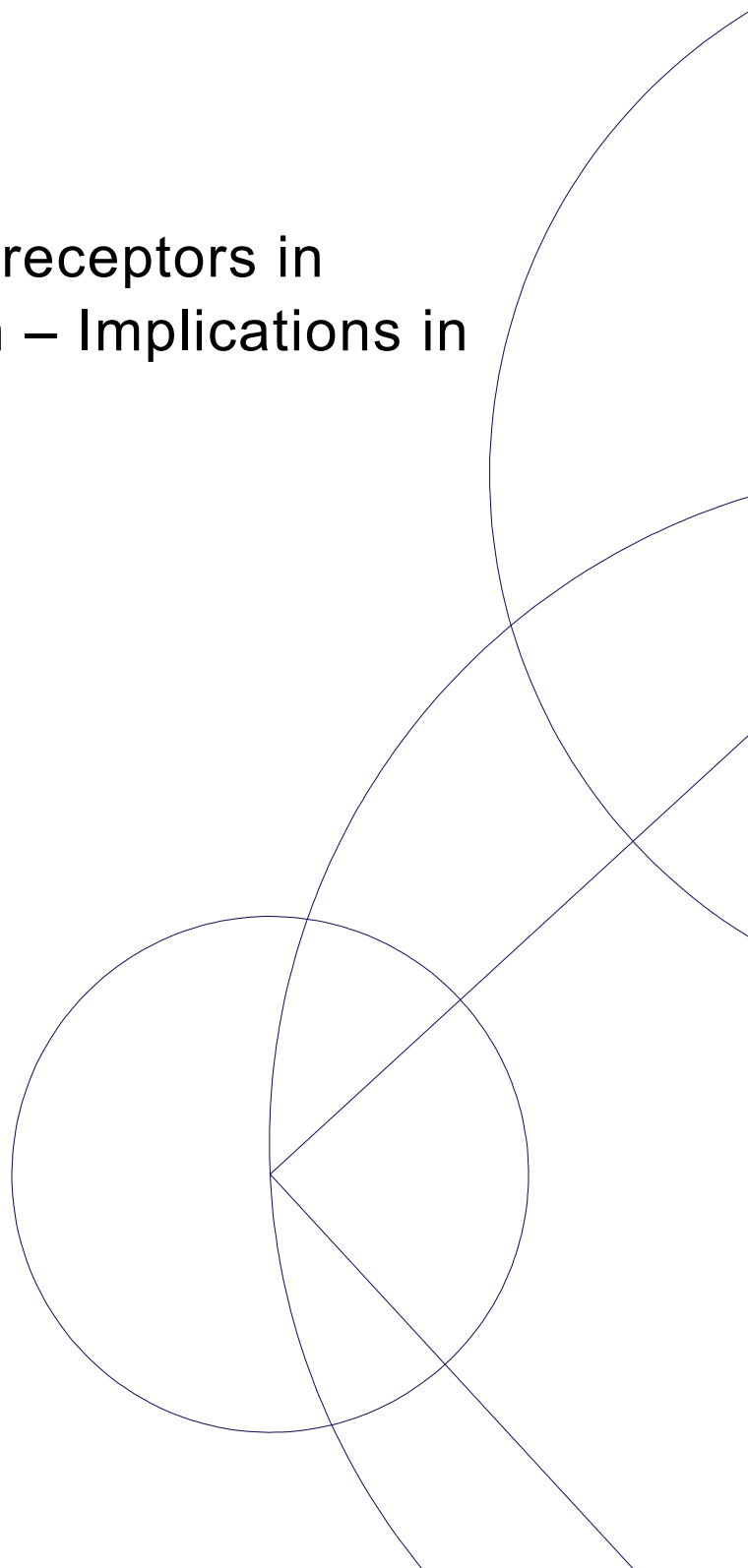
## PhD thesis

Martin Andreas Santini

# The role of serotonin 2A receptors in prefrontal cortex function – Implications in schizophrenia

Academic advisor: Jens D. Mikkelsen

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**Title:** The role of serotonin 2A receptors in prefrontal cortex function – Implications in schizophrenia

**Author:** Martin Andreas Santini, MSc in Human Biology

**Department:** Neurobiology Research Unit, Neuroscience Center, Copenhagen University Hospital Rigshospitalet, Denmark

**Institution:** Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

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**Supervisors:**

Associate Professor Jens Damsgaard Mikkelsen, Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, Denmark

Professor Gitte Moos Knudsen, Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, Denmark

**Review committee:**

Professor Anders Fink-Jensen (Chairperson), Department of Neurology, Psychiatry and Sensory Sciences, University of Copenhagen, Denmark

Associate Professor Gregers Wegener, Department of Clinical Medicine - Centre for Psychiatric Research, Aarhus University, Denmark

Associate Professor Vidita Vaidya, Department of Biological Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai, India

This thesis has been submitted to the Graduate School of The Faculty of Health and Medical Sciences, University of Copenhagen

## Preface

The present PhD thesis is the result of a 3-year PhD programme at the Graduate School of the Faculty of Health and Medical Sciences, University of Copenhagen. The experimental work was carried out at the Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet and at the Department of Psychiatry, McLean Hospital, Harvard Medical School.

The thesis is based on the following four original manuscripts, which in the text are referred to by their Roman numerals:

- I. **Santini MA**, Klein AB, El-Sayed M, Ratner C, Knudsen GM, Mikkelsen JD, Aznar S (2011) Novelty-induced activity-regulated cytoskeletal-associated protein (Arc) expression in frontal cortex requires serotonin 2A receptor activation, *Neuroscience*, 190, 251-257
- II. **Santini MA**, Ratner C, Aznar S, Klein AB, Knudsen GM, Mikkelsen JD (2012) Enhanced prefrontal serotonin 2A receptor signaling in the subchronic PCP mouse model of schizophrenia, *Accepted, Journal of Neuroscience Research*
- III. **Santini MA**, Balu DT, Hill-Smith TE, Lucki I, Mikkelsen JD, Coyle JT (2012) Forebrain NMDA receptor hypofunction attenuates pharmacological and behavioral effects induced by the hallucinogenic 5-HT<sub>2A</sub> receptor agonist DOI, *Manuscript*
- IV. Svensson KA, **Santini MA**, Mikkelsen JD (2012) The mGlu<sub>2/3</sub> Receptor Agonists LY354740 and LY379268 Differentially Regulate Restraint Stress-induced Expression of c-Fos in Rat Cerebral Cortex, *Manuscript*

The thesis includes a general introduction to the theme and the experimental methods as well as an integrated discussion of the results obtained. For specific methodological details and in-depth discussions of the individual experiments, I refer to the original manuscripts above (I-IV).

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Martin Andreas Santini, Copenhagen, December 2012

## Summary in English

The core features of schizophrenia include cognitive and behavioral alterations that are related to abnormal function of the prefrontal cortex (PFC). The prominent innervation by serotonin neurons and the dense expression of serotonergic receptors in the PFC suggest that serotonin is a major modulator of its function. Of the serotonin receptors, especially prefrontal serotonin 2A receptors (5-HT<sub>2A</sub>R) are involved in the pathogenesis and treatment of schizophrenia based on the findings that activation of 5-HT<sub>2A</sub>R by lysergic acid diethylamide (LSD) induces schizophrenia-like psychosis in humans and most atypical antipsychotics exhibit high-affinity blocking of 5-HT<sub>2A</sub>R.

The overall aim of this PhD thesis was to investigate the involvement of 5-HT<sub>2A</sub>R in PFC functionality in relation to schizophrenia. More specifically the aims were to 1) investigate the involvement of 5-HT<sub>2A</sub>R in activation of PFC 2) study the functionality of 5-HT<sub>2A</sub>R in PFC in a rodent model of schizophrenia 3) determine the involvement of the glutamatergic system in 5-HT<sub>2A</sub>R signaling and 4) evaluate the effect of metabotropic glutamate 2 receptor (mGluR2) activation on animal models relevant to antipsychotic and anxiolytic activity.

We show that 5-HT<sub>2A</sub>R is involved in the activation of PFC following exposure to a novel environment. Expression of the immediate-early gene (IEG) activity-regulated cytoskeletal-associated protein (*Arc*) was used as an output of PFC activation. Novelty-exposure up-regulated *Arc* mRNA expression in PFC compared to home-cage controls. This PFC response was inhibited by pretreatment with the 5-HT<sub>2A</sub>R antagonists ketanserin and MDL100907, but not by the selective 5-HT<sub>2C</sub>R antagonist SB242084. Taken together, these results suggest that 5-HT<sub>2A</sub>R is involved in the activation of PFC following exposure to a novel environment.

In a second study, we show that the functionality of cortical 5-HT<sub>2A</sub> receptors is enhanced in the subchronic phencyclidine (PCP) rodent model of schizophrenia, as measured by the 5-HT<sub>2A</sub>R-selective head-twitch response (HTR) and mRNA expression of IEGs in PFC. These changes were not associated with alterations in the level of 5-HT<sub>2A</sub>R binding, however basal *Arc* mRNA levels were increased in the PFC after PCP treatment. Together, these findings indicate that subchronic PCP administration produces changes in the neurotransmission indirectly involving the 5-HT<sub>2A</sub>R, which could contribute to the behavioral deficits observed in this model

Furthermore, we show that glutamate signaling is involved in the functionality of 5-HT<sub>2A</sub> receptors. Using an animal model of glutamatergic hypofunction, the constitutive serine racemase knockout (SR<sup>-/-</sup>) mouse, we observe attenuated functional responses following 5-HT<sub>2A</sub>R activation. This reduction in 5-HT<sub>2A</sub>R functionality was not related to changes in receptor binding, which supports the results following PCP treatment that these two measures not necessarily correlate.

Finally, we evaluated the involvement of the mGluR2 receptor in animal models relevant to antipsychotic and anxiolytic activity. The mGluR2/3 agonist LY354740 attenuated restraint stress-induced increase in c-Fos protein expression in the PFC. However, the structurally similar mGluR2/3 agonist LY379268 had no effect in this response and induced c-Fos expression by itself. Further, LY354740 attenuated the increase in PFC c-Fos expression induced by 5-HT<sub>2A</sub>R activation, a property that has been reported previously for LY379268. These results suggest that restraint stress and 5-HT<sub>2A</sub>R stimulation activate PFC through independent pathways.

Together, the experiments included in this PhD thesis adds further to the understanding of the functionality of 5-HT<sub>2A</sub>R in PFC in relations to schizophrenia. In addition, we contribute to the understanding of the interplay between 5-HT<sub>2A</sub>R and the glutamate system.

## Summary in Danish

Skizofreni er kendetegnet ved kognitive og adfærdsmæssige forstyrrelser der forårsages af en abnormt fungerende præfrontal cortex (PFC). Serotonin er en betydelig modulator af PFC's funktion, baseret på dens udtalte innervering af serotonin neuroner og høje udtryk af serotonin receptorer. Af serotonin receptorerne er især serotonin 2A receptoren (5-HT<sub>2A</sub>R) involveret i patogenesen og behandlingen af skizofreni. Dette baseres på at LSD gennem aktivering af 5-HT<sub>2A</sub>R inducerer en skizofreni-lignende tilstand i mennesker, mens de fleste atypiske neuroleptika udviser høj-affin blokering af 5-HT<sub>2A</sub>R.

Det overordnede formål med dette PhD project var at undersøge 5-HT<sub>2A</sub> receptorens rolle i funktionen af PFC med særligt henblik på skizofreni. Mere specifikt var målene at 1) undersøge 5-HT<sub>2A</sub>R's rolle i PFC aktivering 2) studere funktionaliteten af 5-HT<sub>2A</sub>R i PFC i en skizofreni dyremodel 3) undersøge glutamatsystemets rolle i 5-HT<sub>2A</sub>R signalering og 4) evaluere effekten af metabotrofe glutamat 2 receptor (mGluR2) aktivering på dyremodeller relevante for antipsykotisk og anxiolytisk aktivitet.

Her viser vi, at 5-HT<sub>2A</sub>R er involveret i aktiveringen af PFC efter novelty. Som udtryk for PFC aktivering anvendte vi mRNA ekspressionen af immediate-early genet (IEG) activity-regulated cytoskeletal-associated protein (*Arc*). Novelty opregulerede *Arc* mRNA ekspression i PFC, hvilket kunne hæmmes ved forbehandling med 5-HT<sub>2A</sub>R antagonisterne ketanserin og MDL100907, men ikke med 5-HT<sub>2C</sub>R antagonist SB242084. Samlet set viser disse resultater, at 5-HT<sub>2A</sub>R er involveret i aktiveringen af PFC efter novelty.

I et andet studie viser vi at corticale 5-HT<sub>2A</sub> receptorers funktionalitet er øget i den subkroniske fencyklidin (PCP) dyremodel for skizofreni-lignende symptomer. Dette blev målt ved det 5-HT<sub>2A</sub>R-selektive head-twitch respons og ekspressionen af IEG mRNA i PFC. Ændringerne var ikke associeret med forskelle i 5-HT<sub>2A</sub>R binding, dog førte PCP behandlingen til øget basal ekspression af *Arc* i PFC. Samlet set antyder vores resultater, at subkronisk PCP behandling fører til ændringer i neurotransmissionen der indirekte involverer 5-HT<sub>2A</sub>R, hvilket kan bidrage til de adfærdsforstyrrelser der observeres i denne model.



Yderligere viser vi, at glutamat signalering er involveret i 5-HT<sub>2A</sub>R's funktionalitet. I en dyremodel med glutamaterg hypofunktion, den konstitutive serin racemase knockout mus (SR<sup>-/-</sup>), observerede vi svækket funktionalitet efter 5-HT<sub>2A</sub>R aktivering. Denne reduktion var ikke associeret med ændringer i receptor binding, hvilket støtter at disse to mål ikke nødvendigvis følges som observeret efter PCP behandling.

Til sidst evaluerede vi hvorledes mGluR2 receptoren er involveret i dyremodeller relevante til at måle antipsykotisk og anxiolytisk aktivitet. mGluR2/3 agonisten LY354740 svækkede restraint-stress induceret c-Fos opregulering i PFC i modsætning til den strukturelt lignende agonist LY379268 der yderligere i sig selv inducerede c-Fos i PFC. Derudover nedsatte LY354740 c-Fos opreguleringen af c-Fos i PFC efter 5-HT<sub>2A</sub>R aktivering, hvilket tidligere er observeret for LY379268. Disse resultater indikerer, at restraint stress og 5-HT<sub>2A</sub>R stimulering aktiverer PFC gennem uafhængige veje.

Samlet set føjer resultaterne i denne afhandling til forståelsen af 5-HT<sub>2A</sub>R funktionaliteten i PFC i relation til skizofreni. Derudover bidrager vi med viden omkring interaktionen mellem 5-HT<sub>2A</sub>R og glutamat systemet.

## Abbreviations

5-HT	5-hydroxytryptamine, Serotonin	PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
5-HT <sub>2A</sub> R	Serotonin 2A receptor	PKC	Protein kinase C
AA	Arachidonic acid	PLA <sub>2</sub>	Phospholipase A2
ACTH	Adrenocorticotrophic hormone	PLC-β	Phospholipase C-β
APD	Antipsychotic drug	PLD	Phospholipase D
Arc	Activity-regulated cytoskeleton-associated protein	qPCR	Quantitative polymerase chain reaction
DAG	Diacylglycerol	ROI	Region of interest
DOI	2,5-dimethoxy-4-iodoamphetamine	RT-qPCR	Reverse transcriptase-qPCR
Egr-2	Early growth response gene 2	S1	Somatosensory cortex area 1
EPSP	Excitatory postsynaptic potential	SR <sup>-/-</sup>	Constitutive serine racemase knockout mouse
ERK	Extracellular-signal-regulated kinase		
ESR	Ear-scratch response		
GABA	Gamma-aminobutyric acid		
GAD-67	Glutamic acid decarboxylase 67		
GMS	Glycine modulatory site		
GPCR	G-protein coupled receptor		
HPA	Hypothalamic-pituitary-adrenal		
HTR	Head-twitch response		
IEG	Immediate-early gene		
IP <sub>3</sub>	Inositol triphosphate		
ISH	In situ hybridization		
LSD	Lysergic acid diethylamide		
MAPK	Mitogen-activated protein kinase		
MEK	MAPK kinase		
mGluR2	Metabotropic glutamate 2 receptor		
MP2	Mediating protein 2		
NMDA	N-Methyl-D-aspartic acid		
PCP	Phencyclidine		
PFC	Prefrontal cortex		
PI	Phosphoinositide		

## Introduction

### Schizophrenia

Schizophrenia is a severe mental illness affecting approximately 1% of the population worldwide and it ranks as one of the leading causes of chronic disability (Murray and Lopez, 1997). The symptoms, which typically start in young adulthood, are subdivided into four distinct domains referred to as positive (e.g., delusions, hallucinations, and disorganized speech and behavior), negative (e.g. lack of motivation, reduction in spontaneous speech, social withdrawal, and blunted affect), cognitive (e.g., impaired sustained memory attention, executive function, and working memory) and affective (e.g., anxiety/depression and hostility/aggression) (Andreasen and Carpenter, Jr., 1993). While all available antipsychotics have robust efficacy for positive symptoms, they are much less effective in reducing negative and cognitive symptoms and may even cause a worsening in these symptoms (Leucht et al., 2009; Hill et al., 2010).

The cause of schizophrenia is unknown, but evidence suggests that genetic factors, early environmental influences and social factors contribute (Stefansson et al., 2008; Fox, 1990). Based on adoption and twin studies, the heritability of schizophrenia is estimated at 70-90%, although the genetic transmission involves multiple susceptibility genes, each with a small effect and acting in concert with epigenetic and environmental factors (Rao et al., 1981; Mulle, 2012). Prenatal and perinatal events, such as maternal influenza, rubella and malnutrition during pregnancy and obstetric complications increase the risk of developing schizophrenia later in life (Takei et al., 1996; Thomas et al., 2001). Such factors most likely interact with genetic vulnerability to cause alterations in brain neurochemistry and increase the overall risk of the illness.

Recent evidence indicates that schizophrenia pathophysiology involves widespread perturbations in several closely interacting neurotransmitter systems in cortical and subcortical structures (Lisman et al., 2008). Schizophrenia research has focused on neurochemical hypotheses based largely on the pharmacology of antipsychotic drugs. An interest in dopamine was based on the observation that early antipsychotic drugs shared a capacity to block dopamine D<sub>2</sub> receptors and that amphetamine, which increase synaptic dopamine, aggravated schizophrenic symptoms (Creese et al., 1976; Snyder, 1972). Later, abnormalities of the serotonin system were indicated by the finding that atypical antipsychotic drugs (APDs) fully occupy 5-HT<sub>2A</sub>R at clinical relevant doses and have

lower affinity for D<sub>2</sub> than for 5-HT<sub>2A</sub>R (Meltzer et al., 1989). The role of 5-HT<sub>2A</sub>R in schizophrenia was further supported by the finding that activation of 5-HT<sub>2A</sub> receptors by LSD and LSD-like drugs induce a schizophrenia-like psychosis in humans (Vollenweider et al., 1998).

In addition to dopamine and serotonin, abnormalities in glutamate transmissions have been suggested to be involved in the pathophysiology of schizophrenia. The glutamate hypothesis stemmed primarily from the ability of PCP and ketamine, two *N*-Methyl-D-aspartic acid (NMDA) receptor antagonists, to induce schizophrenia-like symptoms in healthy volunteers (Gouzoulis-Mayfrank et al., 2005; Lahti et al., 1995). Further, acute and subchronic treatment with NMDA receptor antagonists are the most well-established animal models of schizophrenia, due to their ability to evoke positive and negative symptoms and cognitive deficits, which are translatable to the symptoms observed in humans (Morris et al., 2005).

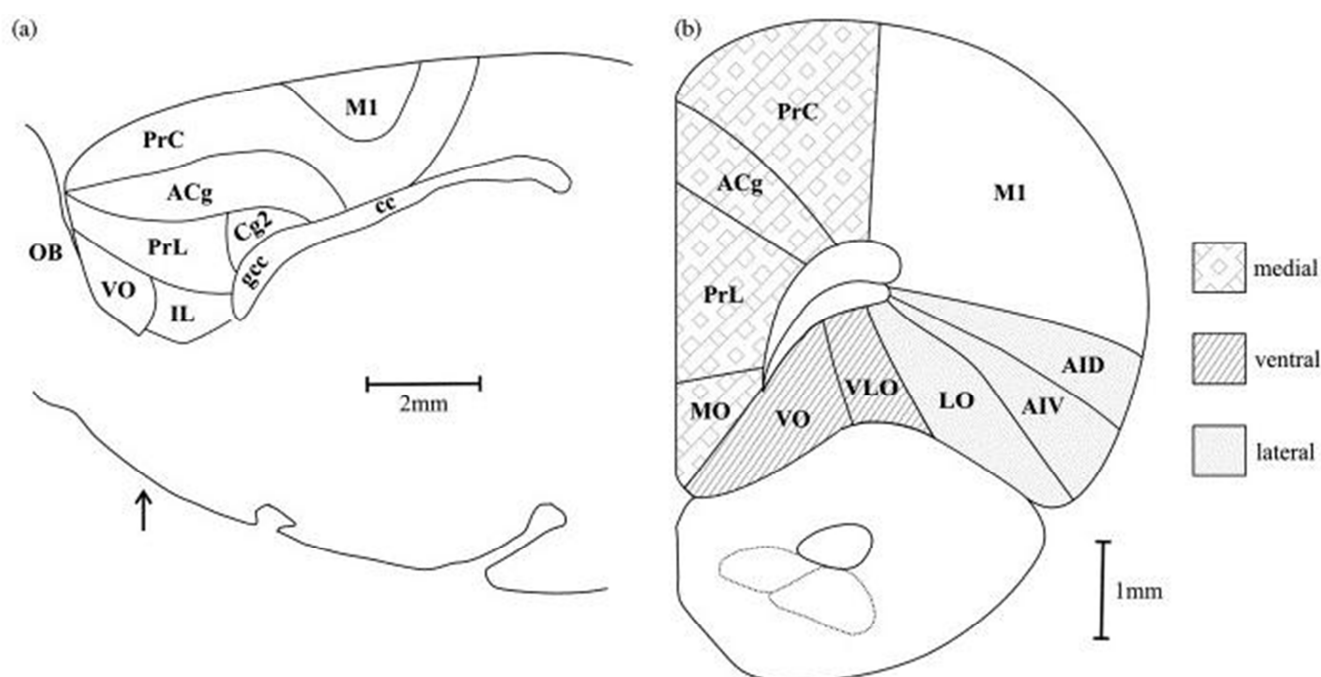
A common trait of the neurochemical hypotheses of schizophrenia is that they are associated with circuit dysregulation in PFC (Marek, 2010; Artigas, 2010). Neuroimaging studies have shown that patients with schizophrenia have a reduced prefrontal bloodflow during cognitive tasks leading to the hypofrontality hypothesis of schizophrenia (Weinberger et al., 1986). However, recent studies have also described unchanged or hyperactive PFC states (Krystal et al., 2003; Paz et al., 2008). Thus, findings of both hypo- and hyperfrontality during cognitive tasks are probably reflections of a general PFC dysfunction in schizophrenia, which may underlie the psychotic, cognitive and emotional manifestations in schizophrenia.

## **Prefrontal Cortex**

The PFC, the most rostral part of the frontal lobe, is critical to many cognitive abilities that are considered particularly human, and forms a large part of a neural system crucial for normal socio-emotional and executive functioning in humans and other primates (Levy and Goldman-Rakic, 2000; Fuster, 1997; Wood, 2003). In the primate, PFC can be divided into ventromedial and dorsolateral regions, each of which is associated with posterior and subcortical brain regions. The ventromedial PFC has connections with brain regions that are associated with emotional processing (amygdala), memory (hippocampus) and higher-order sensory processing (temporal visual association areas), while dorsolateral PFC has connections with brain regions that are associated with motor control (basal ganglia, premotor cortex, supplementary motor area), performance monitoring (cingulate cortex) and higher-order sensory processing (association areas, parietal

cortex). The ventromedial PFC is thus well suited to support functions involving the integration of information about emotion, memory and environmental stimuli, while the dorsolateral PFC to support the regulation of behavior and control of responses to environmental stimuli (Wood and Grafman, 2003).

There has been much debate about whether rodents have what could be considered a PFC and, if they do, what its primate homologue is. Anatomical evidence, however, supports the view that rodents do have a PFC and that it is related to the dorsolateral PFC (Seamans et al., 2008; Uylings et al., 2003). The rat prefrontal cortex is divided into three topologically different regions (Figure 1). First, a medially located cortical region, the medial PFC (mPFC), which constitutes the major portion of the medial wall of the hemisphere and includes the precentral cortex, regions 1 and 3 of the anterior cingulate, prelimbic, infralimbic, and medial orbital cortex. Second, a ventrally located cortical region termed orbitofrontal cortex (OFC), which includes the ventral orbital and ventrolateral cortex, and third, a laterally located cortical region, the lateral PFC, which includes the lateral orbital and agranular insular cortex (Otani, 2004).



**Figure 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 sub-divisions of the PFC (*medial*, *ventral* and *lateral*). Abbreviations: ACg, anterior cingulate cortex; AID, dorsal agranular insular cortex; AIV, ventral agranular insular cortex; 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. Figure from (Dalley et al., 2004).

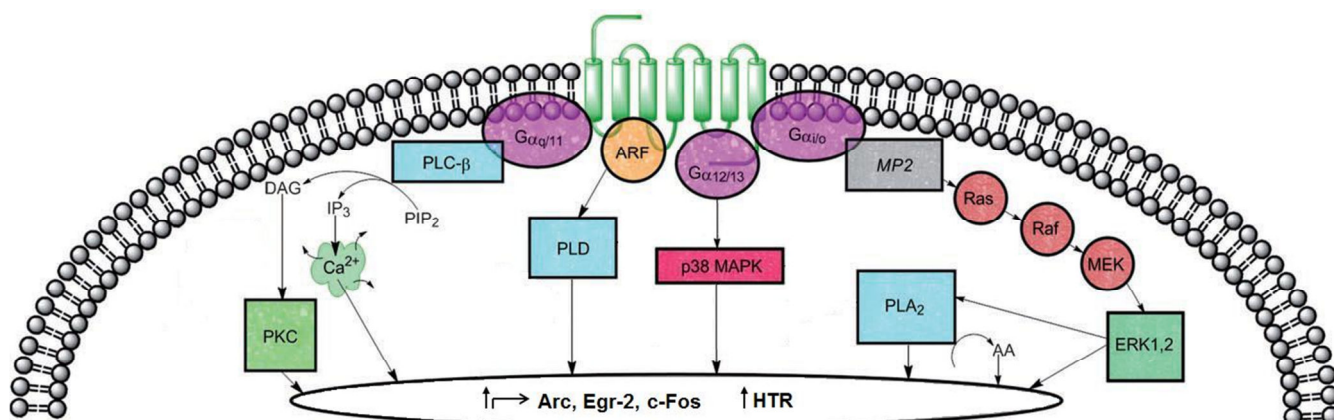
Neurons of the PFC are divided into two main types: “excitatory” glutamate-secreting pyramidal projecting neurons, and “inhibitory”  $\gamma$ -aminobutyric acid (GABA)-secreting local circuit interneurons (Beaulieu, 1993). Autopsy and neuroimaging studies have shown a reduced PFC volume, tight packing of pyramidal neurons and reduced neuropil in the brains of schizophrenic patients (Harrison, 1999; Selemon and Goldman-Rakic, 1999). These anatomical changes translate into profound deficits in PFC function and could account for most of the symptoms observed in schizophrenic patients (Artigas, 2010; Arnsten, 2011).

In order to perform its many higher-order executive tasks, the PFC is highly interconnected with numerous cortical and subcortical structures and (Hoover and Vertes, 2007) receives inputs from neuromodulatory centers in the brainstem and forebrain, including a dense serotonergic innervation originating predominantly from the raphe nuclei (Peyron et al., 1998). Receptor autoradiography studies have documented the presence of most serotonin receptor subtypes in the PFC (Mengod et al., 1996), which suggests that serotonin is a major modulator of its functions.

### The serotonin 2A receptor

Serotonin is involved in the regulation of behavior, cognition and mood, and plays an important role in brain development, regulating both neurite outgrowth, synaptogenesis and cell survival (Gaspar et al., 2003; Martinowich and Lu, 2008). In the CNS, serotonin is produced in neurons originating from the raphe nucleus in the brainstem, from where they project and innervate multiple brain regions (Kroeze et al., 2002).

Serotonin exerts its effects through at least 15 different receptors, divided into 7 receptor subfamilies, 5-HT<sub>1-7</sub>R (Hoyer et al., 1994). All of them are G-protein-coupled receptors (GPCRs), except for 5-HT<sub>3</sub>R, which is a ligand-gated ion channel (reviewed in Barnes and Sharp, 1999). The 5-HT<sub>2A</sub>R couples with  $G\alpha_{q/11}$ ,  $G\alpha_{i/o}$  and  $G\alpha_{12/13}$  proteins both *in vitro* and *in vivo* (Nichols, 2004)(Figure 2). The most prominent and well-understood signaling pathway mediated by 5-HT<sub>2A</sub>R is coupling to  $G\alpha_{q/11}$  leading to phosphoinositide (PI) hydrolysis and subsequent PKC activation (Roth et al., 1984). The activation of 5-HT<sub>2A</sub>R also leads to stimulation of phospholipase A2 (PLA2), which preferentially hydrolyzes arachadonic acid (AA)-containing phospholipids to produce free AA and lysophospholipid. The PLA2 signaling pathway is more complex than the PI turnover cascade, involving multiple G-proteins and the ERK1/2 and p38 mitogen-activated protein kinases, and is, at least in part, mediated through the  $G\alpha_{i/o}$  pathway (Kurrasch-Orbaugh et al., 2003). These second messenger systems ultimately mediate the cellular response to the receptors activation.



**Figure 2: Graphical representation of 5-HT<sub>2A</sub>R signaling pathways** | The 5-HT<sub>2A</sub> receptor couples to various downstream signaling cascades, ultimately affecting immediate-early gene (IEG) expression and head-twitch response (HTR). Abbreviations: PKC, Protein Kinase C; DAG, Diacylglycerol; PIP<sub>2</sub>, Phosphatidylinositol 4,5-bisphosphate; IP<sub>3</sub>, Inositol Triphosphate; PLC-β, Phospholipase C-beta; PLD, Phospholipase D; p38 MAPK, p38 Mitogen-activated Protein Kinase; MP2, Mediating Protein 2; MEK, Mitogen-activated Protein Kinase Kinase; ERK1/2, Extracellular-signal-regulated Kinase 1/2; PLA<sub>2</sub>, Phospholipase A2; Arc, activity-regulated cytoskeleton-associated protein; Egr-2, Early Growth Response Protein 2. Figure modified from (Blaazer et al., 2008).

*In vivo* administration of hallucinogenic 5-HT<sub>2A</sub>R agonists (e.g. LSD and DOI) to rodents results in an induction of IEGs, such as *Arc*, *c-Fos* and early growth response gene 2 (*Egr-2*) in cortical regions (Gonzalez-Maeso et al., 2003; Pei et al., 2004). Both hallucinogenic and non-hallucinogenic 5-HT<sub>2A</sub>R agonists induce *c-Fos* expression through G<sub>αq/11</sub>-dependent PLC activation, while only hallucinogenic drugs induce G<sub>i/o</sub>-dependent expression of *Egr-2* (Gonzalez-Maeso et al., 2007). The circuit involved in IEG induction following systemic DOI administration seems to be cortical-cortical (Beique et al., 2007) as cellular signaling to 5-HT<sub>2A</sub>R agonists is eliminated in 5-HT<sub>2A</sub>R knockout mice, but can be rescued by genetic restoration of 5-HT<sub>2A</sub>R to cortical pyramidal neurons (Gonzalez-Maeso et al., 2007). Together, these data show that the effects of DOI and other hallucinogenic compounds are mediated through cortical 5-HT<sub>2A</sub> receptors.

Both systemic and direct injection of 5-HT<sub>2A</sub>R agonists into the PFC also induce a characteristic head-twitch response (HTR) in rodents (Willins and Meltzer, 1997). This response is absent in 5-HT<sub>2A</sub>R knock-out mice and is, similarly to the cellular response, rescued by reintroduction of 5-HT<sub>2A</sub> receptor expression in cortex (Gonzalez-Maeso et al., 2007). Further, only compounds which cause hallucinations in humans induce HTR, suggesting that this response is an indicator of hallucinogenic action (Gonzalez-Maeso et al., 2007). Thus, both the induction of cortical IEG

mRNA expression and HTR following 5-HT<sub>2A</sub>R activation can be utilized as a 5-HT<sub>2A</sub>R-specific measure of receptor signaling output.

In addition, recent studies suggest, that signaling of 5-HT<sub>2A</sub>R is more complex because the receptor signals not only via canonical pathways involving heterotrimeric G proteins, but also via noncanonical G protein-independent interactions with other signaling protein including  $\beta$ -arrestins and PSD-95 (Gelber et al., 1999; Xia et al., 2003). Further, the density of 5-HT<sub>2A</sub>R is atypically regulated through interaction with intracellular modulating proteins (Schmid et al., 2008). Unlike most other GPCRs, 5-HT<sub>2A</sub>Rs are down-regulated by many antagonists (Leysen et al., 1986; Andree et al., 1986) and have a dynamic subcellular sorting and transportation (Allen et al., 2008).

### *Expression of the 5-HT<sub>2A</sub> receptor in the schizophrenic brain*

Based on the finding that activation of 5-HT<sub>2A</sub> receptors by LSD induces a schizophrenia-like psychosis in humans, it has been hypothesized that schizophrenia is associated with higher 5-HT<sub>2A</sub>R binding in cortical regions (Gonzalez-Maeso and Sealton, 2009). Many laboratories have attempted to determine the density of 5-HT<sub>2A</sub>R in postmortem tissue, and most studies have reported decreased or unchanged 5-HT<sub>2A</sub>R densities (Ebdrup et al., 2011). However, these findings are likely confounded by illness chronicity and a history of treatment with antipsychotics, as both have been shown to decrease 5-HT<sub>2A</sub>R binding (Dean, 2003). In two recent studies, the level of expression of 5-HT<sub>2A</sub>R determined by [<sup>3</sup>H]-ketanserin-binding saturation curves in cortical membrane preparations was significantly higher in the postmortem human brain of antipsychotic-free subjects (Gonzalez-Maeso et al., 2008; Muguruza et al., 2012). Further, the fraction of high-affinity sites of DOI displacing [<sup>3</sup>H]-ketanserin-binding to the 5-HT<sub>2A</sub>R was increased in antipsychotic-free schizophrenic subjects, which suggest that the active conformation of the 5-HT<sub>2A</sub>R is up-regulated in prefrontal cortex of antipsychotic-free schizophrenic subjects (Muguruza et al., 2012).

The 5-HT<sub>2A</sub> receptor density in the schizophrenic brain has also been investigated using positron emission tomography (PET). The majority of these studies has not demonstrated changes in the density of cortical 5-HT<sub>2A</sub>R (Ebdrup et al., 2011). However, in the largest PET study of antipsychotic-naïve first-episode schizophrenia patients to date (30 patients), an overall decreased 5-HT<sub>2A</sub>R binding in cortex, which was most pronounced in the frontal cortex, was reported (Rasmussen H et al., 2009).

Taken together, no obvious pattern of 5-HT<sub>2A</sub>R expression levels in the schizophrenic brain has been identified. This could be because the majority of 5-HT<sub>2A</sub>Rs are located intracellularly in



cortical neurons (Cornea-Hebert et al., 1999). Thus, it is not possible to distinguish between the intracellular pool of receptors (which might be highly dynamic) and functional receptors at the cell membrane, which could make the density of 5-HT<sub>2A</sub>R a poor predictor of actual receptor signaling.

### *5-HT<sub>2A</sub> receptor signaling in animal models of schizophrenia and related disorders*

A variety of animal models are used to mimic the symptoms of schizophrenia. The most widespread and well-established model utilizes the ability of the NMDA receptor antagonists PCP and PCP-like drugs, such as MK-801 and ketamine, to evoke changes in animal behavior, which are comparable to the symptoms observed in humans (Morris et al., 2005). Subchronic administration of PCP produces deficits in a variety of cognitive tests, including object recognition (Hashimoto et al., 2005), attentional setshifting (Laurent and Podhorna, 2004), and prepulse inhibition (Martinez et al., 1999). Furthermore, repeated PCP treatment causes schizophrenia-like changes in the PFC, including decreased levels of parvalbumin and glutamic acid decarboxylase-67 (GAD-67) (two markers of GABAergic neurons)(Amitai et al., 2012) and reduction in spine density (Hajszan et al., 2006).

Interestingly, acute systemic PCP administration evokes head-twitches in rodents similar to those induced by 5-HT<sub>2A</sub>R agonists such as DOI and LSD (Nabeshima et al., 1987). In fact, the PCP-induced HTR is dependent on 5-HT<sub>2A</sub>R activation, as pretreatment with selective 5-HT<sub>2A</sub>R antagonists inhibits the effect of PCP (Kitaichi et al., 1994). This suggests that 5-HT<sub>2A</sub>R is a key regulator of the actions induced by acute PCP. However, selective 5-HT<sub>2A</sub>R antagonists are not able to reverse cognitive deficits induced by subchronic PCP (Snigdha et al., 2010).

Another animal model of schizophrenia utilizes maternal influenza infection. Accumulating evidence from epidemiological studies suggests that maternal immune activation is a risk factor for schizophrenia (Brown, 2011). Preclinically, the offspring of mothers infected with mouse-adapted influenza virus display a series of behavioral and neurochemical abnormalities that are relevant to schizophrenia. These include deficits in PPI, social interaction deficits, abnormal cortical neurogenesis, pyramidal cell atrophy and loss of GABAergic interneurons (Patterson, 2009). In a recent study, the expression and function of 5-HT<sub>2A</sub>R and mGluR2 receptors in this model was investigated. Here, systemic administration of the hallucinogenic 5-HT<sub>2A</sub>R agonist DOI caused a significantly increased HTR and IEG induction in adult mice born to influenza-infected mothers. Further, these responses were associated with higher expression of 5-HT<sub>2A</sub>R and lower expression of mGluR2 receptors.

The functionality of prefrontal 5-HT<sub>2A</sub>Rs has also been assessed after early life stress (ES) of maternal separation, which is a rat model of psychiatric vulnerability resulting in a persistent vulnerability to endophenotypes of anxiety and depression in adulthood (Sanchez et al., 2001). Significant correlations between anxiety symptoms and positive symptoms in schizophrenia have been previously described (Muller et al., 2004). In this model, adult animals with a history of early stress exhibited heightened prefrontal network activity *in vitro*, enhanced *Arc* mRNA expression *in vivo* and potentiated HTR upon stimulation of prefrontal 5-HT<sub>2A</sub> receptors (Benekareddy et al., 2010). This enhanced functionality occurred despite unaltered 5-HT<sub>2A</sub>R mRNA expression in PFC of ES animals and only a small increase in 5-HT<sub>2A</sub>R binding (Benekareddy et al., 2010).

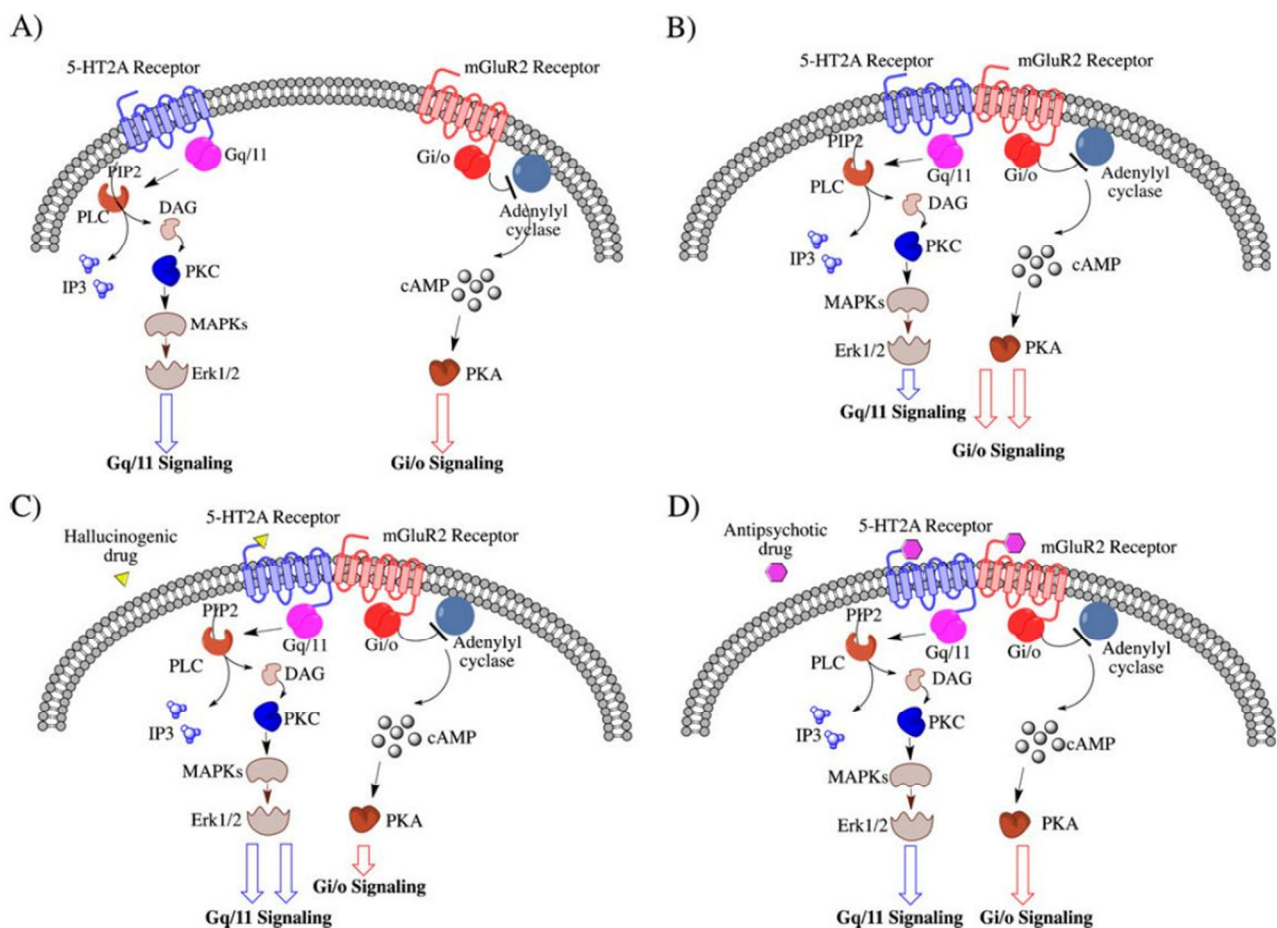
Taken together, these studies suggest that enhanced 5-HT<sub>2A</sub>R signaling is a key property of animal models of schizophrenia.

### **Interaction between mGluR2 and 5-HT<sub>2A</sub>R**

Metabotropic glutamate (mGlu) receptors are a family of G-protein-coupled receptors that are activated by the excitatory amino acid neurotransmitter glutamate. The mGlu receptors are subdivided into three groups (Group I: mGluR1,5; Group II: mGluR2,3; Group III: mGluR4,6,7,8) based on sequence homology, signal transduction pathways and pharmacology (Schoepp et al., 1999). Of these subclasses, Group II receptors are often localized presynaptically on axon terminals, acting as auto- or heteroreceptors to limit the release of glutamate or other neurotransmitters (Cartmell and Schoepp, 2000).

Several lines of evidence have shown that mGluR2/3 agonists may be effective for the treatment of schizophrenia in both animal models and in humans. Orthosteric mGluR2/3 agonists, such as LY354740, have been found to have antipsychotic-like effects in a variety of preclinical rodent *in vivo* paradigms (Rorick-Kehn et al., 2007; Schoepp et al., 1999; Schoepp et al., 2003). When taken to the clinic, LY2140023 monohydrate, the prodrug of a different orthosteric mGluR2/3 agonist, LY404039, recently demonstrated antipsychotic activity in phase II proof of concept study (Patil et al., 2007), although results from a later clinical study with this compound were inconclusive (Kinon et al., 2011).

The neuronal mechanisms by which mGlu2/3 receptor agonists exert their effects are thought to be mediated by acting on the glutamatergic and 5-HTergic pathways (Marek, 2010). A functional interaction between 5-HT<sub>2A</sub>R and mGluR2/3 has been well-established. Activation of mGluR2/3 suppress both DOI-induced excitatory postsynaptic potentials (EPSPs) (Marek et al., 2000), c-Fos expression (Zhai et al., 2003) and HTR (Benneyworth et al., 2007). One proposed hypothesis to explain this mechanism is that activation of the presynaptic mGluR2/3 receptor inhibits glutamate release from cortical terminals of thalamic neurons expressing 5-HT<sub>2A</sub>R (Marek et al., 2001). However, recent studies indicate that the effect of 5-HT<sub>2A</sub>R activation on glutamatergic synaptic activity in PFC is not mediated through a thalamocortical circuit. Instead, 5-HT<sub>2A</sub>R activation leads to an increase in glutamatergic recurrent network activity in the PFC (Beique et al., 2007).



**Figure 3: Model of the intracellular signaling by 5-HT<sub>2A</sub>/mGluR2 heterocomplexes** | (A) Intracellular signaling pathways originating from independent 5-HT<sub>2A</sub> and mGluR2 receptors (B) Putative signaling by multimerized 5-HT<sub>2A</sub>/mGluR2 heterocomplex (mGluR2-mediated G<sub>i/o</sub> dependent signaling predominates) (C) At 5-HT<sub>2A</sub>R activation (putative pro-psychotic condition), the downstream signaling by the 5-HT<sub>2A</sub>/mGluR2 heterocomplex is prevalently mediated by the G<sub>q/11</sub> dependent pathway (D) Antipsychotic administration (targeting either 5-HT<sub>2A</sub>R or mGluR2) may revert the pro-psychotic state by re-balancing downstream signaling through G<sub>q/11</sub>–G<sub>i/o</sub> dependent pathways. Adapted from (Blaazer et al., 2008; Fribourg et al., 2011).

Another hypothesis is based on evidence showing a direct interaction between 5-HT<sub>2A</sub>R and mGluR2, both *in vitro* and *in vivo* (Gonzalez-Maeso et al., 2008). These heteromeric complexes are functional as activation of G $\alpha_{q/11}$  by 5-HT<sub>2A</sub>R is decreased by co-expression of mGluR2, while the activation of G $\alpha_{i/o}$  is markedly enhanced (Figure 3). These mGluR2-dependent effects can be reversed by activation of mGluR2, showing that this receptor complex integrates serotonin and glutamate transmission to dictate the pattern of G-protein regulation. Thus, the attenuating effects of mGluR2/3 agonists on 5-HT<sub>2A</sub>R-mediated signaling and behavior can, at least in part, be attributed to a direct interaction between the two receptors.

### Serine Racemase knock-out mice

Activation of NMDARs requires the binding of either glycine or D-serine at the glycine modulatory site (GMS) on the NR1 subunit (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). The SR enzyme converts L-serine to D-serine and consequently, SR<sup>-/-</sup> mice have >80% reduction in brain D-serine, while the levels of L-serine, glycine and glutamate in the cerebral cortex are equivalent to those of WT controls (Basu et al., 2009).

SR<sup>-/-</sup> mice display reduced cortical volume (Balu et al., 2012) and neurons in the PFC have reduced complexity, total length and spine density of apical dendrites (Devito et al., 2011). Furthermore, pyramidal neurons in the primary somatosensory cortex (S1) of SR<sup>-/-</sup> mice also show reduced complexity, total length and spine density of apical and basal dendrites (Balu et al., 2012). SR<sup>-/-</sup> mice further exhibit reduced global NMDAR-mediated neurotransmission and impaired LTP at the Schaffer collateral-CA1 pyramidal neuron synapse (Basu et al., 2009).

NMDARs are involved in learning and memory. In the Morris water maze paradigm, which assesses hippocampal NMDAR-dependent spatial memory (Morris et al., 1986), SR<sup>-/-</sup> mice show impaired spatial reference memory (Basu et al., 2009). SR<sup>-/-</sup> mice also exhibit disrupted representation of the odor associated with events in distinct experiences monitored by object recognition and the odor sequence test (Devito et al., 2011). Further, SR<sup>-/-</sup> mice show increased locomotor activity but no deficits in prepulse inhibition (PPI) (Basu et al., 2009). Altogether, these observations indicate that targeted deletion of serine racemase leads to NMDAR hypofunction and subsequent cellular and behavioral abnormalities.

## **Aims of thesis**

The overall aim of this PhD thesis was to investigate the involvement of 5-HT<sub>2A</sub>R in PFC functionality in relations to schizophrenia. More specifically the aims were to:

- Investigate the involvement of 5-HT<sub>2A</sub>R in activation of PFC following exposure to a novel environment
  
- Study the functionality of 5-HT<sub>2A</sub>R in PFC in the subchronic PCP rodent model of schizophrenia
  
- Study the involvement of the glutamate system in the cellular and behavioral responses to 5-HT<sub>2A</sub>R activation
  
- Evaluate the effect of mGluR2/3 receptor activation on 5-HT<sub>2A</sub>R stimulation and after restraint stress

## Experimental Methods

### Measuring 5-HT<sub>2A</sub>R-mediated functionality

In order to measure the functionality of cortical 5-HT<sub>2A</sub> receptors, we utilized both a cellular and behavioral output. Administration of the full 5-HT<sub>2A</sub>R agonist DOI leads to a 5-HT<sub>2A</sub>R-selective HTR and induction of IEGs, such as *Arc*, *c-Fos* and *Egr-2* in cortical regions (Gonzalez-Maeso et al., 2003; Pei et al., 2004; Gonzalez-Maeso et al., 2007). Thus, both the induction of IEG expression in the PFC and HTR can be utilized as a measure of 5-HT<sub>2A</sub>R signaling, and HTR as a predictor of hallucinogenic potential of 5-HT<sub>2A</sub>R-agonists. To measure the induction of IEG mRNA, we used reverse transcriptase polymerase chain reaction (RT-qPCR) and *in situ* hybridization.

#### *Reverse transcriptase polymerase chain reaction*

RT-qPCR is a sensitive technique to measure and to compare mRNA levels among samples. RT-qPCR is a combination of three steps: 1) the reverse transcriptase-dependent conversion of mRNA to cDNA, 2) the amplification of the cDNA using PCR and 3) the detection and quantification of amplification products in real time (Nolan et al., 2006). Data obtained from qPCR reflect number of cycles run when the exponential phase of the amplification starts. Samples containing high levels of target cDNA need fewer cycles to run before reaching the exponential phase than do samples containing lower cDNA levels. For data analysis, the cycle threshold (C<sub>T</sub>) value for each sample's gene of interest was normalized to the sample with the lowest C<sub>T</sub> value within the gene of interest and within the housekeeping gene, and fold change was calculated according to the comparative C<sub>T</sub> method (Schmittgen and Livak, 2008).

Because RT-qPCR is extremely sensitive, small variations in the conditions between samples could lead to large changes in the final output. Ideally, the efficiency of the assay should be 100%, meaning that during the logarithmic phase of the reaction, the PCR product of interest is doubling with each cycle. However, several parameters can affect the efficiency of the PCR, including PCR inhibitors (e.g., sample preparation reagents, excessive protein), primer design and qPCR running conditions. To minimize the risk of non-optimal qPCR reactions, primers and running conditions were tested using standard dilutions. Further, contamination with genomic DNA could lead to false positive results, due to the exponential amplification of the starting material. To minimize the risk of contamination with genomic DNA, samples were treated with DNase and primers were constructed to span splice sites so they will only anneal to the spliced RNA.

### *In situ hybridization*

*In situ* hybridization (ISH) is a technique for localizing specific nucleic acid targets within fixed tissues and cells. ISH is not as sensitive as RT-qPCR because the mRNA is not amplified, instead it allows for temporal and spatial information about gene expression. The underlying basis of ISH is that nucleic acids, if preserved adequately within a histologic specimen, can be detected through the application of a complementary strand of nucleic acid to which a reporter molecule is attached. Two major methods for labeling and detection are used: (1) radioisotope labeling with  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$  or  $^{32}\text{P}$ , which is detected with x-ray film; and (2) non-isotope labeling with molecules such as biotin, digoxigenin or alkaline phosphatase, which are visualized by immunohistochemistry detection systems. We used radioisotope labeling with a  $^{35}\text{S}$ -labeled probe, which represents the most sensitive and rapid approach of the isotopes (Wilcox, 1993).

Experimentally, a 45-50mer DNA oligonucleotide probe was 3'-end labeled with [ $\alpha$ - $^{35}\text{S}$ ]-dATP using terminal transferase. After column purification of the labeled probe it was hybridized directly to a tissue section where it forms a DNA-RNA duplex with its cognate mRNA. For visualization, the section was exposed to an x-ray film, where darkening of the film indicates specific hybridization of the radioactive probe and thus gene expression. Image analysis was conducted by hand-drawing regions of interest (ROI) for each brain region, and the mean pixel density in the ROIs was measured. This pixel density was converted to (nCi/mg) using standard [ $^{14}\text{C}$ ]-microscales relating image densitometry to mRNA expression.

For ISH it is important to use optimal conditions for probe hybridization in order to reduce non-specific background binding. Salt concentration, temperature, pH and the washing steps all affect the binding of the probe. Further, the specificity of the signal should be determined performing competition hybridization with excess concentrations of unlabeled oligonucleotides and reproducing the autoradiographic pattern with several independent probes built to hybridize different parts of the same mRNA (Wisden and Morris, 2002).

### *Head-twitch response*

Behavioral animal models cannot capture the perturbations of perception, cognition, and mood produced by hallucinogens in humans. However, rodents might exhibit behavioral proxies of human hallucinogenic effects. Hallucinogenic compounds in rodents elicits several unconditioned effects, including changes in exploratory behavior (Adams and Geyer, 1985), grooming (Trulson and

Howell, 1984), HTR, ear scratch response (ESR), and hyperthermia (Corne and Pickering, 1967; Darmani et al., 1990b; Silva and Calil, 1975). Of these however, only ESR and HTR are specific measures of HCs (Gonzalez-Maeso et al., 2007). We choose HTR as an output of 5-HT<sub>2A</sub>R functionality as it in contrast to ESR is robustly elicited by DOI and normally distributed among individual mice. The HTR is an easily observed and quantifiable movement of the head, similar to the reflexive response observed when the ear is touched or pinched (the pinna reflex response) (Keller and Umbreit, 1956). In our studies mice were injected with vehicle or DOI and moved to a cage without enrichment where they were videotaped for 25 minutes. Scoring of HTR was done in 5 minute bins for 20 min (between 5-25 min after injection).

### Receptor autoradiography

*In vitro* receptor autoradiography is a widely used method for determining the localization and quantitation of receptors in thin brain sections. It employs radioactively labeled ligands, which bind reversibly but with high affinity to specific receptors in their native, properly folded state. Receptor autoradiography offers anatomical resolution and sensitivity compared to biochemical methods such as Western Blotting.

Ligands can be labeled using a broad variety of isotopes; however for our studies we used [<sup>3</sup>H]-labeled ligands, which provide high spatial resolution due to their low-energy  $\beta$ -emission. We employed *in vitro* autoradiography as a method to quantify receptor binding for the 5-HT<sub>2A</sub> and 5-HT<sub>1A</sub> receptors and for the serotonin transporter (SERT) using [<sup>3</sup>H]-MDL100907 (5-HT<sub>2A</sub>R antagonist), [<sup>3</sup>H]-WAY100635 (5-HT<sub>1A</sub>R antagonist) and [<sup>3</sup>H]-escitalopram (SERT blocker), respectively. Non-specific binding was analyzed after blocking 5-HT<sub>2A</sub>R, 5-HT<sub>1A</sub>R or SERT binding with high concentrations of ketanserin, serotonin or paroxetine.

For the experiments, we used a saturating concentration of radioactively labeled ligand (4-6 times  $K_d$ ) where binding levels are a direct measure of the total concentration of receptor binding sites ( $B_{max}$ ). Image analysis was conducted by hand-drawing ROIs for each brain region, and the mean pixel density in the ROIs was measured. This pixel density was converted to (nCi/mg) using standard [<sup>3</sup>H]-microscales relating image densitometry to receptor binding.



## Novel environment

Exposure to a novel environment with an active exploratory component (e.g. open-field arena) is a well-established model of emotional stress resulting from the conflict between innate drives to explore a novel environment and safety need (Weisstaub et al., 2006; Aloyo and Dave, 2007; Prut and Belzung, 2003).

Several groups have shown that exposure to a novel environment leads to an increase in *Arc* mRNA levels in hippocampus and PFC (Vazdarjanova et al., 2002; Elizalde et al., 2010; Pinaud et al., 2001), and *Arc* has been suggested to play a major role in brain areas displaying a prominent emotion-related synaptic plasticity (Ons et al., 2004; Vazdarjanova et al., 2002). Importantly, PFC is a key component of the neuronal circuitry mediating responses to novelty including those directed to emotional control and memory formation (Roozendaal, 2002).

A variety of paradigms have been used to induce novelty in animals, including the hole-board test (Ons et al., 2004), spatial exploration grid (Vazdarjanova et al., 2006; Guzowski et al., 1999) and open-field arena (Eisenstein et al., 2010; Kumakura et al., 2010; Aloyo and Dave, 2007).

We used an open-field arena in a room that was only dimly lit (16.6 lux at the bottom of the field) in order to reduce the evoked anxiety and increase overall exploratory behavior (Bouwknicht et al., 2007). Adult mice were treated with vehicle or experimental drug and returned to their home-cages. After 30 minutes the novelty groups were exposed to a novel environment by placing them in the middle of an open field arena (40×40×80 cm) for 5 minutes, after which novelty-treated mice were returned to their respective home-cage for another 30 minutes. The brains were quickly removed and desired brain regions dissected on ice. Total RNA was extracted from the samples and the expression level of target genes was analyzed using RT-qPCR.

## Restraint stress

Restraint stress is used to induce both psychological and physical stress effects on the nervous system and concomitant interaction with the endocrine system, which causes a wide variety of behavioral and physiological alterations (Chrousos, 1998). Restraint stress is a high-intensity stimulus in terms of activation of the hypothalamo-pituitary adrenocortical (HPA) axis and thus robustly increase circulating levels of adrenocorticotrophic hormone (ACTH) and corticosterone (Mikkelsen and Larsen, 2006). We used restraint stress to induce an anxiety-like state, as this type of stressor has been shown to mediate fear potentiation in the elevated plus-maze (Heinrichs et al., 1994)

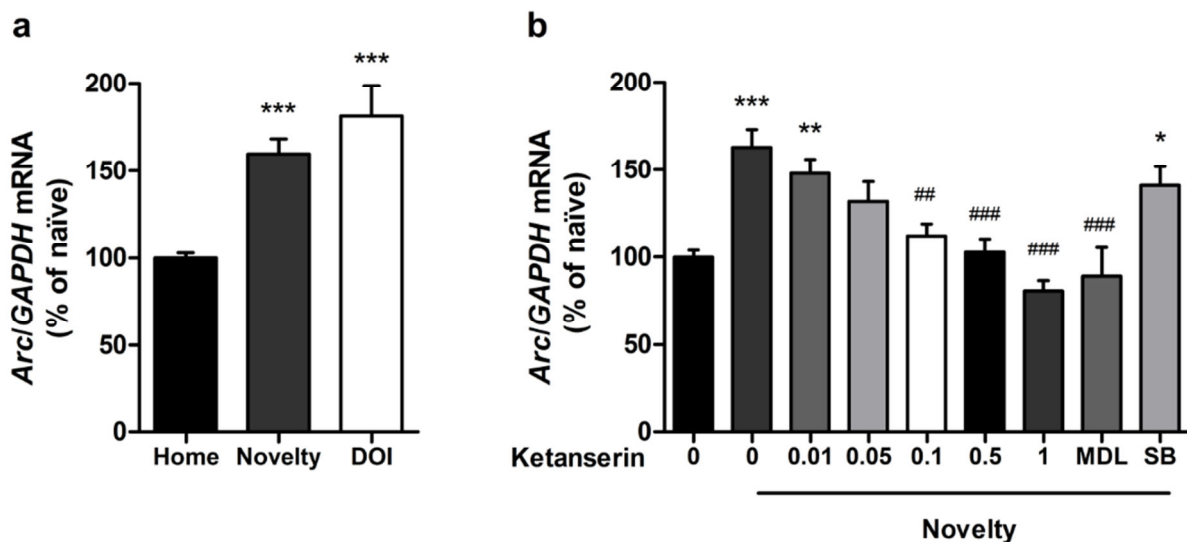
On the day of the study animals were pretreated with vehicle or experimental drug and returned to their home cages. After 30 minutes, animals were restrained in an acrylic flat-bottomed restrainer for 20 minutes and euthanized by decapitation 2 hours after onset of restraint. Whole brains were rapidly removed and immediately immersed in isopentane over dry ice and then stored at -80°C until sectioned.

## Results and Discussion

The 5-HT system, and particularly the 5-HT<sub>2A</sub> receptor, has been proposed to be involved in the regulation of PFC function and in diseases with a marked dysfunction of PFC (Puig and Gullledge, 2011). To investigate the involvement of 5-HT<sub>2A</sub>R in PFC function we made use of two fundamentally different approaches: 1) activate 5-HT<sub>2A</sub>R in PFC with systemic administration of 5-HT<sub>2A</sub>R agonists (manuscript 2 and 3) and 2) expose the animal to a situation that activates PFC and use selective antagonists to affect PFC serotonin signaling (manuscript 1 and 4).

### Novelty-induced *Arc* expression in PFC requires 5-HT<sub>2A</sub>R activation

In paper I we investigated the role of 5-HT<sub>2A</sub>R in the activation of PFC using a novelty paradigm. Exposure to a novel stimulus engages PFC in the evaluation of the stressfulness of the situation (Robbins, 2000) and the exposure to novelty initiates a number of events that integrates risk assessment behavior where both rewarding and aversive outcomes are possible. Accordingly, both the PFC and the HPA axis are activated. To study the activation of PFC following novelty, we utilized the expression of *Arc* mRNA, which is induced by novelty in a number of cortical areas and in the hippocampus (Klebaur et al., 2002; Vazdarjanova et al., 2002).



**Figure 4: *Arc* mRNA expression in the frontal cortex** | (a) Administration of the 5-HT<sub>2A</sub>R agonist DOI (2 mg/kg) induces *Arc* expression to levels comparable to novelty (b) Novelty exposure up-regulates the expression of *Arc*. The 5-HT<sub>2A</sub>R antagonists ketanserin (0.1-1 mg/kg) and MDL100907 (1 mg/kg), but not the 5-HT<sub>2C</sub>R selective antagonist SB242084 (SB; 1 mg/kg) blocks novelty-induced *Arc* up-regulation. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to vehicle-treated animals; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  compared to vehicle/novelty-treated animals, one-way ANOVA with Tukey's post-hoc test. Data presented as % of drug- and novelty-naïve animals  $\pm$  SEM.

We found that exposure to a novel environment robustly upregulated the expression of *Arc* mRNA in PFC compared to home-cage controls (Figure 4a). To study the involvement of 5-HT<sub>2A</sub>R in this response, we pretreated the animals with the 5-HT<sub>2R</sub> antagonist ketanserin, which attenuated novelty-induced *Arc* mRNA expression in a dose-dependent manner (Figure 4b). Because ketanserin also has affinity for 5-HT<sub>2C</sub>R, we further pretreated animals with the highly selective antagonists MDL100907 and SB242084, showing affinity towards 5-HT<sub>2A</sub>R and 5-HT<sub>2C</sub>R respectively. MDL100907 (1 mg/kg) fully inhibited novelty-induced *Arc* induction while SB242084 had no effect, showing that it is the 5-HT<sub>2A</sub>R blockade that mediates the effect of ketanserin in attenuating novelty-induced PFC activation.

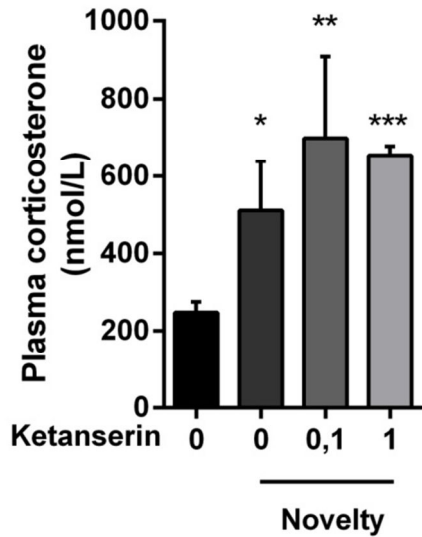
These data suggest that 5-HT is a major modulator of PFC activation through 5-HT<sub>2A</sub>R. This is corroborated by previous studies showing that exposure to a novel environment or stimuli activates 5-HT neurons in a subset of the dorsal raphe nucleus and increases extracellular 5-HT concentrations in the PFC (Adell et al., 1997; Lowry et al., 2005). Similarly, stress-related behavioral paradigms, such as restraint stress and conditioned fear, increase 5-HT metabolism and release in the medial prefrontal cortex, nucleus accumbens, amygdala, and dorsal hippocampus (Inoue et al., 1994; Konstandi et al., 2000). However, most of these stimuli also increase the release of dopamine and glutamate in FC (Ihalainen et al., 1999; Moghaddam, 1993), which in addition could be involved in the cortical IEG up-regulation we observe after novelty. The ability of ketanserin and MDL100907 to fully inhibit novelty-induced *Arc* expression however, suggests that 5-HT<sub>2A</sub>R activation is necessary for activation of the PFC circuits mediating the behavioral response to novelty. In fact, we find that direct activation of 5-HT<sub>2A</sub>R by its agonist DOI leads to increases in *Arc* expression in PFC comparable to that as induced by novelty-exposure. However, DOI does not induce *Arc* directly in 5-HT<sub>2A</sub>R expressing neurons but in neurons containing AMPA and NMDA receptors (Pei et al., 2004). Instead activation of 5-HT<sub>2A</sub>R in PFC results in a robust increase in glutamate receptor activation, indicating that certain actions of 5-HT<sub>2A</sub>R in this region may be mediated by the release of glutamate (Beique et al., 2007). Taken together, these data support the hypothesis that exposure to a novel environment releases 5-HT, which through 5-HT<sub>2A</sub>R activation and subsequent glutamate release induce *Arc* expression in the PFC.

In addition, we observed that exposure to a novel environment induced several IEGs in PFC, including *Arc*, *c-Fos* and *Egr-2*. These are all markers of neuronal activation and show similar

properties in our study where their induction in PFC by novelty is blocked by ketanserin pretreatment. We focused mainly on *Arc* expression, because it in contrast to *c-Fos* and *Egr-2*, is a brain-specific effector IEG product that plays a key role in the activity-dependent synaptic modifications underlying memory consolidation (Steward and Worley, 2002). In the hippocampus, it is well-established that *Arc* is associated with encoding of new information via direct involvement in receptor cycling in the synapse, fitting with the idea that learning processes involve plastic processes requiring alterations in genomic or proteomic processing (Ons et al., 2004; Vazdarjanova et al., 2002; Miyashita et al., 2008; Plath et al., 2006; Shepherd et al., 2006). The role of *Arc* in the PFC is more uncertain because it has not been demonstrated whether the same molecular mechanisms involved in memory consolidation in the hippocampus have separate roles in the PFC. However, animals and humans with either reduced or increased concentrations of intraneuronal *Arc* levels show cognitive disturbances associated with PFC dysfunction (Plath et al., 2006; Greer et al., 2010). In our study, novelty exposure also induced *Arc* expression in the hippocampus, which has been well-described in previous studies and underlies the consolidation of long-term memory (Vazdarjanova et al., 2002). However, ketanserin pretreatment did not block induction of the *Arc* response in hippocampus, pointing to a specific role of the 5-HT<sub>2A</sub>Rs in the novelty-induced *Arc* expression in the PFC.

We further investigated the effect of stress in our experiments because 5-HT<sub>2A</sub>R is involved in anxiety modulation (Weisstaub et al., 2006) and in direct activation of the HPA-axis (Zhang et al., 2002; Mikkelsen et al., 2004). Novel environment exposure is considered an emotional “processing” stressor because it requires cognitive processing but does not represent an immediate physical threat (Herman et al., 1996). However, exposure to a novel environment results in a marked activation of the HPA axis comparable to that seen for severe stressors, such as foot shock and immobilization (Mikkelsen and Larsen, 2006; Shanks et al., 1990). In our experimental setup plasma corticosterone levels were robustly upregulated in animals exposed to novelty compared to home cage controls (Figure 5). However, it is unlikely that 5-HT<sub>2A</sub>Rs effect on PFC activation is through regulation of the HPA axis, because ketanserin at doses blocking increase in *Arc* in the PFC had no effect on circulating corticosterone. In fact, exposure to anxiety or administration of pharmacological agents increasing anxiety induce activation of different subset of neurons in the dorsal raphe nucleus dependent on the stressor (Lowry et al., 2005; Abrams et al., 2005). As a major proportion of serotonergic neurons activated after novelty project to the basolateral amygdaloid

nucleus (Hale et al., 2008), it is conceivable that these neurons operate on the HPA axis independent of 5-HT<sub>2A</sub>R.



**Figure 5: Plasma corticosterone levels after novelty and pre-treatment with ketanserin** | Exposure to a novel environment increases plasma corticosterone levels from  $212.4 \pm 98.9$  nmol/L to  $510.4 \pm 181.1$  nmol/L. No differences were observed between novelty-exposed animals regardless of pretreatment, as 0.1 and 1 mg/kg ketanserin pretreatment yielded  $698.7 \pm 181.1$  and  $654.7 \pm 28.5$  respectively. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , one-way ANOVA with Tukey's post-hoc test. Data presented as mean  $\pm$  SEM.

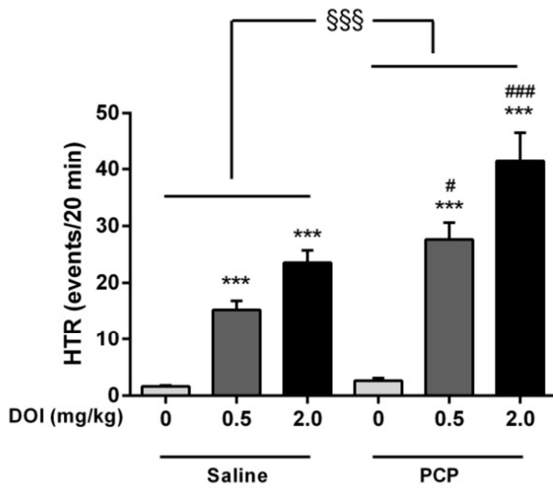
In summary, our results demonstrate that the induction of *Arc* in the PFC following exposure to a novel environment is dependent on 5-HT<sub>2A</sub>R, and suggests that the simultaneous release of corticosterone is regulated via another system independent of 5-HT<sub>2A</sub>R activation. Taken together, the data presented in paper I indicate an important role for the 5-HT<sub>2A</sub>R in the activation of PFC of animals exposed to a novel environment inducing an exploratory behavior.

### Enhanced 5-HT<sub>2A</sub>R functionality in the subchronic PCP model of schizophrenia

After determining that 5-HT<sub>2A</sub>R is involved in the function of the PFC we wanted to study whether 5-HT<sub>2A</sub>R-mediated signaling was affected in an animal model of schizophrenia. We primarily based this hypothesis on studies showing that activation of 5-HT<sub>2A</sub>R by LSD-like drugs induces a schizophrenia-like psychosis in humans (Vollenweider et al., 1998) and that most atypical antipsychotics exhibit high-affinity blocking of 5-HT<sub>2A</sub>Rs (Kapur et al., 1999). In this context 5-HT<sub>2A</sub>R in PFC could be important in the development of both positive and negative/cognitive symptoms in schizophrenia. Thus, the aim of the present study was to investigate if subchronic PCP administration changes expression, binding or functionality of cortical 5-HT<sub>2A</sub> receptors.

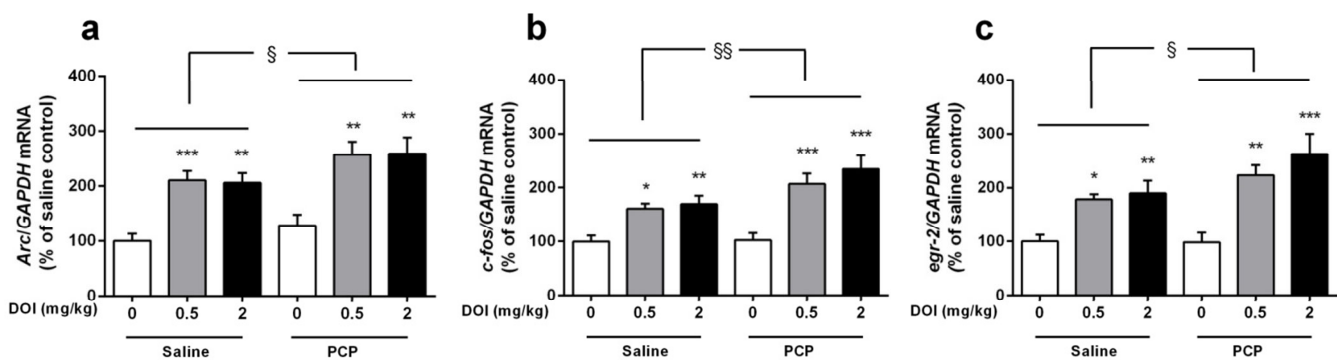
We focused on the repeated PCP administration paradigm in mice to evoke a psychosis-like phenotype. In humans, administration of PCP induces a broad range of behaviors resembling those seen in schizophrenic patients and exacerbate both positive and negative symptoms in patients (Malhotra et al., 1996; Malhotra et al., 1997; Jentsch and Roth, 1999). Consistent with the human literature, systemic administration of PCP to rodents causes molecular and behavioral changes resembling those found in schizophrenia patients (Moghaddam and Jackson, 2003; Mouri et al., 2007). In particular, PCP produce cognitive dysfunctions, such as impaired performance in working memory tasks and cause reduced social behavior (Vigano et al., 2009; Seillier and Giuffrida, 2009). Moreover, subchronic administration of PCP reduces markers for GABAergic interneurons, which are involved in the regulation of cortical circuit activity, especially by restraining excessive pyramidal neuron activity (Homayoun and Moghaddam, 2007; Amitai et al., 2012; Zhu et al., 2004).

Mice were treated with PCP (10 mg/kg) or saline for a total of 10 days. This treatment paradigm was selected based on its ability to induce cellular (reduction in parvalbumin and synaptophysin mRNA in PFC) and behavioral changes (impairment in Y-maze- and novel-object recognition test) relevant to schizophrenia (Thomsen et al., 2009; Hagiwara et al., 2008). Following a 5 day wash-out period the mice were injected with DOI (0.5 or 2.0 mg/kg i.p.) or a corresponding volume of vehicle (saline). As a measure of 5-HT<sub>2A</sub>R functionality we used DOI-induced HTR and expression of IEG mRNA in the PFC.



**Figure 6: Subchronic PCP administration increases DOI-induced HTR** | Animals were treated with PCP or vehicle for 10 days, followed by a five day wash-out period. Shown is graphical representation of total number of head-twitches after DOI administration (0.5 and 2 mg/kg). A significant increase in HTR was observed between the saline and PCP-pretreated groups ( $$$$P = 0.0065$ , two-way ANOVA). For both saline and PCP-pretreated animals, DOI highly induced head-twitches ( $***P < 0.001$ ). However, for both 0.5 and 2 mg/kg DOI, PCP pretreated animals showed a significant higher level of HTR ( $#P < 0.05$  for 0.5 mg/kg DOI and  $###P < 0.001$  for 2 mg/kg DOI, one-way ANOVA with Tukeys post-hoc test). Data presented as mean  $\pm$  SEM.

We found that mice treated subchronically with PCP exhibited enhanced absolute responses to DOI. We first tested whether subchronic PCP administration induced alterations in the response to the 5-HT<sub>2R</sub> agonist DOI. Two-way ANOVA analysis of HTR showed a significant interaction between saline and PCP pretreatment (Figure 6). DOI (both 0.5 and 2 mg/kg) highly induced head-twitches in both saline and PCP pretreated animals. However, DOI induced a significant higher level of head-twitches in mice pre-treated with PCP. We also tested the effect of subchronic PCP on DOI-induced IEG mRNA expression (*Arc*, *c-Fos* and *Egr-2*) in the same animals. As with the HTR, DOI strongly induced IEG expression in both saline and PCP pretreated animals (Figure 7), and two-way ANOVA showed a significant PCP  $\times$  DOI interaction, confirming that PCP-pretreatment augmented DOI-induced IEG expression.



**Figure 7: Repeated PCP pretreatment potentiates DOI-induced IEG mRNA expression** | Shown is a graphical representation of frontal cortex IEG mRNA expression from samples taken 60 minutes following DOI (0.5 and 2 mg/kg) or vehicle treatment. (a-c) For both saline and PCP pretreated animals, DOI highly induced *Arc*, *c-Fos* and *Egr-2* mRNA expression in frontal cortex. A significant increase in IEG mRNA levels was observed between saline and PCP-pretreated groups for *Arc* (Fig. 7a;  $*P = 0.0141$ , two-way ANOVA), *c-Fos* (Fig. 7b;  $##P = 0.0069$ , two-way ANOVA) and *Egr-2* mRNA (Fig. 7c;  $*P = 0.029$ , two-way ANOVA). Data presented as mean  $\pm$  SEM and as % of saline vehicle control.



Interestingly, these changes were not accompanied by differences in 5-HT<sub>2A</sub>R mRNA or 5-HT<sub>2A</sub>R binding levels in the frontal cortex. A correlation between 5-HT<sub>2A</sub>R binding and functionality has previously been described in a maternal influenza animal model of schizophrenia (Moreno et al., 2011) and both PSD-95 and Egr-3 KO mice have decreased 5-HT<sub>2A</sub>R binding and HTR (Abbas et al., 2009; Williams et al., 2012). However, changes in 5-HT<sub>2A</sub>R-mediated functionality without similar changes in binding have been reported in an early life stress model of psychiatric vulnerability (Benekareddy et al., 2010) and in transgene mice overexpressing the 5-HT transporter (Jennings et al., 2008). Further, 5-HT<sub>2A</sub>R levels were unaffected in mGlu2-KO mouse frontal cortex, although these mice exhibited diminished DOI-induced HTR and altered DOI-induced cortical IEG expression (Moreno et al., 2011). Together these studies suggest that changes in 5-HT<sub>2A</sub>R functionality are not necessarily associated with an equivalent change in receptor expression.

There are several mechanisms that can explain the missing correlation between 5-HT<sub>2A</sub>R functionality and the receptor binding potential observed in our study. First, a large pool of 5-HT<sub>2A</sub>Rs are located in intraneuronal endomembranes that bind to [<sup>3</sup>H]-labeled ligands, but are non-functional (Cornea-Hebert et al., 1999; Bhatnagar et al., 2001). Although these receptors contribute to the binding signal, they do not mediate intracellular signaling. Thus, the subchronic PCP treatment could lead to changes in membrane-bound 5-HT<sub>2A</sub>R levels without affecting the total receptor density.

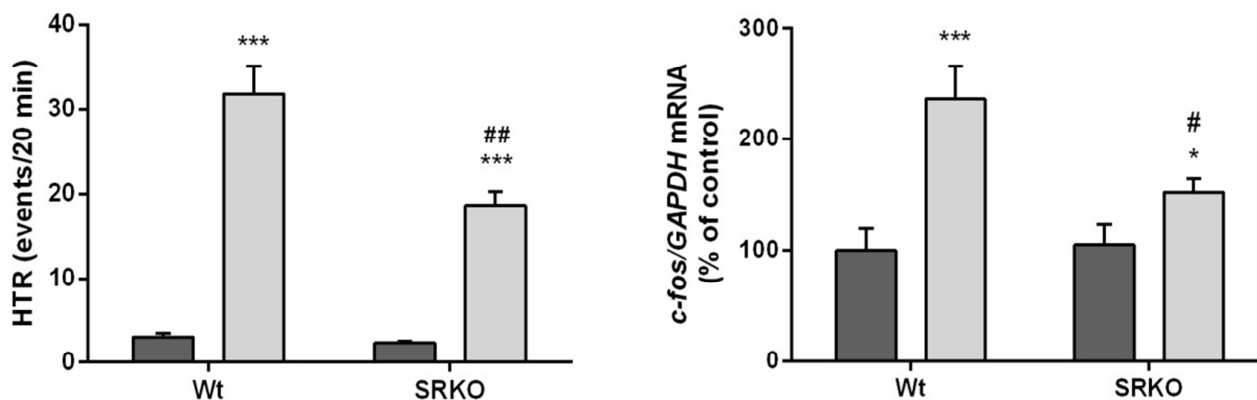
Second, even though activation of the 5-HT<sub>2A</sub> receptor signaling pathway is necessary for HTR, it has been suggested that other receptor systems can modulate the response. For example, HTR induced by DOI can be attenuated by modulating 5-HT<sub>1A</sub>R (Darmani et al., 1990a; Willins and Meltzer, 1997), 5-HT<sub>2C</sub>R (Canal et al., 2010), CB1 receptors (Darmani, 2001) and mGlu2 (Gewirtz and Marek, 2000). Although the neuropharmacological mechanisms underlying all of these interactions are not delineated, these data indicate that 5-HT<sub>2A</sub>R-mediated functionality is modulated through other receptor systems. However, we observed no differences in 5-HT<sub>1A</sub>R and SERT binding or 5-HT<sub>1A</sub>R and 5-HT<sub>2C</sub>R mRNA expression, suggesting that the 5-HT system is not majorly altered following subchronic PCP administration

Finally, the increased HTR and IEG mRNA expression after PCP pretreatment could be explained by changes in the glutamatergic tonus in the PFC. We observed an increase in the basal level of *Arc* mRNA expression in PFC after PCP treatment, which suggests an increase in excitatory tonus. *Arc* is a marker for excitatory neurotransmission, and *Arc* mRNA expression in the hippocampus is directly induced by NMDA receptor activation (Czerniawski et al., 2011). Similarly, DOI-induced HTR and *Arc* expression is dependent upon 5-HT<sub>2A</sub>R-mediated glutamate release and subsequent NMDA receptor activation in the PFC (Gonzalez-Maeso and Sealton, 2009). An enhanced glutamatergic tonus could thus produce an increase in baseline *Arc* mRNA expression and an enhanced DOI-induced HTR and *Arc* gene expression. However, this seems to be in conflict with the hypofrontality hypothesis of schizophrenia. This hypothesis was based on functional neuroimaging studies showing that patients exhibit reduced glucose utilization and blood flow in the prefrontal cortex (hypofrontality) (Franzen and Ingvar, 1974; Weinberger et al., 1986). Decreased glucose utilization in the prefrontal cortex has also been shown after repeated PCP treatment in rats (Cochran et al., 2003). Although this is the primary hypothesis of schizophrenia, a re-formulation has recently emerged, in which hyperactive glutamatergic neurons in several brain regions including the PFC may underlie the psychotic, cognitive and emotional manifestations in schizophrenia (Krystal et al., 2003; Moghaddam and Jackson, 2003). This is based on recent studies conducted in a developmental animal model of schizophrenia that exhibits cortical deficits resembling to those observed in schizophrenia and indicates that the hypofrontal state could be associated with a hyperactive PFC (Tseng et al., 2007; O'Donnell et al., 2002).

Disruption of GABAergic interneurons function may also contribute to initiate and sustain a hyperglutamatergic state in schizophrenia. Repeated PCP treatment causes a deficit of GABAergic interneurons, which leads to disinhibition of cortical pyramidal neurons through alterations in subcortical networks (Lewis, 2000). A reduction of this inhibitory tone may thus contribute to, and sustain, the hyperglutamatergic state in the PFC. Taken together, it is likely that subchronic PCP leads to a hyperglutamatergic PFC that augments the functional response to 5-HT<sub>2A</sub>R activation. In rodents, increases in 5-HT levels produced by the precursor L-5-hydroxytryptophan induce HTR in mice (Schmid et al., 2008), suggesting that subchronic PCP could also lead to a greater degree of 5-HT<sub>2A</sub>R responsiveness to the endogenous 5-HT. In summary, the results presented in manuscript II suggest that subchronic PCP treatment leads to an increased functionality of 5-HT<sub>2A</sub>R, which could account for some of the deficits observed in this animal model of schizophrenia.

### NMDA receptor hypofunction leads to attenuated 5-HT<sub>2A</sub>R functionality

In this part we wanted to study further the interaction between the 5-HT<sub>2A</sub>R and the glutamate system. More specifically we investigated the role of glutamate signaling in the functional responses to 5-HT<sub>2A</sub>R activation. 5-HT<sub>2A</sub>Rs are primarily expressed on apical dendrites of pyramidal neurons that has glutamate as its principal neurotransmitter (Jakab and Goldman-Rakic, 1998; Spratling, 2002). Recent evidence suggests that the cortical activation following 5-HT<sub>2A</sub>R stimulation is dependent on a cortical-cortical circuit (Gonzalez-Maeso et al., 2007). 5-HT<sub>2A</sub>R receptor activation by DOI significantly increases glutamate release and glutamatergic pyramidal neuronal activity in the medial PFC layer V (Celada et al., 2008; Beique et al., 2007; Marek et al., 2001). Further, DOI does not induce *Arc* directly in 5-HT<sub>2A</sub>R expressing neurons but in neurons containing AMPA and NMDA receptors (Pei et al., 2004), which suggests that certain actions of 5-HT<sub>2A</sub>Rs in this region may be mediated by the release of glutamate (Beique et al., 2007).



**Figure 8: DOI-induced HTR and *c-Fos* mRNA expression is reduced in SR<sup>-/-</sup> mice** | (a) Two-way ANOVA showed a significant interaction between treatment [ $F(1,19) = 131.4$ ;  $p < 0.0001$ ] and genotype [ $F(1,19) = 10.06$ ;  $p < 0.0050$ ]. For both WT and SR<sup>-/-</sup> animals, 2 mg/kg DOI highly induced head-twitches ( $^{***}P < 0.001$ ). However, DOI, showed a significant lower level of HTR ( $^{##}P < 0.0054$ ) (b) Two-way ANOVA showed an interaction between the effects of treatment [Fig. 2;  $F(1,26) = 19.81$ ;  $p = 0.0001$ ] and genotype [ $F(1,26) = 4.651$ ;  $p = 0.0405$ ]. DOI induced *c-Fos* mRNA in both WT and SR<sup>-/-</sup> mice ( $^{***}P < 0.001$  and  $^{*}P < 0.05$  for WT and SR<sup>-/-</sup> respectively, students t-test), however, the *post hoc* analysis revealed that DOI induced *c-Fos* mRNA to a lesser extent in SR<sup>-/-</sup> mice compared to wild-type ( $p = 0.0054$ ). Data presented as mean  $\pm$  SEM.

To directly study the involvement of the glutamate system in mediating the cellular and behavioral responses to 5-HT<sub>2A</sub>R activation, we used an animal model of NMDAR hypofunction, the constitutive SR<sup>-/-</sup> mouse. Activation of NMDARs requires the binding of either glycine or D-serine to the GMS of the NR1 subunit. SR<sup>-/-</sup> mice, which lack the ability to convert L-serine to D-serine,

have a >80% reduction in brain D-serine (Basu et al., 2009). Thus, SR<sup>-/-</sup> mice have impaired NMDAR function in the forebrain (Devito et al., 2011).

We found that SR<sup>-/-</sup> mice had attenuated functional responses to 5-HT<sub>2A</sub>R activation (Figure 8). Both cortical *c-Fos* mRNA induction and HTR after DOI treatment were significantly reduced in SR<sup>-/-</sup>, suggesting that glutamate signaling is necessary for at least some of the cellular and behavioral responses induced by 5-HT<sub>2A</sub>R activation. This is consistent with previous reports showing that DOI increases spontaneous and evoked excitatory currents in layer V pyramidal cells (Aghajanian and Marek, 1997; Aghajanian and Marek, 1999) and increases the firing rate of a majority of neurons (Puig et al., 2003). Further, DOI-mediated induction of *Arc* mRNA in rat PFC can be blocked by the NMDAR antagonist MK-801 (Pei et al., 2004), suggesting that the cellular effects of DOI are glutamate release mediated.

The mechanism behind HTR is more complex, but likely also involves glutamatergic signaling. Both systemic and direct injection of 5-HT<sub>2A</sub>R agonists into the PFC produce HTR (Willins and Meltzer, 1997), which is dependent exclusively on 5-HT<sub>2A</sub>R in the pyramidal neurons in the cerebral cortex (Gonzalez-Maeso et al., 2007). It is not – as for *Arc* mRNA (Pei et al., 2004) – possible to block DOI-induced HTR with MK-801, because NMDAR antagonists alone induce HTR. However, HTR and hyperlocomotion induced by acute PCP can be blocked by 5-HT<sub>2A</sub>R antagonists (Kitaichi et al., 1994; O'Neill et al., 1998; Nabeshima et al., 1987), and 5-HT<sub>2A</sub>R antagonists are able to reverse acute PCP-induced cellular disruptions in PFC (Kargieman et al., 2007). Together, these findings indicate that the cellular and behavioral responses induced by acute PCP are mediated through circuits that are modulated by 5-HT<sub>2A</sub>R.

The decreased *c-Fos* mRNA induction and HTR following administration of DOI led us to examine the level of expression of 5-HT<sub>2A</sub> and mGluR2 receptors in the SR<sup>-/-</sup> mice. We found no changes in the binding of [<sup>3</sup>H]-MDL100907 and [<sup>3</sup>H]-LY341495 – measuring 5-HT<sub>2A</sub>R and mGluR2, respectively, in neocortex, S1 cortex or hippocampus. These results suggest that the functional deficits observed in SR<sup>-/-</sup> mice are not caused directly by changes in receptor density, but likely due to decreased secondary activation of post-synaptic NMDARs.

SR<sup>-/-</sup> mice exhibit reduced forebrain NMDAR-mediated neurotransmission leading to impaired LTP at the Schaffer collateral-CA1 pyramidal neuron synapse (Basu et al., 2009). It is conceivable that this reduction in NMDAR-mediated signaling is also present in the cortex, the brain region responsible for the cellular and behavioral effects of 5-HT<sub>2A</sub>R activation (Gonzalez-Maeso et al., 2007).

These results further highlights that the functional responses to 5-HT<sub>2A</sub>R activation can be regulated without concomitant changes in receptor density. Taken together, the results presented in manuscript III suggest a prominent role of NMDARs in regulating the cellular and behavioral responses to 5-HT<sub>2A</sub>R activation, and support the hypothesis presented in paper II that 5-HT<sub>2A</sub>R-mediated functionality is dependent on the glutamatergic tonus in PFC.

### **mGluR2/3 activation attenuates restraint stress- and DOI-induced c-Fos expression**

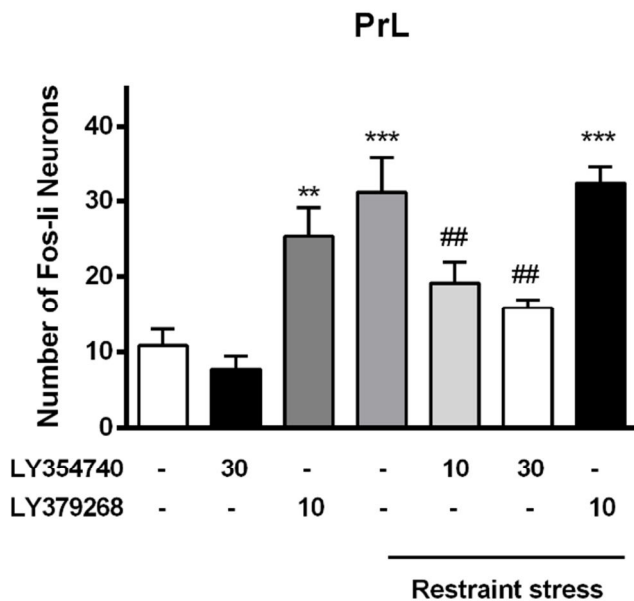
In this study we investigated the interaction between 5-HT<sub>2A</sub>R and mGluR2 in animal models relevant to both antipsychotic and anxiolytic activity. Recent evidence suggesting that hyperactivity of the glutamatergic systems in the limbic cortex may contribute to the symptoms of schizophrenia and anxiety (Harvey and Shahid, 2011; Krystal et al., 1999). Activation of presynaptic mGluR2 receptors with mGluR2/3 agonists negatively modulates the release of glutamate (Anwyl, 1999; Scanziani et al., 1997) and attenuates 5-HT<sub>2A</sub>R-mediated signaling through a direct interaction (Gonzalez-Maeso et al., 2008), together providing feedback that prevents excessive glutamate release.

The first part of the study evaluated the efficacy of two mGluR2/3 agonists (LY354740 and LY379268) in attenuating restraint stress-induced neuronal activation as measured by c-Fos protein expression. Restraint stress mediate fear potentiation in the elevated plus-maze (Heinrichs et al., 1994) and robustly increase circulating levels of ACTH and corticosterone (Mikkelsen and Larsen, 2006). Extensive characterization of restraint-induced c-Fos expression in rat forebrain has previously been reported (Cullinan et al., 1995; Girotti et al., 2006; Imaki et al., 1993; Weinberg et al., 2007) and the suppression of stress-induced c-Fos expression in the rat brain has been associated with the anxiolytic effects of benzodiazepines in the fear conditioning model of anxiety (Beck and Fibiger, 1995).

Consistent with previous reports we observed an increase in c-Fos (Cullinan et al., 1995; Girotti et al., 2006; Trneckova et al., 2006; Weinberg et al., 2007) expression in the forebrain following restraint stress (Figure 9). Interestingly, pretreatment with LY354740 had a strong and dose-dependent inhibitory effect on stress-induced gene expression, while LY379268 had no effect. In fact, LY379268 alone caused c-Fos upregulation in PFC to a comparable degree as restraint stress at high dose. These results clearly show that LY354740 and LY379268 – although highly structurally related – have different properties.

These results are in line with behavioral experiments where the properties of LY354740 and LY379268 seem to differ. LY354740 shows activity in a wide variety of anxiety models (such as fear-induced potentiated startle (Helton et al., 1998), elevated plus maze (Helton et al., 1998; Monn et al., 1997) and stress-induced hyperthermia) and improves the symptoms of patients with generalized anxiety disorder with similar efficacy as a benzodiazepine comparator (Dunayevich et

al., 2008). However, although only a few reports have evaluated the anxiolytic properties of LY379268, the compound does not have identical effects. While LY379268 reduces stress-induced hyperthermia (Satow et al., 2008) and inhibits immobilization-induced hyperprolactinemia (Johnson and Chamberlain, 2002), it has no effect in the elevated plus maze (Satow et al., 2008) and might even have anxiogenic properties, as it increases startle reflex magnitude (Imre et al., 2006).

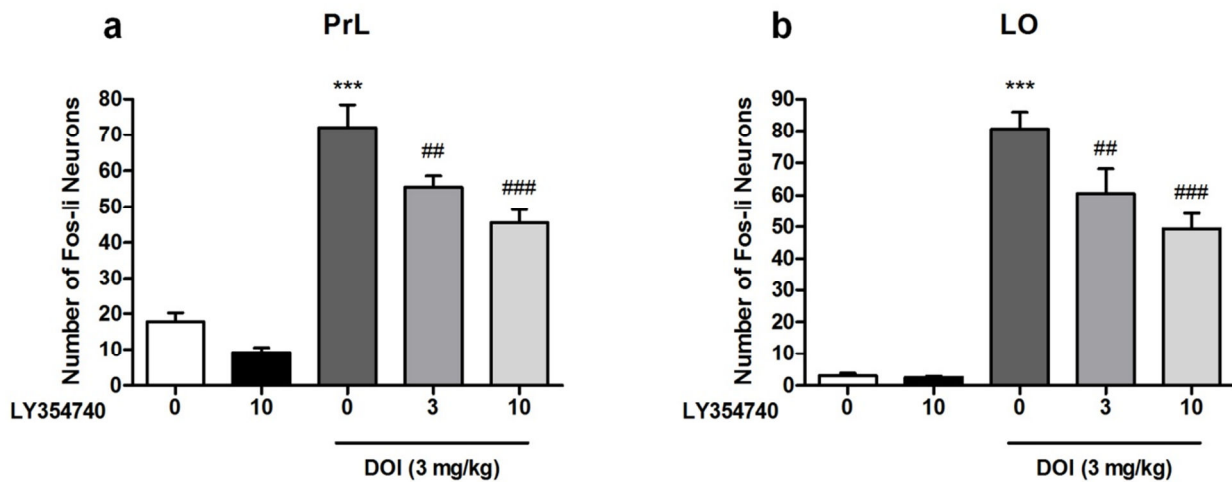


**Figure 9: LY354740 and LY379268 show different effects on restraint stress-induced c-Fos expression |** Restraint stress upregulated c-Fos in the PrL cortex (\*\* $P < 0.001$ ). Pretreatment with LY354740 (10 and 30 mg/kg) attenuated the c-Fos response (\*\* $P < 0.01$ ). Pretreatment with LY379268 (10 mg/kg, i.p.) had no effect on restraint-induced c-Fos expression and in itself induced c-Fos in PrL cortex (\*\* $P < 0.01$ ). One-way ANOVA with Newman-Keuls post-hoc test. Data presented as mean  $\pm$  SEM.

The reason for the difference in *in vivo* efficacy between the two compounds is still speculative. However, LY354740 and LY379268 differ somewhat in their *in vitro* potencies at mGlu2 vs. mGlu3 receptors (Monn et al., 1999). While LY354740 is approximately equipotent at mGlu2 and mGlu3, LY379268 is overall more potent at both receptor subtypes, showing a 5x higher potency at mGlu2 and a 16x higher potency at mGlu3 compared to LY354740 (Monn et al., 1999). This difference in *in vitro* profile may account for differences *in vivo*, including brain 2-deoxyglucose utilization studies in the rat, where the LY354740 shows a more general suppression of glucose use across different brain areas (Lam et al., 1999).

Finally, we investigated the interaction between 5-HT<sub>2A</sub>R and mGlu2/3 receptors, because recent studies suggest that the effects of mGlu2/3 agonists are partly mediated through 5-HT<sub>2A</sub> receptors (Gonzalez-Maeso et al., 2008; Fribourg et al., 2011). Previously, LY379268 has been shown to decrease DOI-induced c-Fos expression in PFC (Wischhof and Koch, 2012). We demonstrated the ability of LY354740 to attenuate the DOI-induced c-Fos expression in the rat PrL and LO cortex (Figure 10). Because increased excitation of the PFC has been implicated in the pathophysiology of

schizophrenia, the ability of LY354740 to reduce the hallucinogenic drug action in this region could be directly related to its antipsychotic-like efficacy (Benneyworth et al., 2007).



**Figure 10: Effect of LY354740 on DOI-induced c-Fos expression** | DOI treatment (3 mg/kg) produced an increase in c-Fos in the PrL (a) and LO (b) cortex. (\*\* $P < 0.001$ ) Pretreatment with LY354740 (3 and 10 mg/kg) attenuated the DOI-induced increase in the PrL and LO cortex (## $P < 0.01$ , ### $P < 0.001$ ). One-way ANOVA with Newman-Keuls post-hoc test. Data presented as mean  $\pm$  SEM.

Antidepressants with anxiolytic properties act in part by blocking the activation of the serotonin 5-HT<sub>2A</sub> receptors (Croom et al., 2009), and mice lacking functional 5-HT<sub>2A</sub> receptors have reduced anxiety-like behaviors (Weisstaub et al., 2006). Further, the ability of both LY354740 and LY379268 to attenuate DOI-induced c-Fos suggests that this c-Fos response is caused by a different mechanism than for restraint stress.

In perspective, the selective modulation of restraint stress- and DOI-induced c-Fos expression in the limbic cortex provides additional evidence that mGlu2/3 agonists may serve as an effective therapeutic strategy for preferentially targeting the glutamatergic dysfunction in schizophrenia and anxiety. Thus, the results from our studies provide further insight in the utility of mGlu2/3 orthosteric agonists in the treatment of a variety of psychiatric conditions including schizophrenia and anxiety.



## Conclusions and Perspectives

The results presented in this thesis have added further knowledge regarding the role of 5-HT<sub>2A</sub>R in PFC function. In manuscript I we show that 5-HT<sub>2A</sub>R is involved in the activation of PFC following exposure to a novel environment. The PFC plays a central role in working memory and cognitive flexibility (Gruber et al., 2010), executive tasks which are impaired in patients with schizophrenia (Manoach, 2003). Thus, the ability of ketanserin and MDL100907 to fully inhibit novelty-induced *Arc* expression suggests that 5-HT<sub>2A</sub>R activation is necessary for activation of the PFC circuits mediating the behavioural response to novelty and highlights the 5-HT<sub>2A</sub>R as important for the normal function of PFC. However, exposure to a novel environment also result in release of dopamine and other neurotransmitters in PFC (Feenstra et al., 1999). This opens up for the possibility that the effect of 5-HT<sub>2A</sub>R antagonism is through modulation of others transmitter systems in the PFC. Further, exposure to a novel environment is considered to have both an exploratory and a stressful component (Prut and Belzung, 2003). To further elucidate in which of these responses 5-HT<sub>2A</sub>R regulate PFC function, various paradigms could be used to isolate the stressful and exploratory component. These include environmental enrichment and novel objects for exploration and restraint stress and physical stress for purely stressful stimuli.

In manuscript II, we demonstrate that the functionality of cortical 5-HT<sub>2A</sub> receptors is enhanced in the subchronic PCP rodent model of schizophrenia. We propose that this is due to glutamatergic hyperactivity in PFC, which has also been observed in patients with schizophrenia (Krystal et al., 2003). However, the changes in the glutamate system could be at receptor, intracellular, release or circuit level, which makes it exceedingly difficult to investigate in further detail. However, as 5-HT<sub>2A</sub>R activation by 5-HT can mediate a hallucinogenic response (Schmid and Bohn, 2010), our results could explain some of the behavioral deficits observed this animal model.

Interestingly, we did not observe a correlation between functional receptor output (HTR and IEG mRNA induction) and 5-HT<sub>2A</sub>R binding. This has also been observed in other studies (Benekareddy et al., 2010; Jennings et al., 2008) and suggests that 5-HT<sub>2A</sub>R binding might be a poor predictor of 5-HT<sub>2A</sub>R signaling *in vivo*. This is particularly important for 5-HT<sub>2A</sub>R due to the high proportion of intracellular receptors (Cornea-Hebert et al., 1999).

We expanded our studies of the role of glutamate signaling in 5-HT<sub>2A</sub>R functionality in manuscript III. Here we show that SR<sup>-/-</sup> mice that exhibit reduced NMDAR signaling also have attenuated

functional 5-HT<sub>2A</sub>R-mediated responses. This further highlights the interaction between the 5-HT<sub>2A</sub>R and the glutamate system. In addition, the reduction in 5-HT<sub>2A</sub>R functionality was not related to changes in binding, underscoring that these two measures not necessarily correlate.

The SR enzyme has been identified as a risk gene for the development of schizophrenia (Labrie et al., 2009) and the SR<sup>-/-</sup> mice were thus generated as a model of schizophrenia (Basu et al., 2009). We observed decreased 5-HT<sub>2A</sub>R functionality in this model, while the reverse was the case in the subchronic PCP model of schizophrenia-like symptoms. However, the discrepancies might reflect differences in the symptoms observed in these models. While PCP treatment is considered to be a model of the positive symptoms of schizophrenia (Enomoto et al., 2007), the deficits of SR<sup>-/-</sup> in hippocampus could reflect negative/cognitive symptoms to a larger degree.

Finally, in manuscript IV, we show that the two orthosteric mGluR2/3 agonists LY354740 and LY379268 exhibit different properties in attenuating restraint stress-induced c-Fos upregulation although they are highly structurally similar and show the same properties in attenuating DOI-induced c-Fos expression. These results indicate that restraint stress and DOI treatment activate the cortex through independent pathways and highlight that the mechanism behind the proposed anxiolytic and antipsychotic properties of mGluR2/3 agonists is very complex.

## References

- Abbas AI, Yadav PN, Yao WD, Arbuckle MI, Grant SG, Caron MG, Roth BL, 2009. PSD-95 is essential for hallucinogen and atypical antipsychotic drug actions at serotonin receptors. *J. Neurosci.* 29: 7124-7136.
- Abrams JK, Johnson PL, Hay-Schmidt A, Mikkelsen JD, Shekhar A, Lowry CA, 2005. Serotonergic systems associated with arousal and vigilance behaviors following administration of anxiogenic drugs. *Neuroscience* 133: 983-997.
- Adams LM, Geyer MA, 1985. Patterns of exploration in rats distinguish lisuride from lysergic acid diethylamide. *Pharmacol. Biochem. Behav.* 23: 461-468.
- Adell A, Casanovas JM, Artigas F, 1997. Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. *Neuropharmacology* 36: 735-741.
- Aghajanian GK, Marek GJ, 1997. Serotonin induces excitatory postsynaptic potentials in apical dendrites of neocortical pyramidal cells. *Neuropharmacology* 36: 589-599.
- Aghajanian GK, Marek GJ, 1999. Serotonin, via 5-HT<sub>2A</sub> receptors, increases EPSCs in layer V pyramidal cells of prefrontal cortex by an asynchronous mode of glutamate release. *Brain Res.* 825: 161-171.
- Allen JA, Yadav PN, Roth BL, 2008. Insights into the regulation of 5-HT<sub>2A</sub> serotonin receptors by scaffolding proteins and kinases. *Neuropharmacology*.
- Aloyo VJ, Dave KD, 2007. Behavioral response to emotional stress in rabbits: role of serotonin and serotonin<sub>2A</sub> receptors. *Behav. Pharmacol.* 18: 651-659.
- Amitai N, Kuczenski R, Behrens MM, Markou A, 2012. Repeated phencyclidine administration alters glutamate release and decreases GABA markers in the prefrontal cortex of rats. *Neuropharmacology* 62: 1422-1431.
- Andreasen NC, Carpenter WT, Jr., 1993. Diagnosis and classification of schizophrenia. *Schizophr. Bull.* 19: 199-214.
- Andree TH, Mikuni M, Tong CY, Koenig JI, Meltzer HY, 1986. Differential effect of subchronic treatment with various neuroleptic agents on serotonin<sub>2</sub> receptors in rat cerebral cortex. *J. Neurochem.* 46: 191-197.
- Arnsten AF, 2011. Prefrontal cortical network connections: key site of vulnerability in stress and schizophrenia. *Int. J. Dev. Neurosci.* 29: 215-223.
- Artigas F, 2010. The prefrontal cortex: a target for antipsychotic drugs. *Acta Psychiatr. Scand.* 121: 11-21.
- Balu DT, Basu AC, Corradi JP, Cacace AM, Coyle JT, 2012. The NMDA receptor co-agonists, D-serine and glycine, regulate neuronal dendritic architecture in the somatosensory cortex. *Neurobiol. Dis.* 45: 671-682.
- Basu AC, Tsai GE, Ma CL, Ehmsen JT, Mustafa AK, Han L, Jiang ZI, Benneyworth MA, Froimowitz MP, Lange N, Snyder SH, Bergeron R, Coyle JT, 2009. Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. *Mol. Psychiatry* 14: 719-727.
- Beaulieu C, 1993. Numerical data on neocortical neurons in adult rat, with special reference to the GABA population. *Brain Res.* 609: 284-292.

- Beique JC, Imad M, Mladenovic L, Gingrich JA, Andrade R, 2007. Mechanism of the 5-hydroxytryptamine 2A receptor-mediated facilitation of synaptic activity in prefrontal cortex. *Proc Natl Acad Sci U S A* 104: 9870-5.
- Benekareddy M, Goodfellow NM, Lambe EK, Vaidya VA, 2010. Enhanced function of prefrontal serotonin 5-HT(2) receptors in a rat model of psychiatric vulnerability. *J. Neurosci.* 30: 12138-12150.
- Benneyworth MA, Xiang Z, Smith RL, Garcia EE, Conn PJ, Sanders-Bush E, 2007. A selective positive allosteric modulator of metabotropic glutamate receptor subtype 2 blocks a hallucinogenic drug model of psychosis. *Mol. Pharmacol.* 72: 477-484.
- Bhatnagar A, Willins DL, Gray JA, Woods J, Benovic JL, Roth BL, 2001. The dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT<sub>2A</sub>) serotonin receptors reveals differential sorting of arrestins and 5-HT<sub>2A</sub> receptors during endocytosis. *J. Biol. Chem.* 276: 8269-8277.
- Blaazer AR, Smid P, Kruse CG, 2008. Structure-activity relationships of phenylalkylamines as agonist ligands for 5-HT(2A) receptors. *ChemMedChem.* 3: 1299-1309.
- Bouwknicht JA, Spiga F, Staub DR, Hale MW, Shekhar A, Lowry CA, 2007. Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors: relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. *Brain Res. Bull.* 72: 32-43.
- Brown AS, 2011. Exposure to prenatal infection and risk of schizophrenia. *Front Psychiatry* 2: 63.
- Canal CE, Olaghere da Silva UB, Gresch PJ, Watt EE, Sanders-Bush E, Airey DC, 2010. The serotonin 2C receptor potently modulates the head-twitch response in mice induced by a phenethylamine hallucinogen. *Psychopharmacology (Berl)* 209: 163-174.
- Cartmell J, Schoepp DD, 2000. Regulation of neurotransmitter release by metabotropic glutamate receptors. *J. Neurochem.* 75: 889-907.
- Celada P, Puig MV, az-Mataix L, Artigas F, 2008. The hallucinogen DOI reduces low-frequency oscillations in rat prefrontal cortex: reversal by antipsychotic drugs. *Biol. Psychiatry* 64: 392-400.
- Chrousos GP, 1998. Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture. *Ann. N. Y. Acad. Sci.* 851: 311-335.
- Cochran SM, Kennedy M, McKerchar CE, Steward LJ, Pratt JA, Morris BJ, 2003. Induction of metabolic hypofunction and neurochemical deficits after chronic intermittent exposure to phencyclidine: differential modulation by antipsychotic drugs. *Neuropsychopharmacology* 28: 265-275.
- Corne SJ, Pickering RW, 1967. A possible correlation between drug-induced hallucinations in man and a behavioural response in mice. *Psychopharmacologia.* 11: 65-78.
- Cornea-Hebert V, Riad M, Wu C, Singh SK, Descarries L, 1999. Cellular and subcellular distribution of the serotonin 5-HT<sub>2A</sub> receptor in the central nervous system of adult rat. *J Comp Neurol* 409: 187-209.
- Creese I, Burt DR, Snyder SH, 1976. Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. *Science* 192: 481-483.
- Croom KF, Perry CM, Plosker GL, 2009. Mirtazapine: a review of its use in major depression and other psychiatric disorders. *CNS. Drugs* 23: 427-452.

- Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ, 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64: 477-505.
- Czerniawski J, Ree F, Chia C, Ramamoorthi K, Kumata Y, Otto TA, 2011. The importance of having Arc: expression of the immediate-early gene Arc is required for hippocampus-dependent fear conditioning and blocked by NMDA receptor antagonism. *J. Neurosci.* 31: 11200-11207.
- Dalley JW, Cardinal RN, Robbins TW, 2004. Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. *Neurosci. Biobehav. Rev.* 28: 771-784.
- Darmani NA, 2001. Cannabinoids of diverse structure inhibit two DOI-induced 5-HT(2A) receptor-mediated behaviors in mice. *Pharmacol. Biochem. Behav.* 68: 311-317.
- Darmani NA, Martin BR, Pandey U, Glennon RA, 1990a. Do functional relationships exist between 5-HT1A and 5-HT2 receptors? *Pharmacol. Biochem. Behav.* 36: 901-906.
- Darmani NA, Martin BR, Pandey U, Glennon RA, 1990b. Pharmacological characterization of ear-scratch response in mice as a behavioral model for selective 5-HT2-receptor agonists and evidence for 5-. *Pharmacol. Biochem. Behav.* 37: 95-99.
- Dean B, 2003. The cortical serotonin2A receptor and the pathology of schizophrenia: a likely accomplice. *J. Neurochem.* 85: 1-13.
- Devito LM, Balu DT, Kanter BR, Lykken C, Basu AC, Coyle JT, Eichenbaum H, 2011. Serine racemase deletion disrupts memory for order and alters cortical dendritic morphology. *Genes Brain Behav.* 10: 210-222.
- Ebdrup BR, Rasmussen H, Arnt JR, Glenth JB, 2011. Serotonin 2A receptor antagonists for treatment of schizophrenia. *Expert. Opin. Investig. Drugs.*
- Eisenstein SA, Clapper JR, Holmes PV, Piomelli D, Hohmann AG, 2010. A role for 2-arachidonoylglycerol and endocannabinoid signaling in the locomotor response to novelty induced by olfactory bulbectomy. *Pharmacol. Res.* 61: 419-429.
- Elizalde N, Pastor PM, Garcia-Garcia AL, Serres F, Venzala E, Huarte J, Ramirez MJ, Del RJ, Sharp T, Tordera RM, 2010. Regulation of markers of synaptic function in mouse models of depression: chronic mild stress and decreased expression of VGLUT1. *J. Neurochem.*
- Enomoto T, Noda Y, Nabeshima T, 2007. Phencyclidine and genetic animal models of schizophrenia developed in relation to the glutamate hypothesis. *Methods Find. Exp. Clin. Pharmacol.* 29: 291-301.
- Feenstra MG, Teske G, Botterblom MH, De Bruin JP, 1999. Dopamine and noradrenaline release in the prefrontal cortex of rats during classical aversive and appetitive conditioning to a contextual stimulus: interference by novelty effects. *Neurosci. Lett.* 272: 179-182.
- Fox JW, 1990. Social class, mental illness, and social mobility: the social selection-drift hypothesis for serious mental illness. *J. Health Soc. Behav.* 31: 344-353.
- Franzen G, Ingvar DH, 1974. [Abnormalities in cerebral blood flow in chronic schizophrenia]. *Lakartidningen* 71: 1300-1302.
- Fribourg M, Moreno JL, Holloway T, Provasi D, Baki L, Mahajan R, Park G, Adney SK, Hatcher C, Eltit JM, Ruta JD, Albizu L, Li Z, Umali A, Shim J, Fabiato A, MacKerell AD, Jr., Brezina V, Sealfon SC,

- Filizola M, Gonzalez-Maeso J, Logothetis DE, 2011. Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell* 147: 1011-1023.
- Fuster JM, 1997. Network memory. *Trends Neurosci.* 20: 451-459.
- Gelber EI, Kroeze WK, Willins DL, Gray JA, Sinar CA, Hyde EG, Gurevich V, Benovic J, Roth BL, 1999. Structure and function of the third intracellular loop of the 5-hydroxytryptamine<sub>2A</sub> receptor: the third intracellular loop is alpha-helical and binds purified arrestins. *J. Neurochem.* 72: 2206-2214.
- Gewirtz JC, Marek GJ, 2000. Behavioral evidence for interactions between a hallucinogenic drug and group II metabotropic glutamate receptors. *Neuropsychopharmacology* 23: 569-576.
- Girotti M, Pace TW, Gaylord RI, Rubin BA, Herman JP, Spencer RL, 2006. Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. *Neuroscience* 138: 1067-1081.
- Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, Zhou M, Okawa Y, Callado LF, Milligan G, Gingrich JA, Filizola M, Meana JJ, Sealfon SC, 2008. Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452: 93-7.
- Gonzalez-Maeso J, Sealfon SC, 2009. Psychedelics and schizophrenia. *Trends Neurosci.* 32: 225-232.
- Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC, Gingrich JA, 2007. Hallucinogens recruit specific cortical 5-HT<sub>2A</sub> receptor-mediated signaling pathways to affect behavior. *Neuron* 53: 439-452.
- Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, Weisstaub N, Hen R, Gingrich JA, Sealfon SC, 2003. Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. *J. Neurosci.* 23: 8836-8843.
- Gouzoulis-Mayfrank E, Heekeren K, Neukirch A, Stoll M, Stock C, Obradovic M, Kovar KA, 2005. Psychological effects of (S)-ketamine and N,N-dimethyltryptamine (DMT): a double-blind, cross-over study in healthy volunteers. *Pharmacopsychiatry* 38: 301-311.
- Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, Kim TK, Griffith EC, Waldon Z, Maehr R, Ploegh HL, Chowdhury S, Worley PF, Steen J, Greenberg ME, 2010. The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell* 140: 704-716.
- Gruber AJ, Calhoun GG, Shusterman I, Schoenbaum G, Roesch MR, O'Donnell P, 2010. More is less: a disinhibited prefrontal cortex impairs cognitive flexibility. *J. Neurosci.* 30: 17102-17110.
- Guzowski JF, McNaughton BL, Barnes CA, Worley PF, 1999. Environment-specific expression of the immediate-early gene *Arc* in hippocampal neuronal ensembles. *Nat. Neurosci.* 2: 1120-1124.
- Hagiwara H, Fujita Y, Ishima T, Kunitachi S, Shirayama Y, Iyo M, Hashimoto K, 2008. Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of the antipsychotic drug perospirone: role of serotonin 5-HT<sub>1A</sub> receptors. *Eur. Neuropsychopharmacol.* 18: 448-454.
- Hajszan T, Leranath C, Roth RH, 2006. Subchronic phencyclidine treatment decreases the number of dendritic spine synapses in the rat prefrontal cortex. *Biol. Psychiatry* 60: 639-644.

- Hale MW, Hay-Schmidt A, Mikkelsen JD, Poulsen B, Bouwknecht JA, Evans AK, Stamper CE, Shekhar A, Lowry CA, 2008. Exposure to an open-field arena increases c-Fos expression in a subpopulation of neurons in the dorsal raphe nucleus, including neurons projecting to the basolateral amygdaloid complex. *Neuroscience* 157: 733-748.
- Harrison PJ, 1999. The neuropathology of schizophrenia. A critical review of the data and their interpretation. *Brain* 122 ( Pt 4): 593-624.
- Harvey BH, Shahid M, 2011. Metabotropic and ionotropic glutamate receptors as neurobiological targets in anxiety and stress-related disorders: Focus on pharmacology and preclinical translational models. *Pharmacol. Biochem. Behav.*
- Hashimoto K, Fujita Y, Shimizu E, Iyo M, 2005. Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of clozapine, but not haloperidol. *Eur. J. Pharmacol.* 519: 114-117.
- Heinrichs SC, Menzaghi F, Pich EM, Baldwin HA, Rassnick S, Britton KT, Koob GF, 1994. Anti-stress action of a corticotropin-releasing factor antagonist on behavioral reactivity to stressors of varying type and intensity. *Neuropsychopharmacology* 11: 179-186.
- Herman JP, Prewitt CM, Cullinan WE, 1996. Neuronal circuit regulation of the hypothalamo-pituitary-adrenocortical stress axis. *Crit Rev. Neurobiol.* 10: 371-394.
- Hill SK, Bishop JR, Palumbo D, Sweeney JA, 2010. Effect of second-generation antipsychotics on cognition: current issues and future challenges. *Expert. Rev. Neurother.* 10: 43-57.
- Homayoun H, Moghaddam B, 2007. NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J. Neurosci.* 27: 11496-11500.
- Hoover WB, Vertes RP, 2007. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Struct. Funct.* 212: 149-179.
- Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP, 1994. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.* 46: 157-203.
- Ihalainen JA, Riekkinen P, Jr., Feenstra MG, 1999. Comparison of dopamine and noradrenaline release in mouse prefrontal cortex, striatum and hippocampus using microdialysis. *Neurosci. Lett.* 277: 71-74.
- Inoue T, Tsuchiya K, Koyama T, 1994. Regional changes in dopamine and serotonin activation with various intensity of physical and psychological stress in the rat brain. *Pharmacol. Biochem. Behav.* 49: 911-920.
- Jakab RL, Goldman-Rakic PS, 1998. 5-Hydroxytryptamine<sub>2A</sub> serotonin receptors in the primate cerebral cortex: possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites. *Proc. Natl. Acad. Sci. U. S. A* 95: 735-740.
- Jennings KA, Sheward WJ, Harmar AJ, Sharp T, 2008. Evidence that genetic variation in 5-HT transporter expression is linked to changes in 5-HT<sub>2A</sub> receptor function. *Neuropharmacology* 54: 776-783.
- Jentsch JD, Roth RH, 1999. The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* 20: 201-225.

- Johnson JW, Ascher P, 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325: 529-531.
- Kapur S, Zipursky RB, Remington G, 1999. Clinical and theoretical implications of 5-HT<sub>2</sub> and D<sub>2</sub> receptor occupancy of clozapine, risperidone, and olanzapine in schizophrenia. *Am. J. Psychiatry* 156: 286-293.
- Kargieman L, Santana N, Mengod G, Celada P, Artigas F, 2007. Antipsychotic drugs reverse the disruption in prefrontal cortex function produced by NMDA receptor blockade with phencyclidine. *Proc. Natl. Acad. Sci. U. S. A* 104: 14843-14848.
- Keller DL, Umbreit WW, 1956. Permanent alteration of behavior in mice by chemical and psychological means. *Science* 124: 723-724.
- Kinon BJ, Zhang L, Millen BA, Osuntokun OO, Williams JE, Kollack-Walker S, Jackson K, Kryzhanovskaya L, Jarkova N, 2011. A multicenter, inpatient, phase 2, double-blind, placebo-controlled dose-ranging study of LY2140023 monohydrate in patients with DSM-IV schizophrenia. *J. Clin. Psychopharmacol.* 31: 349-355.
- Kitaichi K, Yamada K, Hasegawa T, Furukawa H, Nabeshima T, 1994. Effects of risperidone on phencyclidine-induced behaviors: comparison with haloperidol and ritanserin. *Jpn. J. Pharmacol.* 66: 181-189.
- Klebaur JE, Ostrander MM, Norton CS, Watson SJ, Akil H, Robinson TE, 2002. The ability of amphetamine to evoke arc (Arg 3.1) mRNA expression in the caudate, nucleus accumbens and neocortex is modulated by environmental context. *Brain Res.* 930: 30-36.
- Kleckner NW, Dingledine R, 1988. Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science* 241: 835-837.
- Konstandi M, Johnson E, Lang MA, Malamas M, Marselos M, 2000. Noradrenaline, dopamine, serotonin: different effects of psychological stress on brain biogenic amines in mice and rats. *Pharmacol. Res.* 41: 341-346.
- Krystal JH, D'Souza DC, Mathalon D, Perry E, Belger A, Hoffman R, 2003. NMDA receptor antagonist effects, cortical glutamatergic function, and schizophrenia: toward a paradigm shift in medication development. *Psychopharmacology (Berl)* 169: 215-233.
- Krystal JH, D'Souza DC, Petrakis IL, Belger A, Berman RM, Charney DS, bi-Saab W, Madonick S, 1999. NMDA agonists and antagonists as probes of glutamatergic dysfunction and pharmacotherapies in neuropsychiatric disorders. *Harv. Rev. Psychiatry* 7: 125-143.
- Kumakura K, Nomura H, Toyoda T, Hashikawa K, Noguchi T, Takeda K, Ichijo H, Tsunoda M, Funatsu T, Ikegami D, Narita M, Suzuki T, Matsuki N, 2010. Hyperactivity in novel environment with increased dopamine and impaired novelty preference in apoptosis signal-regulating kinase 1 (ASK1)-deficient mice. *Neurosci. Res.* 66: 313-320.
- Kurrasch-Orbaugh DM, Parrish JC, Watts VJ, Nichols DE, 2003. A complex signaling cascade links the serotonin<sub>2A</sub> receptor to phospholipase A<sub>2</sub> activation: the involvement of MAP kinases. *J. Neurochem.* 86: 980-991.
- Labrie V, Fukumura R, Rastogi A, Fick LJ, Wang W, Boutros PC, Kennedy JL, Semeralul MO, Lee FH, Baker GB, Belsham DD, Barger SW, Gondo Y, Wong AH, Roder JC, 2009. Serine racemase is associated with schizophrenia susceptibility in humans and in a mouse model. *Hum. Mol. Genet.* 18: 3227-3243.



- Lahti AC, Koffel B, LaPorte D, Tamminga CA, 1995. Subanesthetic doses of ketamine stimulate psychosis in schizophrenia. *Neuropsychopharmacology* 13: 9-19.
- Lam AG, Monn JA, Schoepp DD, Lodge D, McCulloch J, 1999. Group II selective metabotropic glutamate receptor agonists and local cerebral glucose use in the rat. *J. Cereb. Blood Flow Metab* 19: 1083-1091.
- Laurent V, Podhorna J, 2004. Subchronic phencyclidine treatment impairs performance of C57BL/6 mice in the attentional set-shifting task. *Behav. Pharmacol.* 15: 141-148.
- Leucht S, Arbter D, Engel RR, Kissling W, Davis JM, 2009. How effective are second-generation antipsychotic drugs? A meta-analysis of placebo-controlled trials. *Mol. Psychiatry* 14: 429-447.
- Levy R, Goldman-Rakic PS, 2000. Segregation of working memory functions within the dorsolateral prefrontal cortex. *Exp. Brain Res.* 133: 23-32.
- Lewis DA, 2000. GABAergic local circuit neurons and prefrontal cortical dysfunction in schizophrenia. *Brain Res. Brain Res. Rev.* 31: 270-276.
- LeySEN JE, Van GP, Gommeren W, Woestenborghs R, Janssen PA, 1986. Down regulation of serotonin-S2 receptor sites in rat brain by chronic treatment with the serotonin-S2 antagonists: ritanserin and setoperone. *Psychopharmacology (Berl)* 88: 434-444.
- Lisman JE, Coyle JT, Green RW, Javitt DC, Benes FM, Heckers S, Grace AA, 2008. Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. *Trends Neurosci.* 31: 234-242.
- Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A, 2005. Modulation of anxiety circuits by serotonergic systems. *Stress.* 8: 233-246.
- Malhotra AK, Pinals DA, Adler CM, Elman I, Clifton A, Pickar D, Breier A, 1997. Ketamine-induced exacerbation of psychotic symptoms and cognitive impairment in neuroleptic-free schizophrenics. *Neuropsychopharmacology* 17: 141-150.
- Malhotra AK, Pinals DA, Weingartner H, Sirocco K, Missar CD, Pickar D, Breier A, 1996. NMDA receptor function and human cognition: the effects of ketamine in healthy volunteers. *Neuropsychopharmacology* 14: 301-307.
- Manoach DS, 2003. Prefrontal cortex dysfunction during working memory performance in schizophrenia: reconciling discrepant findings. *Schizophr. Res.* 60: 285-298.
- Marek GJ, 2010. Metabotropic glutamate<sub>2/3</sub> (mGlu<sub>2/3</sub>) receptors, schizophrenia and cognition. *Eur. J. Pharmacol.* 639: 81-90.
- Marek GJ, Wright RA, Gewirtz JC, Schoepp DD, 2001. A major role for thalamocortical afferents in serotonergic hallucinogen receptor function in the rat neocortex. *Neuroscience* 105: 379-392.
- Marek GJ, Wright RA, Schoepp DD, Monn JA, Aghajanian GK, 2000. Physiological antagonism between 5-hydroxytryptamine(2A) and group II metabotropic glutamate receptors in prefrontal cortex. *J. Pharmacol. Exp. Ther.* 292: 76-87.
- Martinez ZA, Ellison GD, Geyer MA, Swerdlow NR, 1999. Effects of sustained phencyclidine exposure on sensorimotor gating of startle in rats. *Neuropsychopharmacology* 21: 28-39.

- Meltzer HY, Matsubara S, Lee JC, 1989. Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin<sub>2</sub> pKi values. *J. Pharmacol. Exp. Ther.* 251: 238-246.
- Mengod G, Vilaro MT, Raurich A, Lopez-Gimenez JF, Cortes R, Palacios JM, 1996. 5-HT receptors in mammalian brain: receptor autoradiography and in situ hybridization studies of new ligands and newly identified receptors. *Histochem. J.* 28: 747-758.
- Mikkelsen JD, Hay-Schmidt A, Kiss A, 2004. Serotonergic stimulation of the rat hypothalamo-pituitary-adrenal axis: interaction between 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptors. *Ann. N. Y. Acad. Sci.* 1018: 65-70.
- Mikkelsen JD, Larsen MH, 2006. Effects of stress and adrenalectomy on activity-regulated cytoskeleton protein (Arc) gene expression. *Neurosci. Lett.* 403: 239-243.
- Miyashita T, Kubik S, Lewandowski G, Guzowski JF, 2008. Networks of neurons, networks of genes: an integrated view of memory consolidation. *Neurobiol. Learn. Mem.* 89: 269-284.
- Moghaddam B, 1993. Stress preferentially increases extraneuronal levels of excitatory amino acids in the prefrontal cortex: comparison to hippocampus and basal ganglia. *J. Neurochem.* 60: 1650-1657.
- Moghaddam B, Jackson ME, 2003. Glutamatergic animal models of schizophrenia. *Ann. N. Y. Acad. Sci.* 1003: 131-137.
- Monn JA, Valli MJ, Massey SM, Hansen MM, Kress TJ, Wepsiec JP, Harkness AR, Grutsch JL, Jr., Wright RA, Johnson BG, Andis SL, Kingston A, Tomlinson R, Lewis R, Griffey KR, Tizzano JP, Schoepp DD, 1999. Synthesis, pharmacological characterization, and molecular modeling of heterobicyclic amino acids related to (+)-2-aminobicyclo[3.1.0] hexane-2,6-dicarboxylic acid (LY354740): identification of two new potent, selective, and systemically active agonists for group II metabotropic glutamate receptors. *J. Med. Chem.* 42: 1027-1040.
- Moreno JL, Holloway T, Albizu L, Sealfon SC, Gonzalez-Maeso J, 2011. Metabotropic glutamate mGlu<sub>2</sub> receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT<sub>2A</sub> receptor agonists. *Neurosci. Lett.* 493: 76-79.
- Morris BJ, Cochran SM, Pratt JA, 2005. PCP: from pharmacology to modelling schizophrenia. *Curr. Opin. Pharmacol.* 5: 101-106.
- Morris RG, Anderson E, Lynch GS, Baudry M, 1986. Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* 319: 774-776.
- Mouri A, Noda Y, Enomoto T, Nabeshima T, 2007. Phencyclidine animal models of schizophrenia: approaches from abnormality of glutamatergic neurotransmission and neurodevelopment. *Neurochem. Int.* 51: 173-184.
- Muguruza C, Moreno JL, Umali A, Callado LF, Meana JJ, Gonzalez-Maeso J, 2012. Dysregulated 5-HT<sub>2A</sub> receptor binding in postmortem frontal cortex of schizophrenic subjects. *Eur. Neuropsychopharmacol.*
- Mulle JG, 2012. Schizophrenia genetics: progress, at last. *Curr. Opin. Genet. Dev.* 22: 238-244.
- Muller JE, Koen L, Soraya S, Emsley RA, Stein DJ, 2004. Anxiety disorders and schizophrenia. *Curr. Psychiatry Rep.* 6: 255-261.
- Murray CJ, Lopez AD, 1997. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 349: 1498-504.

- Nabeshima T, Ishikawa K, Yamaguchi K, Furukawa H, Kameyama T, 1987. Phencyclidine-induced head-twitch responses as 5-HT<sub>2</sub> receptor-mediated behavior in rats. *Neurosci. Lett.* 76: 335-338.
- Nichols DE, 2004. Hallucinogens. *Pharmacol. Ther.* 101: 131-181.
- Nolan T, Hands RE, Bustin SA, 2006. Quantification of mRNA using real-time RT-PCR. *Nat Protoc* 1: 1559-82.
- O'Donnell P, Lewis BL, Weinberger DR, Lipska BK, 2002. Neonatal hippocampal damage alters electrophysiological properties of prefrontal cortical neurons in adult rats. *Cereb. Cortex* 12: 975-982.
- O'Neill MF, Hicks CA, Shaw G, Parameswaran T, Cardwell GP, O'Neill MJ, 1998. Effects of 5-hydroxytryptamine<sub>2</sub> receptor antagonism on the behavioral activation and immediate early gene expression induced by dizocilpine. *J. Pharmacol. Exp. Ther.* 287: 839-846.
- Ons S, Marti O, Armario A, 2004. Stress-induced activation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA. *J. Neurochem.* 89: 1111-1118.
- Otani S, 2004 *Prefrontal Cortex - From synaptic plasticity to cognition.* Kluwer Academic Publishers.
- Patterson PH, 2009. Immune involvement in schizophrenia and autism: etiology, pathology and animal models. *Behav. Brain Res.* 204: 313-321.
- Paz RD, Tardito S, Atzori M, Tseng KY, 2008. Glutamatergic dysfunction in schizophrenia: from basic neuroscience to clinical psychopharmacology. *Eur. Neuropsychopharmacol.* 18: 773-786.
- Pei Q, Tordera R, Sprakes M, Sharp T, 2004. Glutamate receptor activation is involved in 5-HT<sub>2</sub> agonist-induced Arc gene expression in the rat cortex. *Neuropharmacology* 46: 331-339.
- Peyron C, Petit JM, Rampon C, Jouviet M, Luppi PH, 1998. Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience* 82: 443-468.
- Pinaud R, Penner MR, Robertson HA, Currie RW, 2001. Upregulation of the immediate early gene arc in the brains of rats exposed to environmental enrichment: implications for molecular plasticity. *Brain Res. Mol. Brain Res.* 91: 50-56.
- Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A, Mahlke C, Welzl H, Kobalz U, Stawrakakis A, Fernandez E, Waltereit R, Bick-Sander A, Therstappen E, Cooke SF, Blanquet V, Wurst W, Salmen B, Bosl MR, Lipp HP, Grant SG, Bliss TV, Wolfer DP, Kuhl D, 2006. Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron* 52: 437-444.
- Prut L, Belzung C, 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur. J. Pharmacol.* 463: 3-33.
- Puig MV, Celada P, az-Mataix L, Artigas F, 2003. In vivo modulation of the activity of pyramidal neurons in the rat medial prefrontal cortex by 5-HT<sub>2A</sub> receptors: relationship to thalamocortical afferents. *Cereb. Cortex* 13: 870-882.
- Puig MV, Gullledge AT, 2011. Serotonin and prefrontal cortex function: neurons, networks, and circuits. *Mol. Neurobiol.* 44: 449-464.

- Rao DC, Morton NE, Gottesman II, Lew R, 1981. Path analysis of qualitative data on pairs of relatives: application to schizophrenia. *Hum. Hered.* 31: 325-333.
- Rasmussen H, Erritzoe D, Andersen R, Ebdrup B, Aggernaes B, Oranje B, Kalbitzer J, Madsen J, Pinborg L, Baaré W, Svarer C, Lublin H, Knudsen GM, Glenthoj B, 2009. Decreased frontal 5-HT<sub>2A</sub> receptor binding in antipsychotic-naïve first-episode schizophrenia patients. *Arch Gen Psychiatry.*
- Robbins TW, 2000. From arousal to cognition: the integrative position of the prefrontal cortex. *Prog. Brain Res.* 126: 469-483.
- Roozendaal B, 2002. Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. *Neurobiol. Learn. Mem.* 78: 578-595.
- Roth BL, Nakaki T, Chuang DM, Costa E, 1984. Aortic recognition sites for serotonin (5HT) are coupled to phospholipase C and modulate phosphatidylinositol turnover. *Neuropharmacology* 23: 1223-1225.
- Sanchez MM, Ladd CO, Plotsky PM, 2001. Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Dev. Psychopathol.* 13: 419-449.
- Schmid CL, Bohn LM, 2010. Serotonin, but not N-methyltryptamines, activates the serotonin 2A receptor via a ss-arrestin2/Src/Akt signaling complex in vivo. *J. Neurosci.* 30: 13513-13524.
- Schmid CL, Raehal KM, Bohn LM, 2008. Agonist-directed signaling of the serotonin 2A receptor depends on beta-arrestin-2 interactions in vivo. *Proc. Natl. Acad. Sci. U. S. A* 105: 1079-1084.
- Schmittgen TD, Livak KJ, 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3: 1101-1108.
- Schoepp DD, Jane DE, Monn JA, 1999. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* 38: 1431-1476.
- Seamans JK, Lapish CC, Durstewitz D, 2008. Comparing the prefrontal cortex of rats and primates: insights from electrophysiology. *Neurotox. Res.* 14: 249-262.
- Seillier A, Giuffrida A, 2009. Evaluation of NMDA receptor models of schizophrenia: divergences in the behavioral effects of sub-chronic PCP and MK-801. *Behav. Brain Res.* 204: 410-415.
- Selemon LD, Goldman-Rakic PS, 1999. The reduced neuropil hypothesis: a circuit based model of schizophrenia. *Biol. Psychiatry* 45: 17-25.
- Shanks N, Griffiths J, Zalzman S, Zacharko RM, Anisman H, 1990. Mouse strain differences in plasma corticosterone following uncontrollable footshock. *Pharmacol. Biochem. Behav.* 36: 515-519.
- Shepherd JD, Rumbaugh G, Wu J, Chowdhury S, Plath N, Kuhl D, Huganir RL, Worley PF, 2006. Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. *Neuron* 52: 475-484.
- Silva MT, Calil HM, 1975. Screening hallucinogenic drugs: systematic study of three behavioral tests. *Psychopharmacologia.* 42: 163-171.
- Snigdha S, Horiguchi M, Huang M, Li Z, Shahid M, Neill JC, Meltzer HY, 2010. Attenuation of phencyclidine-induced object recognition deficits by the combination of atypical antipsychotic drugs and pimavanserin (ACP 103), a 5-hydroxytryptamine(2A) receptor inverse agonist. *J. Pharmacol. Exp. Ther.* 332: 622-631.

- Snyder SH, 1972. Catecholamines in the brain as mediators of amphetamine psychosis. *Arch Gen Psychiatry* 27: 169-179.
- Spratling MW, 2002. Cortical region interactions and the functional role of apical dendrites. *Behav. Cogn Neurosci. Rev.* 1: 219-228.
- Stefansson H, Rujescu D, Cichon S, Pietilainen OP, Ingason A, Steinberg S, Fossdal R, Sigurdsson E, Sigmundsson T, Buizer-Voskamp JE, Hansen T, Jakobsen KD, Muglia P, Francks C, Matthews PM, Gylfason A, Halldorsson BV, Gudbjartsson D, Thorgeirsson TE, Sigurdsson A, Jonasdottir A, Jonasdottir A, Bjornsson A, Mattiasdottir S, Blondal T, Haraldsson M, Magnusdottir BB, Giegling I, Moller HJ, Hartmann A, Shianna KV, Ge D, Need AC, Crombie C, Fraser G, Walker N, Lonnqvist J, Suvisaari J, Tuulio-Henriksson A, Paunio T, Touloupoulou T, Bramon E, Di FM, Murray R, Ruggeri M, Vassos E, Tosato S, Walshe M, Li T, Vasilescu C, Muhleisen TW, Wang AG, Ullum H, Djurovic S, Melle I, Olesen J, Kiemenev LA, Franke B, Sabatti C, Freimer NB, Gulcher JR, Thorsteinsdottir U, Kong A, Andreassen OA, Ophoff RA, Georgi A, Rietschel M, Werge T, Petursson H, Goldstein DB, Nothen MM, Peltonen L, Collier DA, St CD, Stefansson K, 2008. Large recurrent microdeletions associated with schizophrenia. *Nature* 455: 232-236.
- Steward O, Worley P, 2002. Local synthesis of proteins at synaptic sites on dendrites: role in synaptic plasticity and memory consolidation? *Neurobiol. Learn. Mem.* 78: 508-527.
- Takei N, Mortensen PB, Klaening U, Murray RM, Sham PC, O'Callaghan E, Munk-Jorgensen P, 1996. Relationship between in utero exposure to influenza epidemics and risk of schizophrenia in Denmark. *Biol. Psychiatry* 40: 817-824.
- Thomas HV, Dalman C, David AS, Gentz J, Lewis G, Allebeck P, 2001. Obstetric complications and risk of schizophrenia. Effect of gender, age at diagnosis and maternal history of psychosis. *Br. J. Psychiatry* 179: 409-414.
- Thomsen MS, Christensen DZ, Hansen HH, Redrobe JP, Mikkelsen JD, 2009. alpha(7) Nicotinic acetylcholine receptor activation prevents behavioral and molecular changes induced by repeated phencyclidine treatment. *Neuropharmacology* 56: 1001-1009.
- Trneckova L, Armario A, Hynie S, Sida P, Klenerova V, 2006. Differences in the brain expression of c-fos mRNA after restraint stress in Lewis compared to Sprague-Dawley rats. *Brain Res.* 1077: 7-15.
- Trulson ME, Howell GA, 1984. Ontogeny of the behavioral effects of lysergic acid diethylamide in cats. *Dev. Psychobiol.* 17: 329-346.
- Tseng KY, Lewis BL, Lipska BK, O'Donnell P, 2007. Post-pubertal disruption of medial prefrontal cortical dopamine-glutamate interactions in a developmental animal model of schizophrenia. *Biol. Psychiatry* 62: 730-738.
- Uylings HB, Groenewegen HJ, Kolb B, 2003. Do rats have a prefrontal cortex? *Behav. Brain Res.* 146: 3-17.
- Vazdarjanova A, McNaughton BL, Barnes CA, Worley PF, Guzowski JF, 2002. Experience-dependent coincident expression of the effector immediate-early genes arc and Homer 1a in hippocampal and neocortical neuronal networks. *J. Neurosci.* 22: 10067-10071.
- Vazdarjanova A, Ramirez-Amaya V, Insel N, Plummer TK, Rosi S, Chowdhury S, Mikhael D, Worley PF, Guzowski JF, Barnes CA, 2006. Spatial exploration induces ARC, a plasticity-related immediate-early gene, only in calcium/calmodulin-dependent protein kinase II-positive principal excitatory and inhibitory neurons of the rat forebrain. *J. Comp Neurol.* 498: 317-329.

- Vigano D, Guidali C, Petrosino S, Realini N, Rubino T, Di M, V, Parolaro D, 2009. Involvement of the endocannabinoid system in phencyclidine-induced cognitive deficits modelling schizophrenia. *Int. J. Neuropsychopharmacol.* 12: 599-614.
- Vollenweider FX, Vollenweider-Scherpenhuyzen MF, Babler A, Vogel H, Hell D, 1998. Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2 agonist action. *Neuroreport* 9: 3897-3902.
- Weinberg MS, Girotti M, Spencer RL, 2007. Restraint-induced fra-2 and c-fos expression in the rat forebrain: relationship to stress duration. *Neuroscience* 150: 478-486.
- Weinberger DR, Berman KF, Zec RF, 1986. Physiologic dysfunction of dorsolateral prefrontal cortex in schizophrenia. I. Regional cerebral blood flow evidence. *Arch Gen Psychiatry* 43: 114-124.
- Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP, Sibille E, Underwood M, Itohara S, Dauer WT, Ansorge MS, Morelli E, Mann JJ, Toth M, Aghajanian G, Sealfon SC, Hen R, Gingrich JA, 2006. Cortical 5-HT<sub>2A</sub> receptor signaling modulates anxiety-like behaviors in mice. *Science* 313: 536-540.
- Wilcox JN, 1993. Fundamental principles of in situ hybridization. *J. Histochem. Cytochem.* 41: 1725-1733.
- Williams AA, Ingram WM, Levine S, Resnik J, Kamel CM, Lish JR, Elizalde DI, Janowski SA, Shoker J, Kozlenkov A, Gonzalez-Maeso J, Gallitano AL, 2012. Reduced levels of serotonin 2A receptors underlie resistance of Egr3-deficient mice to locomotor suppression by clozapine. *Neuropsychopharmacology* 37: 2285-2298.
- Willins DL, Meltzer HY, 1997. Direct injection of 5-HT<sub>2A</sub> receptor agonists into the medial prefrontal cortex produces a head-twitch response in rats. *J. Pharmacol. Exp. Ther.* 282: 699-706.
- Wischhof L, Koch M, 2012. Pre-treatment with the mGlu<sub>2/3</sub> receptor agonist LY379268 attenuates DOI-induced impulsive responding and regional c-Fos protein expression. *Psychopharmacology (Berl)* 219: 387-400.
- Wisden W, Morris BJ, 2002. In situ hybridization with oligonucleotide probes. *Int. Rev. Neurobiol.* 47: 3-59.
- Wood JN, 2003. Social cognition and the prefrontal cortex. *Behav. Cogn Neurosci. Rev.* 2: 97-114.
- Wood JN, Grafman J, 2003. Human prefrontal cortex: processing and representational perspectives. *Nat. Rev. Neurosci.* 4: 139-147.
- Xia Z, Gray JA, Compton-Toth BA, Roth BL, 2003. A direct interaction of PSD-95 with 5-HT<sub>2A</sub> serotonin receptors regulates receptor trafficking and signal transduction. *J Biol Chem* 278: 21901-8.
- Zhai Y, George CA, Zhai J, Nisenbaum ES, Johnson MP, Nisenbaum LK, 2003. Group II metabotropic glutamate receptor modulation of DOI-induced c-fos mRNA and excitatory responses in the cerebral cortex. *Neuropsychopharmacology* 28: 45-52.
- Zhang Y, Damjanoska KJ, Carrasco GA, Dudas B, D'Souza DN, Tetzlaff J, Garcia F, Hanley NR, Scripathirathan K, Petersen BR, Gray TS, Battaglia G, Muma NA, Van de Kar LD, 2002. Evidence that 5-HT<sub>2A</sub> receptors in the hypothalamic paraventricular nucleus mediate neuroendocrine responses to (-)DOI. *J. Neurosci.* 22: 9635-9642.
- Zhu Y, Stornetta RL, Zhu JJ, 2004. Chandelier cells control excessive cortical excitation: characteristics of whisker-evoked synaptic responses of layer 2/3 nonpyramidal and pyramidal neurons. *J. Neurosci.* 24: 5101-5108.

## Appendices

### Manuscripts I-IV

- I. Santini MA, Klein AB, El-Sayed M, Ratner C, Knudsen GM, Mikkelsen JD, Aznar S (2011) Novelty-induced activity-regulated cytoskeletal-associated protein (Arc) expression in frontal cortex requires serotonin 2A receptor activation, *Neuroscience*, 190, 251-257
- II. Santini MA, Ratner C, Aznar S, Klein AB, Knudsen GM, Mikkelsen JD (2012) Enhanced prefrontal serotonin 2A receptor signaling in the subchronic PCP mouse model of schizophrenia, *Accepted, Journal of Neuroscience Research*
- III. Santini MA, Balu DT, Hill-Smith TE, Lucki I, Mikkelsen JD, Coyle JT (2012) Forebrain NMDA receptor hypofunction attenuates pharmacological and behavioral effects induced by the hallucinogenic 5-HT<sub>2A</sub> receptor agonist DOI, *Manuscript*
- IV. Svensson KA, Santini MA, Mikkelsen JD (2012) The mGlu<sub>2/3</sub> Receptor Agonists LY354740 and LY379268 Differentially Regulate Restraint Stress-induced Expression of c-Fos in Rat Cerebral Cortex, *Manuscript*

### Co-author statements

# *Manuscript I*

Santini MA, Klein AB, El-Sayed M, Ratner C, Knudsen GM, Mikkelsen JD, Aznar S (2011) Novelty-induced activity-regulated cytoskeletal-associated protein (Arc) expression in frontal cortex requires serotonin 2A receptor activation, *Neuroscience*, 190, 251-257



## NOVELTY-INDUCED ACTIVITY-REGULATED CYTOSKELETAL-ASSOCIATED PROTEIN (ARC) EXPRESSION IN FRONTAL CORTEX REQUIRES SEROTONIN 2A RECEPTOR ACTIVATION

M. A. SANTINI,\* A. B. KLEIN, M. EL-SAYED, C. RATNER, G. M. KNUDSEN, J. D. MIKKELSEN AND S. AZNAR

Neurobiology Research Unit and Center for Integrated Molecular Brain Imaging (Cimbi), Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark

**Abstract**—Many psychiatric disorders are characterized by cognitive and emotional alterations that are related to abnormal function of the frontal cortex (FC). FC is involved in working memory and decision making and is activated following exposure to a novel environment. The serotonin 2A receptor (5-HT<sub>2A</sub>R) is highly expressed in the FC where its activation induces hallucinations, while blockade of 5-HT<sub>2A</sub>Rs contributes to the therapeutic effects of atypical antipsychotic drugs. The purpose of the present study was to investigate the involvement of 5-HT<sub>2A</sub>R in FC activation following exposure to a novel environment. As an output of FC activation we measured expression of activity-regulated cytoskeletal-associated protein (*Arc*). Novelty-exposure (open-field arena) robustly up-regulated FC *Arc* mRNA expression (~160%) in mice compared to home-cage controls. This response was inhibited with the 5-HT<sub>2A</sub>R antagonists ketanserin and MDL100907, but not with the selective 5-HT<sub>2C</sub>R antagonist SB242084. Novelty-exposure also induced *Arc* mRNA expression in hippocampus (~150%), but not in cerebellum or brainstem. Pretreatment with 5-HT<sub>2A</sub>R antagonist ketanserin did not repress the *Arc* induction in hippocampus, indicating that the involvement of 5-HT<sub>2A</sub>R in this response is restricted to the FC. Similarly, the novelty-induced stress as determined by increasing levels of plasma corticosterone, was not influenced by 5-HT<sub>2A</sub>R antagonism suggesting that *Arc* mRNA and stress are activated via distinct mechanisms. Taken together, our results demonstrate that the induction of *Arc* in the FC following exposure to a novel environment is dependent on the 5-HT<sub>2A</sub>R, and that the simultaneous release of corticosterone is regulated via another system independent of 5-HT<sub>2A</sub>R activation. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** 5-HT<sub>2A</sub>, serotonin, novelty, IEG, c-fos, mouse.

The serotonin 2A receptor (5-HT<sub>2A</sub>R) is highly expressed in the frontal cortex (FC) (Cornea-Hebert et al., 1999), a crucial brain region involved in working memory, attention and decision making (Robbins, 2000). Recent studies have revealed that as in hippocampus FC networks can

undergo dynamic neuronal adaptation processes through the induction of synaptic plasticity. The actions of 5-HT<sub>2A</sub>R signaling in FC are at least partly mediated through release of glutamate (Beique et al., 2007). This excitatory activity can in turn lead to increases in the expression of immediate-early genes (IEGs), which act as the earliest genomic response to synaptic activity (Clayton, 2000). The cascade of events initiated by rapid induction of these gene products are important for the long-term functions of FC such as synaptic plasticity (Goto et al., 2010), and compromised induction of synaptic plasticity in the FC is seen in schizophrenia (Goto et al., 2010). These studies support that 5-HT<sub>2A</sub>R signaling is important for FC mediated behaviour and that this receptor is an important pharmacological target for the treatment of neuropsychiatric disorders.

Activity-regulated cytoskeletal-associated protein (*Arc*) is a brain-specific effector IEG product that plays a key role in the activity-dependent synaptic modifications underlying memory consolidation (Steward and Worley, 2002). Because of its engagement in structural synaptic plasticity, *Arc* is suitable for investigation of neuronal networks that underlie information processing (Bramham et al., 2008). *Arc* induction in response to physiological stimuli takes place in several cortical and subcortical areas in the rodent brain and shows a more region-specific pattern of expression than *c-fos*, a widely used IEG for brain activation mapping (Mikkelsen and Larsen, 2006; Ons et al., 2004, 2010).

Exposure to a novel environment induces *Arc* expression in a number of cortical subregions including the medial prefrontal cortex (mPFC), parietal cortex and the hippocampus (Klebaur et al., 2002; Vazdarjanova et al., 2002). Under the exposure to the open field, the FC is engaged in the evaluation of the stressfulness of the situation (Robbins, 2000). Thus, the exposure to novelty initiates a number of events that integrates risk assessment behaviour where both rewarding and aversive outcomes are possible. Accordingly, both the FC and the hypothalamo-pituitary adrenocortical (HPA) axis will be activated. Exposure to a novel environment activates 5-HT neurons in a subset of the dorsal raphe nucleus and increases extracellular 5-HT concentrations in the FC (Adell et al., 1997; Lowry et al., 2005). These data support the hypothesis that exposure to a novel environment requiring active exploration releases 5-HT which in turn induces *Arc* expression in the FC. However, it is not known which 5-HT receptor in the FC mediate the changes in IEG expression, but the 5-HT<sub>2A</sub>R was considered to be an attractive candidate due to its role in FC neuronal control. The aim of this

\*Corresponding author. Tel: +45-3545-6736; fax: +45-3545-6713. E-mail address: santini@nru.dk (M. A. Santini).

**Abbreviations:** ACTH, adrenocorticotrophic hormone; *Arc*, activity-regulated cytoskeletal-associated protein; BDNF, brain-derived neurotrophic factor; CREB, cyclic AMP response element binding protein; Egr-2, early growth response gene-2; FC, frontal cortex; HPA, hypothalamic-pituitary-adrenal axis; IEG, immediate-early genes; LSD, lysergic acid diethylamide; mPFC, medial prefrontal cortex; PFC, prefrontal cortex; 5-HT<sub>2A</sub>R, serotonin 2A receptor.

study was therefore to characterize to what extent 5-HT<sub>2A</sub>Rs are involved in FC activation induced by exploration of a novel environment.

## EXPERIMENTAL PROCEDURES

### Animals

All animal experiments were carried out in accordance with the regulations provided by the Danish Animal Experimentation Inspectorate under the Ministry of Justice. Eight weeks-old male B6D2F1 mice (Charles River Laboratories, Sulzfeld, Germany) were group housed (six in each cage) and kept in a controlled environment with a 12/12 h light/dark cycle, provided with standard rodent diet and water *ad libitum*. The animals were allowed to acclimatize in the animal facility for at least 5 days prior to the day of the experiment.

### Drugs

Ketanserin tartrate [3-{2-[4-(4-fluorobenzoyl)piperidin-1-yl]ethyl}quinazoline-2,4(1*H*,3*H*)-dione] (Sigma-Aldrich, St Louis, MO, USA), MDL100907 [(R)-(+)-alpha-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol] (ABX, Radeberg, Germany) and SB242084 hydrochloride [6-chloro-5-methyl-N-[6-[(2-methylpyridin-3-yl)oxy]pyridin-3-yl]indoline-1-carboxamide] (Tocris Ltd, Bristol, UK) were dissolved 1:20 in dimethyl sulfoxide and 0.9% saline. DOI (Sigma-Aldrich) hydrochloride [(±)-2,5-dimethoxy-4-iodoamphetamine] was dissolved in 0.9% saline.

### Experimental paradigm

Animals were randomly assigned into treatment groups, which were either subjected to a novel environment or placed in their respective home-cages to function as baseline controls. Each treatment group was placed in separate cages to prevent the response of one group to influence the response of another. The experiments were repeated several times to rule out cage-specific effects.

The mice were injected (10 ml/kg, i.p.) with vehicle or experimental drug and returned to their home-cage. After 30 min the novelty groups were exposed to a novel environment by placing them in the middle of an open field (40×40×80 cm<sup>3</sup>), situated in a dimly lit room (16.6 lux at the bottom of the field), while the controls stayed in their home-cages. After 5 min the novelty-treated mice were returned to their respective home-cage for another 30 min before being euthanized.

For DOI treatment, the mice were injected with DOI (2 mg/kg, 10 ml/kg, i.p.) or vehicle (10 ml/kg, i.p.) and returned to their cages for 60 min before being euthanized.

All mice were killed by cervical dislocation and the brains quickly removed and dissected on ice. Trunk blood from the same animals were collected in K<sub>2</sub>EDTA-coated tubes and centrifuged for 10 min at 3000 g to obtain plasma. The blood collection and brain dissection was performed in a room separate from the experiments.

### Quantitative assessment of mRNA levels

Total RNA was isolated with Trizol Reagent (Sigma-Aldrich). The RNA samples were dissolved in RNase-free water and RNA was quantified with UV-spectrophotometry at 260 nm. Extracted RNA was reverse transcribed into single-stranded cDNA using the procedure of the supplier (cat.#: A3800, ImProm-II<sup>TM</sup> reverse transcription system, Promega, Madison, WI, USA). In brief, experimental RNA solution was combined with oligo(dT)<sub>15</sub> primers and heated at 70 °C for 5 min. The reverse transcription reaction mixture contained 20% ImProm-II<sup>TM</sup> 5× reaction buffer, 6 mM MgCl<sub>2</sub>, 0.5 mM dNTP mix and 20 units RNase inhibitor. The reverse transcription reaction was performed at 42 °C for 60 min, followed by heating at 70 °C for 15 min.

The real-time qPCR reactions were performed by adding the sample cDNA to a reaction mixture consisting of 1× Brilliant II SYBR green mastermix (Stratagene, La Jolla, CA, USA) and 15 pmol of each primer (DNA technology, Aarhus, Denmark) and adjusting the volume to 20 μl with DNase free water (Invitrogen, Carlsbad, CA, USA). PCR was performed with a 10 min preincubation at 94 °C followed by 40 cycles of 30 s at 94 °C, 45 s at 60 °C and 1.5 min at 72 °C (Roche Light Cycler 480, Roche, Indianapolis, IN, USA). The PCR primers and method were validated by using serially diluted cDNA to establish a standard curve. To quantify the gene expression of each sample, the C<sub>T</sub> value for each sample was obtained and fold change was calculated according to the comparative C<sub>T</sub> method as earlier described (Schmittgen and Livak, 2008). For each sample, the amount of targeted mRNA was normalized to that of the reference gene GAPDH. Specific primers designed based on GenBank (NCBI, NIH, MD) data were as follows: *Arc* primers, Forward: 5'-GCA GGT GGG TGG CTC TGA AGA ATA-3', Reverse: 5'-TCC CGC TTA CGC CAG AGG AACT-3'; *c-fos* primers, Forward: 5'-CAA AGT AGA GCA GCT ATC TCC-3', Reverse: 5'-CTC GTC TTC AAG TTG ATC TGT-3'; *GAPDH* primers, Forward: 5'-CAT CAA GAA GGT GGT GAA GCA-3', Reverse: 5'-CTG TTG AAG TCA CAG GAG ACA-3'; *Egr-2* primers, Forward: 5'-TGTTAA-CAGGGTCTGCATGTG-3', Reverse: 5'-AGCGGCAGTGACATT-GAAG-3'; *CREB* primers, Forward: 5'-ACTGGCTTGGCACAAC-CAGA-3', Reverse: 5'-GGCAGAAGTCTCTTCATGATT-3'.

### Locomotor activity

Locomotor activity while exposed to the open field was recorded by a camera located on the ceiling above the apparatus. The distance the animals moved was analyzed with the video-tracking program EthoVision (version 3.1, Noldus, Wageningen, The Netherlands).

### Plasma corticosterone levels

Plasma corticosterone was measured with a commercial ELISA kit (cat.# IB79112, Immuno-Biological Laboratories, Minneapolis, MN, USA). In brief, standards, control and samples were added to the microtiter wells in duplicates and incubated in enzyme conjugate for 60 min. After three rinses with diluted wash solution, the wells were incubated in substrate solution for 15 min at room temperature. Stop solution were added and the OD read at 450 nm within 10 min. Sample corticosterone concentrations were calculated from the standard curve using a four parameter logistics curve fit. The corticosterone standards contain corticosterone concentrations between 0 and 240 nmol/L, and samples were diluted to fit this range.

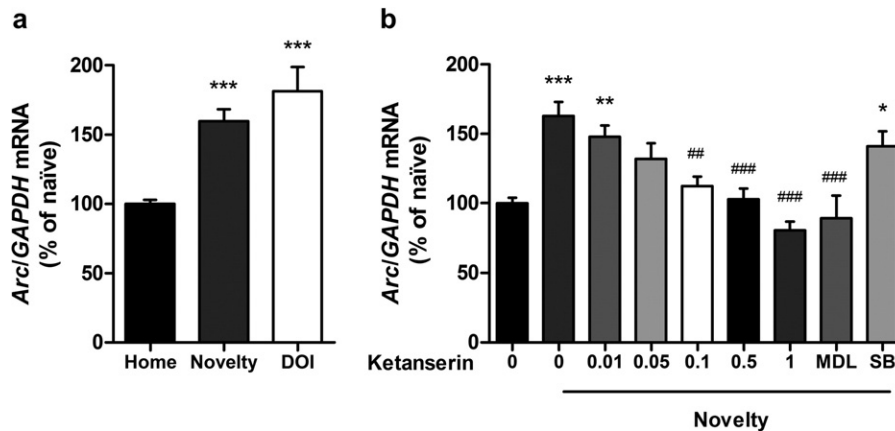
### Data analysis and statistics

The data were analyzed using one-way ANOVA with Tukey's post hoc test. The level of statistical significance was set to  $P < 0.05$ . Data are presented as mean ± SEM if not otherwise stated. For RT-qPCR, data from individual experiments were normalized to the novelty- and drug-naïve samples, which were set to 100%.

## RESULTS

### 5-HT<sub>2A</sub> receptor activation is necessary for FC *Arc* expression following exposure to a novel environment

Five minutes of exposure to a novel environment robustly up-regulated the expression of *Arc* mRNA in FC compared to home-cage controls ( $P < 0.01$ ) (Fig. 1a).



**Fig. 1.** Activity-regulated cytoskeletal-associated protein (*Arc*) gene expression in the frontal cortex. (a) Administration of the 5-HT<sub>2A</sub>R agonist DOI (2 mg/kg) induces *Arc* to levels comparable as novelty. (b) Novelty exposure up-regulates the expression of *Arc*, which is inhibited by the 5-HT<sub>2A</sub>R antagonists ketanserin (0.1–1 mg/kg) to basal level. Also another 5-HT<sub>2A</sub>R antagonist MDL100907 (1 mg/kg), but not the 5-HT<sub>2C</sub>R selective antagonist SB242084 (SB; 1 mg/kg) block novelty-induced *Arc* up-regulation. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared to vehicle-treated animals; ##  $P < 0.01$ , ###  $P < 0.001$  compared to vehicle/novelty-treated animals, one-way ANOVA with Tukey's post-hoc test,  $n = 6–10$ . Data presented as % of drug- and novelty-naive animals  $\pm$  SEM.

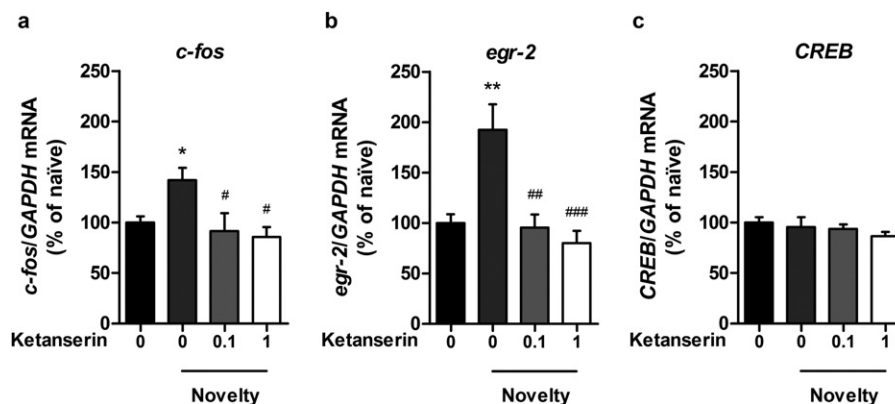
The level of increase in *Arc* mRNA after exposure to novelty was similar to the level produced after a single treatment with the 5-HT<sub>2A</sub>R agonist DOI at a dose of 2 mg/kg (Fig. 1a). Pretreatment with ketanserin before exposure to the novel environment inhibited *Arc* expression in a dose-dependent manner, reaching a significant reduction at 0.1 mg/kg (Fig. 1b). Ketanserin had no effect alone as no differences *Arc* mRNA levels were observed between ketanserin-pretreated animals and drug- and home-cage-controls. Also, *Arc* mRNA levels were not different from home-cage controls, when pretreated with another 5-HT<sub>2A</sub>R antagonist MDL100907 (1 mg/kg). As ketanserin also has affinity for the 5-HT<sub>2C</sub>R, we cannot exclude that non-5-HT<sub>2A</sub>R are involved. Therefore, another group of animals exposed to novelty were pretreated with the highly selective 5-HT<sub>2C</sub>R antagonist SB242084 to assess for a possible involvement of this receptor in the response. SB242084 pretreatment had no effect on the *Arc* mRNA levels (Fig. 1b).

#### 5-HT<sub>2A</sub>R activation mediates the expression of other IEGs following novelty-exposure

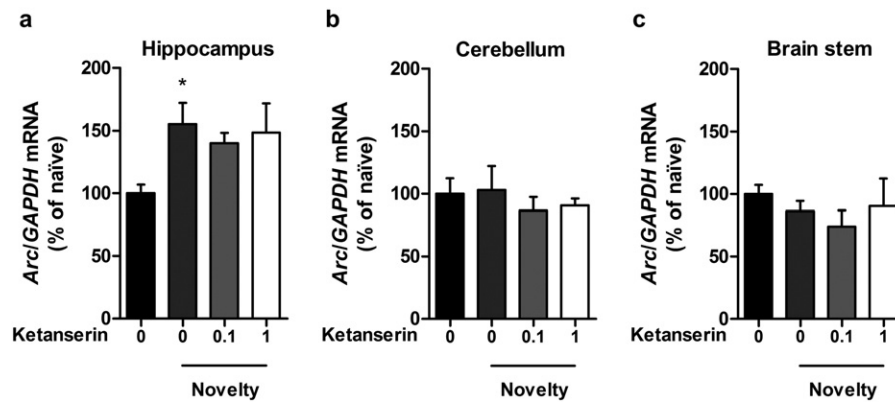
Exposure to a novel environment induced *c-fos* expression in the FC around 140% compared to control (Fig. 2a). Similar to *Arc*, the expression of *c-fos* in FC was completely blocked by ketanserin pretreatment at the same doses and effects as seen for *Arc* mRNA. In FC, early growth response gene 2 (*Egr-2*) mRNA was increased two-fold by novelty exposure and this effect was also blocked by ketanserin pretreatment at both 0.1 and 1 mg/kg doses (Fig. 2b). In contrast, cyclic AMP response element binding protein (*CREB*) mRNA was not affected by exposure to a novel environment or by 5-HT<sub>2A</sub>R antagonism.

#### 5-HT<sub>2A</sub>R mediated novelty-induced *Arc* expression is specific for FC

Consistent with previous work, *Arc* mRNA expression was increased by novelty exposure in the hippocampus (Vaz-



**Fig. 2.** Novelty-induced expression of immediate-early genes. (a, b) Novelty-exposure up-regulates *c-fos* and *Egr-2* expression, both of which are significantly inhibited by ketanserin pretreatment. (c) No effect of novelty-exposure on cyclic AMP response element binding protein (*CREB*) expression is observed. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to vehicle-treated animals; #  $P < 0.05$ , ##  $P < 0.01$ , ###  $P < 0.001$  compared to vehicle and novelty-treated animals, one-way ANOVA with Tukey's post-hoc test,  $n = 6–10$ . Data presented as % of drug- and novelty-naive animals  $\pm$  SEM.

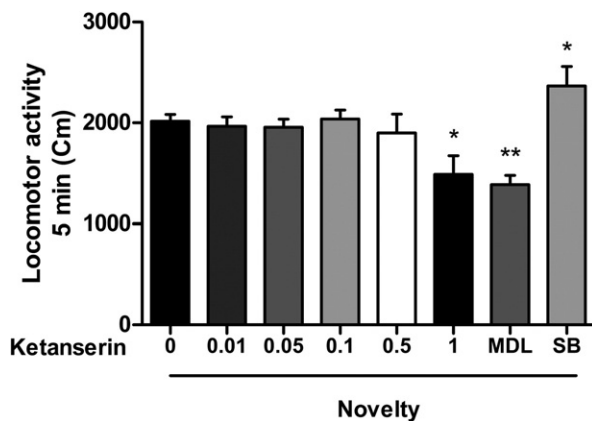


**Fig. 3.** Regional expression of *Arc* mRNA following novelty-exposure. (a) In the hippocampus, novelty-exposure up-regulates *Arc* gene expression. No difference is observed between vehicle and ketanserin pretreated animals. (b, c) *Arc* is not induced in the cerebellum or brainstem after novelty-exposure. \*  $P < 0.05$ , one-way ANOVA with Tukey's post-hoc test,  $n = 6-10$ . Data presented as % of drug- and novelty-naïve animals  $\pm$  SEM.

darjanova et al., 2002) (Fig. 3a). The magnitude was the same as seen in the FC. By contrast, no *Arc* induction was observed in cerebellum and brainstem (Fig. 3b, c). In contrast to the FC, the increase in *Arc* mRNA after novelty in the hippocampus was not affected by pretreatment with ketanserin (Fig. 3a).

#### Ketanserin blockage of *Arc* gene expression is not due to decreased locomotor activity

To investigate whether effect of ketanserin on *Arc* expression was due to a sedative or anxiogenic effect thereby inhibiting the animal's exploratory behaviour and thus its novelty exposure, we measured the locomotor activity of the animals during the 5 min they were placed in the open-field arena. As described previously (Maj et al., 1996), a higher dose of ketanserin (>1 mg/kg) decreased locomotor activity, but at lower doses inhibiting novelty-induced *Arc*-expression, no locomotor effects were observed (Fig. 4).



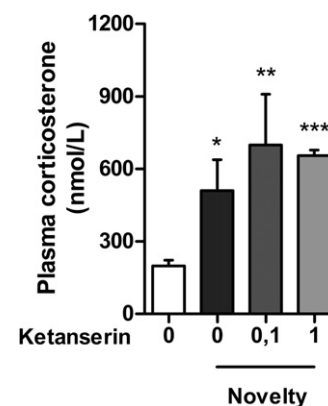
**Fig. 4.** Locomotor activity during 5 min in an open-field arena. There is no effect on locomotion activity of ketanserin pretreatment, when compared to vehicle-treated animals, at doses up to 0.5 mg/kg. Both ketanserin and MDL100907 (MDL) slightly decreased locomotor activity at 1 mg/kg, while SB242084 (SB; 1 mg/kg) increased it. \*  $P < 0.05$ , \*\*  $P < 0.01$ , one-way ANOVA with Tukey's post-hoc test,  $n = 6-12$ . Data presented as mean  $\pm$  SEM.

#### Ketanserin has no effect on novelty-mediated corticosterone hormone levels

As expected, novelty exposed animals showed increased corticosterone plasma levels when compared to home-cage controls. However, no differences in corticosterone levels at the investigated time point were observed between vehicle and ketanserin pretreated animals after novelty (Fig. 5).

## DISCUSSION

The major finding of the present study was that antagonism of 5-HT<sub>2A</sub>R blocked *Arc* gene transcription in the FC of mice exposed to a novel environment. This effect was found to be dose-dependent with significant inhibitory effect at relatively low doses of the 5-HT<sub>2A</sub>R antagonist ketanserin, as a dose of 0.1 mg/kg produced a significant reduction in novelty-induced *Arc* gene expression. These



**Fig. 5.** Plasma corticosterone levels after novelty and pretreatment with ketanserin. Exposure to a novel environment increases plasma corticosterone levels from  $212.4 \pm 98.9$  nmol/L to  $510.4 \pm 181.1$  nmol/L. No significant differences were observed between novelty-exposed animals regardless of pretreatment, as 0.1 and 1 mg/kg ketanserin pretreatment yielded  $698.7 \pm 181.1$  and  $654.7 \pm 28.5$  respectively. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared to home-cage controls, one-way ANOVA with Tukey's post-hoc test,  $n = 4-12$ . Data presented as mean  $\pm$  SEM.

results show that the 5-HT<sub>2A</sub>R is necessary for novelty-induced *Arc* expression. The exposure to a novel stimulus with an active exploratory component is a well-established model of emotional stress resulting from the conflict between innate drives to explore a novel environment and safety need (Aloyo and Dave, 2007; Weisstaub et al., 2006), and it has been shown by several groups that this leads to an increase in *Arc* mRNA levels in hippocampus and cerebral cortex (Elizalde et al., 2010; Pinaud et al., 2001; Vazdarjanova et al., 2002). In the hippocampus, it is well-established that *Arc* is associated with encoding of new information via direct involvement in receptor cycling in the synapse and fitting with the idea that learning processes involve plastic processes requiring alterations in genomic or proteomic processing (Miyashita et al., 2008; Ons et al., 2004; Plath et al., 2006; Shepherd et al., 2006; Vazdarjanova et al., 2002). The role of *Arc* in the FC is more uncertain because it has not been demonstrated whether the same molecular mechanisms involved in memory consolidation in the hippocampus have separate roles in the FC. However, animals and humans with either reduced or increased concentrations of intraneuronal *Arc* levels have shown cognitive disturbances that depend on the FC (Greer et al., 2010; Plath et al., 2006).

In our experimental setup, increased plasma corticosterone levels were seen in animals exposed to novelty compared to home cage. Novel environment exposure is considered an emotional “processing” stressor in that respect it requires cognitive processing but does not represent an immediate physical threat (Herman et al., 1996). The 5-HT<sub>2A</sub>R is involved in anxiety modulation (Weisstaub et al., 2006) and in direct activation of the HPA-axis response at the level of the hypothalamic paraventricular nucleus (Mikkelsen et al., 2004; Zhang et al., 2002), but we find no evidence that these mechanisms are involved after novelty, because ketanserin at doses blocking increase in *Arc* in the FC had no effect on circulating corticosterone. It is known that exposure to anxiety or administration of pharmacological agents increasing anxiety induce activation of different subset of neurons in the dorsal raphe nucleus dependent on the stressor (Abrams et al., 2005; Lowry et al., 2005). A major proportion of serotonergic neurons activated after novelty project to the basolateral amygdaloid nucleus (Hale et al., 2008), and it is conceivable that these neurons operate on the HPA axis independent of 5-HT<sub>2A</sub>R.

To assess a possible pharmacologically induced alteration in locomotion we monitored the movement of the animals while exploring the open-field arena. As earlier described (Barr et al., 2004; Fletcher et al., 2009) a decrease (~20%) in total distance travelled was seen with the highest dose of the 5-HT<sub>2A</sub>R antagonists ketanserin and MDL100907, while an increase in travelled distance was observed after blockade of the 5-HT<sub>2C</sub>R. There was no correlation between the doses of ketanserin inhibiting *Arc* expression and those that reduced locomotor effects. Reduction in travel distance is likely mediated via a 5-HT<sub>2A</sub>R mechanism as MDL100907 had the same effect as ketanserin. However, the relatively high dose of

5-HT<sub>2A</sub>R antagonists eliciting locomotor effects shows that the reduction in *Arc* expression after novelty is not confounded by locomotor behaviour.

Treatment with the 5-HT<sub>2A/2C</sub> agonist DOI leads to increases in *Arc* expression in FC (Gonzalez-Maeso et al., 2003; Pei et al., 2004) comparable to that as induced by novelty-exposure. This effect is mediated through the 5-HT<sub>2A</sub>R as it can be blocked by the 5-HT<sub>2A</sub>R antagonist MDL100907 but not the 5-HT<sub>2C</sub>R antagonist SB206553 (Pei et al., 2004). In FC most (~73%) 5-HT<sub>2A</sub>R immunoreactive neurons have postsynaptic profiles composed of either dendritic shafts or spines, while ~24% had presynaptic profiles and 4% were glial processes (Miner et al., 2003). However, *Arc* is not directly induced in 5-HT<sub>2A</sub>R expressing neurons but in neurons containing AMPA and NMDA receptors (Pei et al., 2004). Instead activation of 5-HT<sub>2A</sub>R in FC results in a robust increase in glutamate receptor activation which means that certain actions of 5-HT<sub>2A</sub>R in this region may be mediated by the release of glutamate (Beique et al., 2007). Indeed, as shown by Pei et al. (2004) 5-HT<sub>2A</sub>R-mediated *Arc* up-regulation can be inhibited by the NMDA antagonist MK801 (Pei et al., 2004).

Exposure to the novel environment also induced the expression of *c-fos* in the FC, which similarly to *Arc* could be blocked by ketanserin pretreatment. *Arc* and *c-fos* have been reported to respond differently to various kinds of stimuli in regard of their anatomical distribution. Exposure to stress induces *c-fos* expression throughout the brain, while *Arc* expression is restricted to the hippocampus and cortical regions (Ons et al., 2004). Thus, *Arc* transcription is specifically linked to neural activity associated with information processing, and not to a nonspecific response to stress (Guzowski et al., 1999). In our study, novelty exposure also induced *Arc* expression in the hippocampus, which has been described in previous studies and underlies the consolidation of long-term memory (Vazdarjanova et al., 2002). However, ketanserin pretreatment did not block induction of the *Arc* response in hippocampus, pointing to a specific role of the 5-HT<sub>2A</sub>R in the novelty-induced *Arc* expression in the FC. Our observations make us conclude that *Arc* expression in FC, is not directly related to the stressfulness of the situation, but to the exploratory potential of the novel experience, supporting a role of the FC, and possible 5-HT<sub>2A</sub>R, in the evaluation of a novel environment.

Another interesting observation is the involvement of 5-HT<sub>2A</sub>R in novelty-induced *Egr-2* expression in the FC. 5-HT<sub>2A</sub>R signaling involves multiple intracellular pathways including the activation of MAPK, PLC- $\beta$  and PLA<sub>2</sub> (Kurasch-Orbaugh et al., 2003), which leads to long-term transcriptional changes. Gonzalez-Maeso and colleagues (2003) found that *Egr-2* gene induction is specific for the hallucinogenic agonists effects of 5-HT<sub>2A</sub>R activation as it is associated with induction of the head-twitch behavioral response (Gonzalez-Maeso et al., 2003). Our observation that exposure to novelty results in a robust up-regulation of *Egr-2*, which can be blocked with 5-HT<sub>2A</sub>R antagonists, indicates that exploratory behaviour activates the same intracellular pathway as hallucinogenic compounds.

Taken together, our data suggest an important role for the 5-HT<sub>2A</sub>R in the activation of the FC of animals exposed to a novel environment inducing an exploratory behaviour.

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## REFERENCES

- Abrams JK, Johnson PL, Hay-Schmidt A, Mikkelsen JD, Shekhar A, Lowry CA (2005) Serotonergic systems associated with arousal and vigilance behaviors following administration of anxiogenic drugs. *Neuroscience* 133:983–997.
- Adell A, Casanovas JM, Artigas F (1997) Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. *Neuropharmacology* 36:735–741.
- Aloyo VJ, Dave KD (2007) Behavioral response to emotional stress in rabbits: role of serotonin and serotonin2A receptors. *Behav Pharmacol* 18:651–659.
- Barr AM, Lehmann-Masten V, Paulus M, Gainetdinov RR, Caron MG, Geyer MA (2004) The selective serotonin-2A receptor antagonist M100907 reverses behavioral deficits in dopamine transporter knockout mice. *Neuropsychopharmacology* 29:221–228.
- Beique JC, Imad M, Mladenovic L, Gingrich JA, Andrade R (2007) Mechanism of the 5-hydroxytryptamine 2A receptor-mediated facilitation of synaptic activity in prefrontal cortex. *Proc Natl Acad Sci U S A* 104:9870–9875.
- Bramham CR, Worley PF, Moore MJ, Guzowski JF (2008) The immediate early gene *Arc/arg3.1*: regulation, mechanisms, and function. *J Neurosci* 28:11760–11767.
- Clayton DF (2000) The genomic action potential. *Neurobiol Learn Mem* 74:185–216.
- Cornea-Hebert V, Riad M, Wu C, Singh SK, Descarries L (1999) Cellular and subcellular distribution of the serotonin 5-HT<sub>2A</sub> receptor in the central nervous system of adult rat. *J Comp Neurol* 409:187–209.
- Elizalde N, Pastor PM, Garcia-Garcia AL, Serres F, Venzala E, Huarte J, Ramirez MJ, Del RJ, Sharp T, Tordera RM (2010) Regulation of markers of synaptic function in mouse models of depression: chronic mild stress and decreased expression of VGLUT1. *J Neurochem* 114:1302–1314.
- Fletcher PJ, Tampakeras M, Sinyard J, Slassi A, Isaac M, Higgins GA (2009) Characterizing the effects of 5-HT<sub>2C</sub> receptor ligands on motor activity and feeding behaviour in 5-HT<sub>2C</sub> receptor knockout mice. *Neuropharmacology* 57:259–267.
- Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, Weisstaub N, Hen R, Gingrich JA, Sealfon SC (2003) Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. *J Neurosci* 23:8836–8843.
- Goto Y, Yang CR, Otani S (2010) Functional and dysfunctional synaptic plasticity in prefrontal cortex: roles in psychiatric disorders. *Biol Psychiatry* 67:199–207.
- Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, Kim TK, Griffith EC, Waldon Z, Maehr R, Ploegh HL, Chowdhury S, Worley PF, Steen J, Greenberg ME (2010) The Angelman syndrome protein Ube3A regulates synapse development by ubiquitinating *Arc*. *Cell* 140:704–716.
- Guzowski JF, McNaughton BL, Barnes CA, Worley PF (1999) Environment-specific expression of the immediate-early gene *Arc* in hippocampal neuronal ensembles. *Nat Neurosci* 2:1120–1124.
- Hale MW, Hay-Schmidt A, Mikkelsen JD, Poulsen B, Bouwknecht JA, Evans AK, Stamper CE, Shekhar A, Lowry CA (2008) Exposure to an open-field arena increases c-Fos expression in a subpopulation of neurons in the dorsal raphe nucleus, including neurons projecting to the basolateral amygdaloid complex. *Neuroscience* 157:733–748.
- Herman JP, Prewitt CM, Cullinan WE (1996) Neuronal circuit regulation of the hypothalamo-pituitary-adrenocortical stress axis. *Crit Rev Neurobiol* 10:371–394.
- Klebaur JE, Ostrander MM, Norton CS, Watson SJ, Akil H, Robinson TE (2002) The ability of amphetamine to evoke *Arc* (Arg 3.1) mRNA expression in the caudate, nucleus accumbens and neocortex is modulated by environmental context. *Brain Res* 930:30–36.
- Kurrasch-Orbaugh DM, Parrish JC, Watts VJ, Nichols DE (2003) A complex signaling cascade links the serotonin2A receptor to phospholipase A2 activation: the involvement of MAP kinases. *J Neurochem* 86:980–991.
- Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A (2005) Modulation of anxiety circuits by serotonergic systems. *Stress* 8:233–246.
- Maj J, Rogoz Z, Skuza G, Wedzony K (1996) The synergistic effect of fluoxetine on the locomotor hyperactivity induced by MK-801, a non-competitive NMDA receptor antagonist. *J Neural Transm* 103:131–146.
- Mikkelsen JD, Hay-Schmidt A, Kiss A (2004) Serotonergic stimulation of the rat hypothalamo-pituitary-adrenal axis: interaction between 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptors. *Ann N Y Acad Sci* 1018:65–70.
- Mikkelsen JD, Larsen MH (2006) Effects of stress and adrenalectomy on activity-regulated cytoskeleton protein (*Arc*) gene expression. *Neurosci Lett* 403:239–243.
- Miner LA, Backstrom JR, Sanders-Bush E, Sesack SR (2003) Ultrastructural localization of serotonin2A receptors in the middle layers of the rat prelimbic prefrontal cortex. *Neuroscience* 116:107–117.
- Miyashita T, Kubik S, Lewandowski G, Guzowski JF (2008) Networks of neurons, networks of genes: an integrated view of memory consolidation. *Neurobiol Learn Mem* 89:269–284.
- Ons S, Marti O, Armario A (2004) Stress-induced activation of the immediate early gene *Arc* (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA. *J Neurochem* 89:1111–1118.
- Ons S, Rotllant D, Marin-Blasco IJ, Armario A (2010) Immediate-early gene response to repeated immobilization: Fos protein and *Arc* mRNA levels appear to be less sensitive than c-fos mRNA to adaptation. *Eur J Neurosci* 31:2043–2052.
- Pei Q, Tordera R, Sprakes M, Sharp T (2004) Glutamate receptor activation is involved in 5-HT<sub>2</sub> agonist-induced *Arc* gene expression in the rat cortex. *Neuropharmacology* 46:331–339.
- Pinaud R, Penner MR, Robertson HA, Currie RW (2001) Upregulation of the immediate early gene *Arc* in the brains of rats exposed to environmental enrichment: implications for molecular plasticity. *Brain Res Mol Brain Res* 91:50–56.
- Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A, Mahlke C, Weizl H, Kobalz U, Stawrakakis A, Fernandez E, Waltereit R, Bick-Sander A, Therstappen E, Cooke SF, Blanquet V, Wurst W, Salmen B, Bosl MR, Lipp HP, Grant SG, Bliss TV, Wolfer DP, Kuhl D (2006) *Arc/Arg3.1* is essential for the consolidation of synaptic plasticity and memories. *Neuron* 52:437–444.
- Robbins TW (2000) From arousal to cognition: the integrative position of the prefrontal cortex. *Prog Brain Res* 126:469–483.
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108.
- Shepherd JD, Rumbaugh G, Wu J, Chowdhury S, Plath N, Kuhl D, Haganir RL, Worley PF (2006) *Arc/Arg3.1* mediates homeostatic synaptic scaling of AMPA receptors. *Neuron* 52:475–484.
- Steward O, Worley P (2002) Local synthesis of proteins at synaptic sites on dendrites: role in synaptic plasticity and memory consolidation? *Neurobiol Learn Mem* 78:508–527.

- Vazdarjanova A, McNaughton BL, Barnes CA, Worley PF, Guzowski JF (2002) Experience-dependent coincident expression of the effector immediate-early genes *Arc* and *Homer 1a* in hippocampal and neocortical neuronal networks. *J Neurosci* 22:10067–10071.
- Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP, Sibille E, Underwood M, Itohara S, Dauer WT, Ansorge MS, Morelli E, Mann JJ, Toth M, Aghajanian G, Sealton SC, Hen R, Gingrich JA (2006) Cortical 5-HT<sub>2A</sub> receptor signaling modulates anxiety-like behaviors in mice. *Science* 313:536–540.
- Zhang Y, Damjanoska KJ, Carrasco GA, Dudas B, D'Souza DN, Tetzlaff J, Garcia F, Hanley NR, Scripathirathan K, Petersen BR, Gray TS, Battaglia G, Muma NA, Van de Kar LD (2002) Evidence that 5-HT<sub>2A</sub> receptors in the hypothalamic paraventricular nucleus mediate neuroendocrine responses to (–)DOI. *J Neurosci* 22: 9635–9642.

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# *Manuscript II*

Santini MA, Ratner C, Aznar S, Klein AB, Knudsen GM, Mikkelsen JD (2012) Enhanced prefrontal serotonin 2A receptor signaling in the subchronic PCP mouse model of schizophrenia, *Accepted, Journal of Neuroscience Research*



**Enhanced prefrontal serotonin 2A receptor signaling in the  
subchronic PCP mouse model of schizophrenia**

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Keywords:	Serotonergic receptors, Prefrontal cortex, Immediate early genes, N-Methyl-D-aspartate, Schizophrenia

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Manuscripts

1 **Enhanced prefrontal serotonin 2A receptor signaling in the subchronic PCP mouse model**  
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8 Martin A. Santini, Cecilia Ratner, Susana Aznar, Anders B. Klein, Gitte M. Knudsen, and Jens D. Mikkelsen  
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11 **Running Title:** Enhanced 5-HT<sub>2A</sub>R signaling after subchronic PCP  
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17 *Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark*  
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31 \* Corresponding Author:  
32

33 Jens D. Mikkelsen, MD PhD  
34

35 Neurobiology Research Unit  
36

37 Blegdamsvej 9  
38  
39

40 Rigshospitalet, building 9201  
41  
42

43 DK-2100 Copenhagen,  
44

45 Denmark  
46

47 Phone (+45) 35456701 / Fax: (+45) 35456713  
48  
49

50 Email: jens.mikkelsen@nru.dk  
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**Abstract**

Prefrontal serotonin 2A receptors (5-HT<sub>2A</sub>R) have been linked to the pathogenesis and treatment of schizophrenia. Many antipsychotics fully occupy 5-HT<sub>2A</sub>R at clinical relevant doses and activation of 5-HT<sub>2A</sub>R receptors by lysergic acid diethylamide (LSD) and LSD-like drugs induces a schizophrenia-like psychosis in humans. Subchronic phencyclidine (PCP) administration is a well-established model for schizophrenia-like symptoms in rodents. The aim of the present study was to investigate if subchronic PCP administration changes expression, binding or functionality of cortical 5-HT<sub>2A</sub>R receptors. As a measure of 5-HT<sub>2A</sub>R functionality we used 2,5-dimethoxy-4-iodoamphetamine (DOI)-induced head-twitch response (HTR) and mRNA expression of the immediate-early genes (IEGs) activity-related cytoskeletal associated-protein (*Arc*), *c-fos* and early growth response protein 2 (*egr-2*) in the frontal cortex. Mice were treated with PCP (10 mg/kg) or saline for 10 days followed by a three day wash-out period. The PCP pretreatment increased the overall induction of HTR and frontal cortex IEG mRNA expression following a single challenge with the 5-HT<sub>2A/2C</sub>R agonist DOI as revealed using qPCR. These functional changes were not associated with changes in 5-HT<sub>2A</sub>R binding. Also, binding of the 5-HT<sub>1A</sub>R and the 5-HT transporter was unaffected. Finally, basal mRNA level of *Arc* was increased in the prefrontal cortex after subchronic PCP administration as revealed with in situ hybridisation. Together, these findings indicate that PCP administration produces changes in the brain that results in an increase in the absolute effect of DOI. Therefore, neurotransmission involving the 5-HT<sub>2A</sub>R could contribute to the behavioral deficits observed after PCP treatment.

Keywords: Serotonergic receptors, Prefrontal cortex, Immediate early genes, N-Methyl-D-aspartate, Schizophrenia

## 1 Introduction

2  
3 Activation of serotonin 2A receptors (5-HT<sub>2A</sub>R) by lysergic acid diethylamide (LSD)-like drugs induces  
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5 schizophrenia-like psychosis in humans (Vollenweider et al., 1998) and most atypical antipsychotics exhibit  
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7 high-affinity blocking of 5-HT<sub>2A</sub>Rs (Kapur et al., 1999). These cellular and behavioral effects of LSD-like  
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9 drugs are mediated through cortical 5-HT<sub>2A</sub> receptors (Gonzalez-Maeso et al., 2007). Thus, the role of the 5-  
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11 HT<sub>2A</sub>R in the prefrontal cortex (PFC) could be important in the development of both positive and  
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13 negative/cognitive symptoms in schizophrenia. Imaging and post-mortem binding studies in antipsychotic-naïve  
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15 schizophrenics have reported both increased and decreased cortical 5-HT<sub>2A</sub>R binding (Ebdrup et al., 2011;  
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17 Rasmussen et al., 2010; Gonzalez-Maeso et al., 2008). Thus, it remains unclear whether increased 5-HT<sub>2A</sub>R  
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19 binding in the PFC is a marker of schizophrenia (Dean, 2003).  
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27 In rodents, administration of hallucinogenic 5-HT<sub>2A</sub>R agonists results in an induction of immediate-early genes  
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29 (IEGs), such as *Arc*, *c-fos* and *Egr-2* in cortical regions (Gonzalez-Maeso et al., 2003; Pei et al., 2004). This  
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31 activation is dependent exclusively on 5-HT<sub>2A</sub>R in the pyramidal neurons in the cerebral cortex, because region-  
32  
33 specific rescue of 5-HT<sub>2A</sub>R expression in these particular cells in transgene animals is sufficient to restore  
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35 induction of transcriptional factors after systemic administration of the 5-HT<sub>2A</sub> agonist 2,5-dimethoxy-4-  
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37 iodoamphetamine (DOI) (Gonzalez-Maeso et al., 2007). Further, both systemic and direct injection of 5-HT<sub>2A</sub>R  
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39 agonists into the PFC produce a head-twitch response (HTR) (Willins and Meltzer, 1997). More interestingly,  
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41 only compounds which causes hallucinations in humans induces HTR in rodents, suggesting that this response  
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43 is an indicator of the hallucinogenic action (Gonzalez-Maeso et al., 2007). In summary, both the induction of  
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45 IEG expression in the PFC and HTR can be utilized as functional outputs of 5-HT<sub>2A</sub>R-dependent activation.  
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53 The pathophysiology of schizophrenia involves abnormal N-Methyl-D-aspartate (NMDA) receptor-mediated  
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55 neurotransmission in the PFC (Coyle, 2006; Pratt et al., 2008). In humans, administration of non-competitive  
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57 NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, induces a broad range of behaviors  
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1 resembling those seen in schizophrenics, and they also exacerbate both positive and negative symptoms in  
2 patients (Malhotra et al., 1996; Malhotra et al., 1997; Jentsch and Roth, 1999). Systemic administration of PCP  
3 to adult rodents causes a complex behavioral profile that mimics some symptoms seen seen in schizophrenia  
4 (Moghaddam and Jackson, 2003). In particular, PCP produce cognitive dysfunctions, such as impaired  
5 performance in working memory tasks and cause reduced social behavior (Vigano et al., 2009; Seillier and  
6 Giuffrida, 2009). Moreover, subchronic administration of PCP has been shown to reduce markers for gamma-  
7 aminobutyric acid (GABA)-ergic interneurons, which are involved in the regulation of cortical circuit activity,  
8 especially by restraining excessive pyramidal neuron activity (Homayoun and Moghaddam, 2007; Amitai et al.,  
9 2012; Zhu et al., 2004). In animals, hyperlocomotion and HTR induced by acute PCP can be blocked by 5-  
10 HT<sub>2A</sub>R antagonists suggesting that 5-HT<sub>2A</sub>R are important in the pathophysiology produced by PCP (Kitaichi et  
11 al., 1994; O'Neill et al., 1998; Nabeshima et al., 1987). Also, 5-HT<sub>2A</sub>R antagonists are able to reverse acute  
12 PCP-induced cellular disruptions in PFC (Kargieman et al., 2007). This indicates that the cellular and  
13 behavioral responses induced by acute PCP are mediated through circuits that are modulated by 5-HT<sub>2A</sub>R,  
14 which lend credence to the idea that the schizophrenia-like phenotype induced in rodents by subchronic PCP  
15 treatment is caused by alterations in 5-HT<sub>2A</sub>R-mediated signaling. We here determined 5-HT<sub>2A</sub>R binding in the  
16 frontal cortex after subchronic PCP treatment and compared these to receptor functionality, as measured by  
17 DOI-induced IEG expression and HTR.  
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## 1 **Methods**

### 2 3 *Animals*

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5 Eight weeks old male C57BL/6J mice (Charles River Laboratories, Sulzfeld, Germany) were kept in a  
6 controlled environment with a 12/12 h light/dark cycle, provided with standard rodent diet and water *ad libitum*.  
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8  
9 All experiments were approved the Animal Experimentation Inspectorate, Ministry of Justice, Denmark.  
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11

### 12 13 14 *Drugs*

15  
16 DOI [(±)-2,5-dimethoxy-4-iodoamphetamine] hydrochloride and PCP [1-(1-phenylcyclohexyl)piperidine]  
17 hydrochloride (both synthesized at the Department of Medicinal Chemistry, Faculty of Pharmaceutical  
18 Sciences, Copenhagen University) were dissolved in 0.9 % saline.  
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### 24 25 26 *Treatment paradigm*

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28 PCP (10 mg/kg; 10 ml/kg) or its vehicle (saline) was administered s.c. for 10 days (once daily on days 1–5 and  
29 8–12, with no treatment on days 6, 7, 13, and 14). This treatment paradigm was selected based on its ability to  
30 induce cellular (reduction in parvalbumin and synaptophysin mRNA in PFC) and behavioral changes  
31 (impairment in Y-maze- and novel-object recognition test) relevant to schizophrenia (Thomsen et al., 2009;  
32 Hagiwara et al., 2008).  
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41 Following a 5 day wash-out period the mice were injected with DOI (0.5 or 2.0 mg/kg i.p.) or a corresponding  
42 volume of vehicle (saline). For RT-qPCR and binding studies, brains were removed 1 h after DOI treatment.  
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### 46 47 48 *Head-twitch response*

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50 The head-twitch response (HTR) scoring was performed as previously reported (Darmani, 2001) with minor  
51 modifications. In brief, animals ( $n = 8$  per group) were injected with DOI (0.5 or 2 mg/kg i.p.) or a  
52 corresponding volume of saline. After drug administration the mice were immediately moved to a standard cage  
53 (15 x 24 cm) without enrichment material and videotaped for 25 min. Subsequently the total number of head-  
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1 twitches was scored in the interval between 5 and 25 minutes after drug administration by an observer not aware  
2  
3 of the treatment of the animal.  
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#### 5 6 7 8 *Receptor autoradiography* 9

10 To determine receptor binding, animals ( $n = 8$  per group) were decapitated and brains were quickly removed,  
11 frozen in powdered dry ice and stored at  $\pm 80$  °C until sectioning. Brains were cut in 12  $\mu\text{m}$  coronal sections and  
12 mounted on Super Frost Plus slides and stored at  $\pm 80$  °C until further processing. 5-HT<sub>2A</sub>R autoradiography was  
13 performed using [<sup>3</sup>H]-MDL100907 [R(+)- $\alpha$ -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)-ethyl]-4-piperidin-  
14 methanol] (a gift from Professor Halldin, Karolinska Institute, Stockholm) and non-specific binding was  
15 determined using 10  $\mu\text{M}$  ketanserin tartrate (Sigma). For 5-HT<sub>1A</sub>R autoradiography we used [<sup>3</sup>H]-WAY100635  
16 (GE Healthcare, UK) and measured non-specific binding with 10  $\mu\text{M}$  5-HT (Sigma). Briefly, sections were  
17 allowed to thaw for 1 h at room temperature (RT) and then pre-incubated with 50 mM Tris-HCl (Sigma), pH  
18 7.4 containing 0.01% ascorbic acid (Sigma) for 15 min at RT under constant gentle shaking. Sections were then  
19 incubated for 60 min at RT using the same buffer containing either 2 nM of [<sup>3</sup>H]-MDL100907 or 1.5 nM [<sup>3</sup>H]-  
20 WAY100635 with or without the respective cold ligand. Following incubation, slides were washed for 2 $\times$ 5 min  
21 in ice-cold 50 mM Tris-HCl, pH 7.4, and then for 20 s in ice-cold H<sub>2</sub>O, and dried for 1 h under a gentle stream  
22 of air.  
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41 For 5-HT transporter (SERT) autoradiography, sections were pre-incubated for 20 min in 50 mM Tris-  
42 HCl, 120 mM NaCl, 5 mM KCl, pH 7.4 and subsequently incubated with 2.0 nM [[<sup>3</sup>H]]-escitalopram (donated  
43 by H. Lundbeck A/S, Copenhagen, Denmark) for 60 min diluted in the same buffer. Nonspecific binding was  
44 determined in the presence of 10  $\mu\text{M}$  paroxetine (GSK, Harlow, UK). After incubation, sections were washed  
45 for 3 $\times$ 2 min in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4 (4 °C) and for 20 s in ice-cold dH<sub>2</sub>O.  
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53 All sections were placed at 4 °C overnight in a fixator containing paraformaldehyde vapour and then put in an  
54 excicator box for 3 h before slides were exposed together with [<sup>3</sup>H]-microscales (GE Healthcare, UK) to a BAS-  
55 TR2040 Imaging Plate (Science Imaging Scandinavia AB, Nacka, Sweden) for 7-14 days at 4 °C. Finally, the  
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1 imaging plate was scanned on a BAS-2500 phosphoimage scanner (Fujifilm Europe GmbH, Düsseldorf,  
2 Germany) and specific and non-specific binding was determined in the entire frontal cortex or hippocampus  
3 using ImageJ and expressed as fmol/mg tissue equivalents (TE).  
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#### 10 *Quantitative assessment of mRNA levels using RT qPCR*

11 Total RNA from samples of the frontal cortex was isolated with Trizol Reagent (Sigma-Aldrich). The RNA  
12 samples were dissolved in RNase-free water and RNA was quantified with UV-spectrophotometry at 260 nm.  
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The real-time qPCR reactions were performed by adding the sample cDNA to a reaction mixture consisting of 1x Brilliant II SYBR green mastermix (Stratagene, La Jolla, CA) and 15 pmol of each primer (DNA technology, Aarhus, Denmark) and adjusting the volume to 20  $\mu$ l with DNase free water (Invitrogen, Carlsbad, CA). PCR was performed with a 10 minute preincubation at 94°C followed by 40 cycles of 30 seconds at 94°C, 45 seconds at 60°C and 1.5 minutes at 72 °C (Roche Light Cycler 480, Roche, Indianapolis, IN). For each sample, the amount of targeted mRNA was normalized to that of the reference transcript GAPDH mRNA. Specific primers were as follows: *Arc* primers, Forward: 5'-GCAGGT GGGTGGCTCTGAAGAATA-3', Reverse: 5'-TCCCGCTTACGCCAGAGGAACT-3'; *c-fos* primers, Forward: 5'-CAA AGT AGA GCA GCT ATC TCC-3', Reverse: 5'-CTC GTC TTC AAG TTG ATC TGT-3'; *Egr-2* primers, Forward: 5'-TGTTAACAGGGTCTGCATGTG-3', Reverse: 5'-AGCGGCAGTGACATTGAAG-3'; *GAPDH* primers, Forward: 5'-CATCAAGAAGGTGGTGAAGCA-3', Reverse: 5'-CTGTTGAAGTCACAGGAGACA-3'; 5-*HT<sub>2A</sub>R* primers, Forward: 5'-CCG CTT CAA CTC CAG AAC CAA AGC-3', Reverse: 5'-



1 CTTCGAATCATCCTGTACCCGAA-3'; *5-HT<sub>2C</sub>R* primers, Forward: 5'-  
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3 TAATGGTGAACCTGGGCACTGCGG-3', Reverse: 5'-TAAAAGTGTTCAGTTACTATAGCTGC-3'; 5-  
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5 *HT<sub>1A</sub>R* primers, Forward: 5'-CTGTTTATCGCCCTGGATG-3', Reverse: 5'-  
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7 ATGAGCCAAGTGAGCGAGAT-3'.  
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### 10 11 12 *In situ hybridization*

13 Slides were incubated for 5 min in 4% paraformaldehyde in 0.2 M PBS, and washed twice for 1 min in PBS.  
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15 This was followed by acetylation (0.25% acetic anhydride, 0.1 M triethanolamine in 0.9% NaCl, pH 8.0) for 10  
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17 min at room temperature. The slides were then delipidated and dehydrated in a series of ethanol's (70%/5 min;  
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19 80%/1 min; 95%/2 min; and 99%/1 min) and finally incubated for 5 min in chloroform. Excess chloroform was  
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21 washed off the slides in 99% and 96% ethanol, and the slides air-dried. A synthetic oligonucleotide DNA probe  
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23 complimentary to the rat *Arc* (targeting bases 789–839) was used (DNA Technology, Aarhus, Denmark) (Pei et  
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25 al., 2004; Thomsen et al., 2010b). The probe was labelled at the 3'-end with  $\alpha$ -[<sup>35</sup>S]ATP (>1250 Ci/mmol,  
26  
27 Perkin Elmer) using terminal deoxynucleotidyl transferase (Sigma) and purified with Illustra ProbeQuant G-50  
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29 micro columns (GE Healthcare, WI) according to the manufacturer's instructions. Labeled probe was added at a  
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31 specific activity of  $1 \times 10^6$  cpm/100  $\mu$ l to the hybridization buffer [45% formamid (v/v), 4 x saline sodium  
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33 citrate (SSC) (1 x SSC is 0.15 M NaCl, 0,015 M sodium citrate, pH 7.2), 1x Denhardts solution (0,02% Ficoll,  
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35 polyvinylpyrrolidone and bovine serum albumin), salmon sperm ssDNA (0.5 mg/ml), 0.25 mg/ml yeast transfer  
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37 RNA and 10% (w/v) dextran sulfate and 10 mM dithiotreitol] and was applied to the sections at 37°C overnight  
38  
39 in a humid chamber. The sections were then transferred to 4 rapid consecutive washes in 0.5x SSC (RT) and  
40  
41 then washed for 4 times 15 min in 0.5x SSC (55 °C) and for 2 times 30 min in 0.5x SSC at RT. Finally, the  
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43 sections were airdried and simultaneously exposed together with <sup>14</sup>C standards to a Kodak BiomaxMR film for  
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45 2 weeks.  
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55 Optical densities from the *in situ* hybridization were quantified in the PFC (as described by (Thomsen et  
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57 al., 2010a)) using a computer image analysis system (Quantity-One<sup>®</sup> software, Bio-Rad, Hercules CA). The  
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1 individual value for each animal was the average of two individual sections measured bilaterally within the area  
2  
3 of interest. Background values were measured for each slide individually in an area without tissue. The  
4  
5 presented values are the measured value minus the background converted to nCi/g using the  $^{14}\text{C}$  standards.  
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### 10 *Statistical analyses*

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12 All analyses were performed using GraphPad 5. Data are presented as mean  $\pm$  SEM. The data were analyzed  
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14 using Student's *t*-test if comparing two groups, one-way ANOVA with Tukey's *post hoc* test when comparing  
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16 multiple groups, and two-way ANOVA with Tukey's *post hoc* test, when comparing the groups with more than  
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18 one variable. The level of statistical significance was set to  $P < 0.05$ .  
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## Results

### *Subchronic PCP administration increases basal Arc mRNA levels and DOI-induced head-twitch response and IEG mRNA expression*

To assess the effects of subchronic PCP treatment on the functionality of 5-HT<sub>2A</sub>R we first analyzed the behavioral HTR following acute DOI challenge. Animals were treated for 10 days with PCP or vehicle and allowed a five day wash-out period before being challenged with a single dose of DOI (0.5 and 2 mg/kg). For both saline and PCP pretreated animals, 0.5 and 2 mg/kg DOI highly induced head-twitches (Fig. 1). However, two-way ANOVA analysis of HTR showed a significant interaction between saline and PCP pretreatment [ $F(2,40) = 5.733$ ;  $P = 0.0065$ ] and the *post hoc* analysis revealed that DOI induced a significant higher level of head-twitches in mice pre-treated with PCP ( $P < 0.05$  for 0.5 mg/kg DOI and  $P < 0.001$  for 2 mg/kg DOI).

We further sought to determine whether subchronic PCP administration increased the activity of the frontal cortex following DOI administration. To visualize *in vivo* cortex circuit activity, we examined the mRNA expression of the IEGs, *Arc*, *c-fos* and *Egr-2*, which have been previously shown to be strongly upregulated following systemic DOI administration (Gonzalez-Maeso et al., 2007; Pei et al., 2000). Again, animals were pretreated for 10 days with PCP or vehicle and allowed a five day was-out period. Frontal cortex samples were taken for RT-qPCR analysis 60 minutes after acute challenge with DOI (0.5 and 2 mg/kg) or vehicle. We observed that mRNA expression for all three IEGs in PCP pretreated animals was significantly potentiated compared with saline pretreated controls; Two-way ANOVA showed a significant PCP x DOI interaction for *Arc* ( $[F(1,38) = 6.63$ ;  $P = 0.0141$ ] (Fig. 2a)), *c-fos* ( $[F(1,41) = 8.105$ ;  $P = 0.0069$ ] (Fig. 2b)) and *egr-2* ( $[F(1,41) = 5.108$ ;  $P = 0.0292$ ] (Fig. 2c)).

To investigate the possibility of an increased basal glutamatergic activity in PFC following subchronic PCP treatment, we examined the expression of *Arc* mRNA in PFC using *in situ* hybridization, which has a higher regional resolution than RT-qPCR. Here, we found that repeated PCP treatment significantly increased baseline PFC *Arc* mRNA expression ( $P < 0.01$ ; Fig. 3), suggesting that PCP causes an increase in basal PFC excitability.

Collectively, these experiments indicate that subchronic PCP treatment results in enhanced circuit activation of PFC in response to 5-HT<sub>2A</sub>R stimulation.

#### *Influence of PCP on 5-HT<sub>2A</sub>R, 5-HT<sub>2C</sub>R, 5-HT<sub>1A</sub>R and SERT binding and expression*

Previous studies have shown that 5-HT<sub>2A</sub>R functionality can be regulated by expression levels of 5-HT<sub>2A</sub>R (Abbas et al., 2009) and interaction with 5-HT<sub>2C</sub>R (Vickers et al., 2001), 5-HT<sub>1A</sub>R (Willins and Meltzer, 1997) and SERT (Jennings et al., 2008). To investigate if the observed changes in 5-HT<sub>2A</sub>R functionality were caused by changes in these receptors systems, we determined their expression levels using *in vitro* receptor autoradiography and/or RT-qPCR.

The densities of 5-HT<sub>2A</sub>, 5-HT<sub>1A</sub> receptors and SERT in the cortex (as shown in Fig. 4a), were determined using quantitative *in vitro* receptor autoradiography. No difference in [<sup>3</sup>H]-MDL100907 binding was observed between saline and PCP-treated animals (92.3±4.3 vs. 90.6±2.3 fmol/mg TE, Fig. 4a). Also, no changes were observed in the binding of [<sup>3</sup>H]-WAY-100635 (40.9±0.9 vs. 42.6±2.3 fmol/mg TE, Fig. 4b) or [<sup>3</sup>H]-Escitalopram (9.4±0.3 vs. 10.0±0.4 fmol/mg TE, Fig. 4c), measuring 5-HT<sub>1A</sub>R and SERT receptor binding, respectively. Further, the 5-HT receptor mRNA levels were investigated using RT-qPCR. No changes were observed in 5-HT<sub>2A</sub>R, 5-HT<sub>2C</sub>R or 5-HT<sub>1A</sub>R mRNA expression levels between saline and PCP pretreated groups (Fig. 5a-c).

Together, these data suggest that the observed changes in 5-HT<sub>2A</sub>R functionality following PCP treatment are not caused by changes in receptor expression.

## Discussion

In this study we report that mice treated subchronically with PCP exhibit enhanced absolute responses to DOI. This increased functionality was measured as DOI-induced increases in the IEGs *Arc*, *c-fos* and *egr-2* mRNA levels and HTR. These changes were not accompanied by differences in 5-HT<sub>2A</sub>R mRNA or 5-HT<sub>2A</sub>R binding levels in the frontal cortex. A correlation between 5-HT<sub>2A</sub>R binding and functionality has previously been described in a maternal influenza model of schizophrenia and in PSD-95 KO mice (Moreno et al., 2011; Abbas et al., 2009). However, in an early life stress model, the 5-HT<sub>2A</sub>R-dependent HTR and *Arc* gene expression were highly enhanced in animals subjected to stress, without a corresponding increase in 5-HT<sub>2A</sub>R binding (Benekareddy et al., 2010). The same has been reported in transgene mice overexpressing the 5-HT transporter, where DOI-induced *Arc* mRNA expression and HTR is increased without changes in 5-HT<sub>2A</sub>R mRNA or binding (Jennings et al., 2008). There are several mechanisms that can explain the poor correlation between 5-HT<sub>2A</sub>R functionality and the receptor binding potential. First, a large pool of 5-HT<sub>2A</sub>Rs are located in intraneuronal endomembranes that bind to ligands, but are non-functional (Bhatnagar et al., 2001). Although these receptors contribute to the binding, they do not mediate intracellular signaling. This is particularly relevant in imaging and post-mortem binding studies where changes in cortical 5-HT<sub>2A</sub>R binding may not be correlated with functional impact (Gonzalez-Maeso et al., 2008; Ebdrup et al., 2011; Rasmussen et al., 2010).

Second, the enhanced responses in PCP-treated animals could be the result of altered PFC 5-HT release. Acute PCP treatment increases the release of 5-HT (Takahashi et al., 2001), but no studies have until now looked into whether 5-HT release is altered after subchronic PCP treatment. Subchronic PCP treatment affects dopamine and glutamate release in PFC (Bubenikova-Valesova et al., 2008). However, we measured SERT and 5-HT<sub>1A</sub>R binding and mRNA expression of 5-HT<sub>2A</sub>R or 5-HT<sub>1A</sub>R, but did not find any changes, suggesting that the effects on 5-HT<sub>2A</sub>R activation were not caused by major alterations in the neocortical serotonergic system in general.

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3 Finally, the increased HTR and IEG expression after PCP pretreatment could be explained by changes in the  
4 glutamatergic tonus in the PFC. Repeated PCP treatment causes a deficit of GABAergic interneurons, which  
5 leads to disinhibition of cortical pyramidal neurons through alterations in subcortical networks (Lewis, 2000).  
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8 The increase in basal levels of *Arc* mRNA expression in PFC after PCP treatment suggests an increase in  
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Finally, the increased HTR and IEG expression after PCP pretreatment could be explained by changes in the glutamatergic tonus in the PFC. Repeated PCP treatment causes a deficit of GABAergic interneurons, which leads to disinhibition of cortical pyramidal neurons through alterations in subcortical networks (Lewis, 2000). The increase in basal levels of *Arc* mRNA expression in PFC after PCP treatment suggests an increase in excitatory tonus. *Arc* is a marker for excitatory neurotransmission, and plays an important role in synaptic changes required for memory consolidation in the hippocampus (Guzowski et al., 2000; Bramham et al., 2008). *Arc* mRNA expression therefore provides an indirect measure of pyramidal cell excitability downstream of the receptor. *Arc* mRNA expression in the hippocampus is directly induced by NMDA receptor activation. Similarly, HTR is dependent upon 5-HT<sub>2A</sub>R-mediated glutamate release and subsequent NMDA receptor activation in the PFC (Gonzalez-Maeso and Sealfon, 2009). An enhanced glutamatergic tonus could produce an increase in baseline *Arc* mRNA expression and an enhanced DOI-induced HTR and *Arc* gene expression. Neuronal hyperexcitability may also account for the increase in 5-HT<sub>2A</sub>R functionality after embryonic stress (Benekareddy et al., 2010). Exposure to early life stress had an impact on cellular development, signal transduction, and G-protein signaling and caused a more immature, excitatory 5-HT phenotype in adult PFC (Benekareddy et al., 2010). Thus, the changes in 5-HT<sub>2A</sub>R-mediated functionality were caused by secondary alterations. Further, increase in serotonin as produced by the precursor L-5-hydroxytryptophan induces HTR in mice (Schmid et al., 2008), why subchronic PCP could lead to a greater degree of responsiveness to the normal levels of serotonin.

Taken together, we report that the subchronic treatment with PCP leads to increased sensitivity of 5-HT<sub>2A</sub>R signalling that is not paralleled by changes in 5-HT<sub>2A</sub>R levels, but may be due to alterations in downstream glutamatergic signaling.

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For Peer Review

## Reference List

- Abbas AI, Yadav PN, Yao WD, Arbuckle MI, Grant SG, Caron MG, Roth BL, 2009. PSD-95 is essential for hallucinogen and atypical antipsychotic drug actions at serotonin receptors. *J. Neurosci.* 29: 7124-7136.
- Amitai N, Kuczenski R, Behrens MM, Markou A, 2012. Repeated phencyclidine administration alters glutamate release and decreases GABA markers in the prefrontal cortex of rats. *Neuropharmacology* 62: 1422-1431.
- Benekareddy M, Goodfellow NM, Lambe EK, Vaidya VA, 2010. Enhanced function of prefrontal serotonin 5-HT(2) receptors in a rat model of psychiatric vulnerability. *J. Neurosci.* 30: 12138-12150.
- Bhatnagar A, Willins DL, Gray JA, Woods J, Benovic JL, Roth BL, 2001. The dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT<sub>2A</sub>) serotonin receptors reveals differential sorting of arrestins and 5-HT<sub>2A</sub> receptors during endocytosis. *J. Biol. Chem.* 276: 8269-8277.
- Bramham CR, Worley PF, Moore MJ, Guzowski JF, 2008. The immediate early gene *arc/arg3.1*: regulation, mechanisms, and function. *J. Neurosci.* 28: 11760-11767.
- Bubenikova-Valesova V, Horacek J, Vrajova M, Hoschl C, 2008. Models of schizophrenia in humans and animals based on inhibition of NMDA receptors. *Neurosci. Biobehav. Rev.* 32: 1014-1023.
- Coyle JT, 2006. Glutamate and schizophrenia: beyond the dopamine hypothesis. *Cell Mol. Neurobiol.* 26: 365-384.
- Darmani NA, 2001. Cannabinoids of diverse structure inhibit two DOI-induced 5-HT(2A) receptor-mediated behaviors in mice. *Pharmacol. Biochem. Behav.* 68: 311-317.
- Dean B, 2003. The cortical serotonin<sub>2A</sub> receptor and the pathology of schizophrenia: a likely accomplice. *J. Neurochem.* 85: 1-13.
- Ebdrup BR, Rasmussen H, Arnt JR, Glenth JB, 2011. Serotonin 2A receptor antagonists for treatment of schizophrenia. *Expert. Opin. Investig. Drugs.*



1 Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, Zhou M, Okawa Y, Callado  
2 LF, Milligan G, Gingrich JA, Filizola M, Meana JJ, Sealfon SC, 2008. Identification of a serotonin/glutamate  
3 receptor complex implicated in psychosis. *Nature* 452: 93-7.

4  
5  
6  
7 Gonzalez-Maeso J, Sealfon SC, 2009. Psychedelics and schizophrenia. *Trends Neurosci.* 32: 225-232.

8  
9  
10 Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q,  
11 Sealfon SC, Gingrich JA, 2007. Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling  
12 pathways to affect behavior. *Neuron* 53: 439-452.

13  
14  
15  
16  
17 Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, Weisstaub N, Hen R, Gingrich JA,  
18 Sealfon SC, 2003. Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-  
19 hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. *J. Neurosci.* 23: 8836-8843.

20  
21  
22  
23  
24 Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA, 2000. Inhibition  
25 of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term  
26 potentiation and the consolidation of long-term memory. *J Neurosci* 20: 3993-4001.

27  
28  
29  
30 Hagiwara H, Fujita Y, Ishima T, Kunitachi S, Shirayama Y, Iyo M, Hashimoto K, 2008. Phencyclidine-induced  
31 cognitive deficits in mice are improved by subsequent subchronic administration of the antipsychotic drug  
32 perospirone: role of serotonin 5-HT1A receptors. *Eur. Neuropsychopharmacol.* 18: 448-454.

33  
34  
35  
36  
37 Homayoun H, Moghaddam B, 2007. NMDA receptor hypofunction produces opposite effects on prefrontal  
38 cortex interneurons and pyramidal neurons. *J. Neurosci.* 27: 11496-11500.

39  
40  
41  
42  
43 Jennings KA, Sheward WJ, Harmar AJ, Sharp T, 2008. Evidence that genetic variation in 5-HT transporter  
44 expression is linked to changes in 5-HT2A receptor function. *Neuropharmacology* 54: 776-783.

45  
46  
47  
48  
49 Jentsch JD, Roth RH, 1999. The neuropsychopharmacology of phencyclidine: from NMDA receptor  
50 hypofunction to the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* 20: 201-225.

51  
52  
53  
54  
55  
56  
57  
58  
59  
60 Kapur S, Zipursky RB, Remington G, 1999. Clinical and theoretical implications of 5-HT2 and D2 receptor  
occupancy of clozapine, risperidone, and olanzapine in schizophrenia. *Am. J. Psychiatry* 156: 286-293.

- 1 Kargieman L, Santana N, Mengod G, Celada P, Artigas F, 2007. Antipsychotic drugs reverse the disruption in  
2 prefrontal cortex function produced by NMDA receptor blockade with phencyclidine. *Proc. Natl. Acad. Sci. U.*  
3 *S. A* 104: 14843-14848.
- 4  
5  
6  
7 Kitaichi K, Yamada K, Hasegawa T, Furukawa H, Nabeshima T, 1994. Effects of risperidone on phencyclidine-  
8 induced behaviors: comparison with haloperidol and ritanserin. *Jpn. J. Pharmacol.* 66: 181-189.
- 9  
10  
11  
12 Lewis DA, 2000. GABAergic local circuit neurons and prefrontal cortical dysfunction in schizophrenia. *Brain*  
13 *Res. Brain Res. Rev.* 31: 270-276.
- 14  
15  
16  
17 Malhotra AK, Pinals DA, Adler CM, Elman I, Clifton A, Pickar D, Breier A, 1997. Ketamine-induced  
18 exacerbation of psychotic symptoms and cognitive impairment in neuroleptic-free schizophrenics.  
19 *Neuropsychopharmacology* 17: 141-150.
- 20  
21  
22  
23 Malhotra AK, Pinals DA, Weingartner H, Sirocco K, Missar CD, Pickar D, Breier A, 1996. NMDA receptor  
24 function and human cognition: the effects of ketamine in healthy volunteers. *Neuropsychopharmacology* 14:  
25 301-307.
- 26  
27  
28  
29  
30 Moghaddam B, Jackson ME, 2003. Glutamatergic animal models of schizophrenia. *Ann. N. Y. Acad. Sci.* 1003:  
31 131-137.
- 32  
33  
34  
35 Moreno JL, Kurita M, Holloway T, Lopez J, Cadagan R, Martinez-Sobrido L, Garcia-Sastre A, Gonzalez-  
36 Maeso J, 2011. Maternal influenza viral infection causes schizophrenia-like alterations of 5-HTA and mGlu  
37 receptors in the adult offspring. *J. Neurosci.* 31: 1863-1872.
- 38  
39  
40  
41 Nabeshima T, Ishikawa K, Yamaguchi K, Furukawa H, Kameyama T, 1987. Phencyclidine-induced head-  
42 twitch responses as 5-HT<sub>2</sub> receptor-mediated behavior in rats. *Neurosci. Lett.* 76: 335-338.
- 43  
44  
45  
46 O'Neill MF, Hicks CA, Shaw G, Parameswaran T, Cardwell GP, O'Neill MJ, 1998. Effects of 5-  
47 hydroxytryptamine<sub>2</sub> receptor antagonism on the behavioral activation and immediate early gene expression  
48 induced by dizocilpine. *J. Pharmacol. Exp. Ther.* 287: 839-846.
- 49  
50  
51  
52 Pei Q, Lewis L, Sprakes ME, Jones EJ, Grahame-Smith DG, Zetterstrom TS, 2000. Serotonergic regulation of  
53 mRNA expression of Arc, an immediate early gene selectively localized at neuronal dendrites.  
54 *Neuropharmacology* 39: 463-470.
- 55  
56  
57  
58  
59  
60

- 1 Pei Q, Tordera R, Sprakes M, Sharp T, 2004. Glutamate receptor activation is involved in 5-HT<sub>2</sub> agonist-  
2 induced Arc gene expression in the rat cortex. *Neuropharmacology* 46: 331-339.  
3  
4  
5  
6 Pratt JA, Winchester C, Egerton A, Cochran SM, Morris BJ, 2008. Modelling prefrontal cortex deficits in  
7 schizophrenia: implications for treatment. *Br. J. Pharmacol.* 153 Suppl 1: S465-S470.  
8  
9  
10 Rasmussen H, Erritzoe D, Andersen R, Ebdrup BH, Aggernaes B, Oranje B, Kalbitzer J, Madsen J, Pinborg LH,  
11 Baare W, Svarer C, Lublin H, Knudsen GM, Glenthøj B, 2010. Decreased frontal serotonin<sub>2A</sub> receptor binding  
12 in antipsychotic-naïve patients with first-episode schizophrenia. *Arch Gen Psychiatry* 67: 9-16.  
13  
14  
15  
16  
17 Schmid CL, Raehal KM, Bohn LM, 2008. Agonist-directed signaling of the serotonin 2A receptor depends on  
18 beta-arrestin-2 interactions in vivo. *Proc. Natl. Acad. Sci. U. S. A* 105: 1079-1084.  
19  
20  
21  
22 Seillier A, Giuffrida A, 2009. Evaluation of NMDA receptor models of schizophrenia: divergences in the  
23 behavioral effects of sub-chronic PCP and MK-801. *Behav. Brain Res.* 204: 410-415.  
24  
25  
26 Takahashi S, Horikomi K, Kato T, 2001. MS-377, a novel selective sigma(1) receptor ligand, reverses  
27 phencyclidine-induced release of dopamine and serotonin in rat brain. *Eur. J. Pharmacol.* 427: 211-219.  
28  
29  
30  
31 Thomsen MS, Christensen DZ, Hansen HH, Redrobe JP, Mikkelsen JD, 2009. alpha(7) Nicotinic acetylcholine  
32 receptor activation prevents behavioral and molecular changes induced by repeated phencyclidine treatment.  
33 *Neuropharmacology* 56: 1001-1009.  
34  
35  
36  
37 Thomsen MS, Hansen HH, Mikkelsen JD, 2010a. alpha7 nicotinic receptor agonism mitigates phencyclidine-  
38 induced changes in synaptophysin and Arc gene expression in the mouse prefrontal cortex. *Neurochem. Int.* 57:  
39 756-761.  
40  
41  
42  
43  
44 Thomsen MS, Hansen HH, Mikkelsen JD, 2010b. Opposite effect of phencyclidine on activity-regulated  
45 cytoskeleton-associated protein (Arc) in juvenile and adult limbic rat brain regions. *Neurochem. Int.* 56: 270-  
46 275.  
47  
48  
49  
50  
51 Vickers SP, Easton N, Malcolm CS, Allen NH, Porter RH, Bickerdike MJ, Kennett GA, 2001. Modulation of 5-  
52 HT(2A) receptor-mediated head-twitch behaviour in the rat by 5-HT(2C) receptor agonists. *Pharmacol.*  
53 *Biochem. Behav.* 69: 643-652.  
54  
55  
56  
57  
58  
59  
60

1 Vigano D, Guidali C, Petrosino S, Realini N, Rubino T, Di M, V, Parolaro D, 2009. Involvement of the  
2 endocannabinoid system in phencyclidine-induced cognitive deficits modelling schizophrenia. *Int. J.*  
3 *Neuropsychopharmacol.* 12: 599-614.

4  
5  
6  
7 Vollenweider FX, Vollenweider-Scherpenhuyzen MF, Babler A, Vogel H, Hell D, 1998. Psilocybin induces  
8 schizophrenia-like psychosis in humans via a serotonin-2 agonist action. *Neuroreport* 9: 3897-3902.

9  
10  
11 Willins DL, Meltzer HY, 1997. Direct injection of 5-HT<sub>2A</sub> receptor agonists into the medial prefrontal cortex  
12 produces a head-twitch response in rats. *J. Pharmacol. Exp. Ther.* 282: 699-706.

13  
14  
15  
16  
17 Zhu Y, Stornetta RL, Zhu JJ, 2004. Chandelier cells control excessive cortical excitation: characteristics of  
18 whisker-evoked synaptic responses of layer 2/3 nonpyramidal and pyramidal neurons. *J. Neurosci.* 24: 5101-  
19 5108.  
20  
21  
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## Figure legends

**Fig. 1. Subchronic PCP administration increases absolute levels of DOI-induced HTR.** Animals were treated with PCP or vehicle for 10 days, followed by a five day wash-out period. Shown is graphical representation of total number of head-twitches after DOI administration (0.5 and 2 mg/kg, i.p.; scored between 5-20 minutes after treatment). A significant increase in HTR was observed between the saline and PCP-pretreated groups ( $^{§§§}P = 0.0065$ , two-way ANOVA). For both saline and PCP-pretreated animals, 0.5 and 2 mg/kg DOI highly induced head-twitches ( $^{***}P < 0.001$ ). However, for both 0.5 and 2 mg/kg DOI, PCP pretreated animals showed a significant higher level of HTR ( $^{\#}P < 0.05$  for 0.5 mg/kg DOI and  $^{\#\#}P < 0.001$  for 2 mg/kg DOI, one-way ANOVA with Tukeys post-hoc test).  $N = 7-8$ . Data presented as mean  $\pm$  SEM.

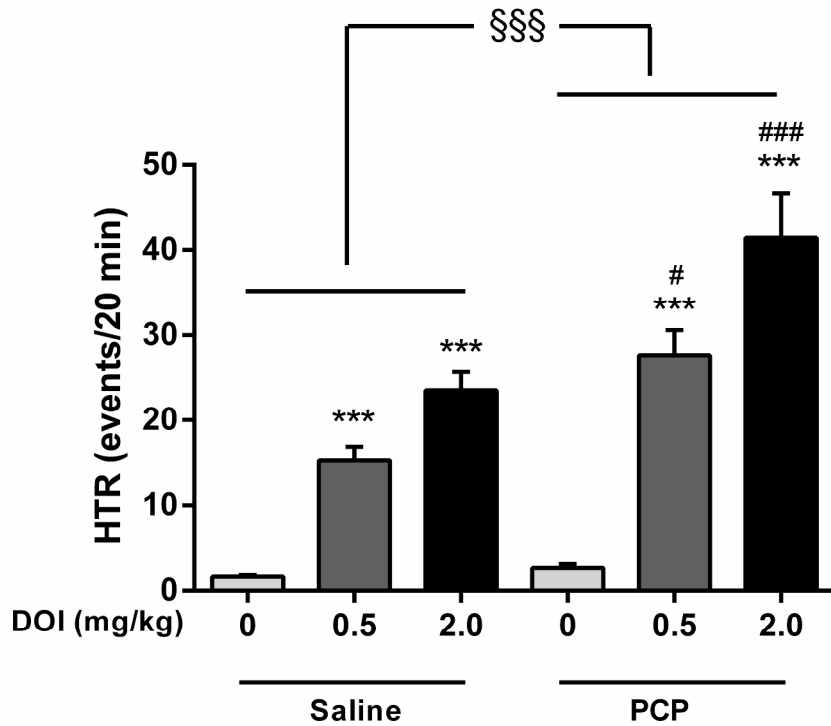
**Fig. 2. Repeated PCP pretreatment potentiates DOI-induced IEG mRNA expression.** Animals were administered PCP or vehicle for 10 days, followed by a five day wash-out period. Shown is graphical representation of frontal cortex IEG mRNA expression from samples taken 60 minutes following DOI (0.5 and 2 mg/kg, i.p.) or vehicle treatment. (a-c) For both saline and PCP pretreated animals, 0.5 and 2 mg/kg DOI highly induced *Arc*, *c-fos* and *egr-2* mRNA expression in frontal cortex. A significant increase in IEG mRNA levels was observed between saline and PCP-pretreated groups for *Arc* (Fig. 2a;  $^{\$}P = 0.0141$ , two-way ANOVA), *c-fos* (Fig. 2b;  $^{§§}P = 0.0069$ , two-way ANOVA) and *egr-2* mRNA (Fig. 2c;  $^{\$}P = 0.029$ , two-way ANOVA).  $N = 7-8$ . Data presented as mean  $\pm$  SEM and as % of saline vehicle control.

**Fig. 3. PCP pretreatment increases baseline PFC *Arc* mRNA expression.** Animals were treated with PCP or vehicle for 10 days, followed by a five day wash-out period. Shown are representative *in situ hybridization* autoradiograms of PFC and graphical representation of the densitometric analysis. Repeated PCP treatment significantly up-regulated *Arc* mRNA expression in the PFC ( $^{**}P < 0.01$ ). Students t-test.  $N = 7-8$ . Data presented as % of saline control  $\pm$  SEM.

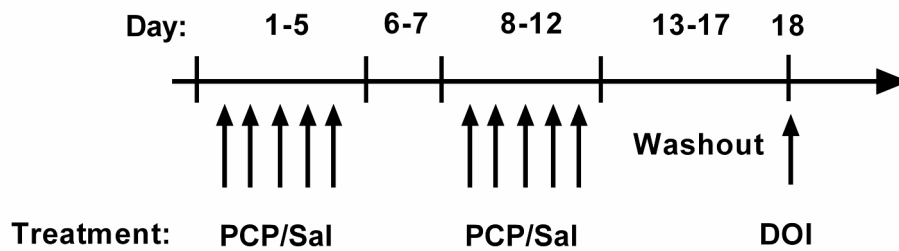
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3 **Fig. 4. Influence of subchronic PCP administration on 5-HT<sub>2A</sub>R, 5-HT<sub>1A</sub>R and SERT binding in cortex.**

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5 Animals were pretreated with PCP or vehicle for 10 days, followed by a five day wash-out period. Shown are  
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8 representative autoradiograms and graphical representation of the densitometric analysis. (a) No difference in  
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10 the 5-HT<sub>2A</sub>R antagonist [<sup>3</sup>H]-MDL100907 binding in cortex was observed between saline and PCP pretreated  
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12 animals. (b) No difference was observed in 5-HT<sub>1A</sub>R antagonist [<sup>3</sup>H]-WAY100635 binding in cortex (c) No  
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14 difference was observed in [<sup>3</sup>H]-Escitalopram binding in the frontal cortex. Students t-test. *N* = 8. Data  
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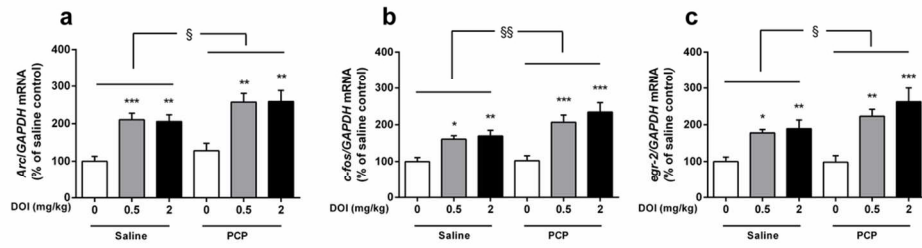
24 **Fig. 5. Subchronic PCP administration has no effect on 5-HT receptor mRNA expression.** Animals were  
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26 treated with PCP or vehicle for 10 days, followed by a five day wash-out period. No difference was observed  
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28 between saline and PCP pretreated animals in the frontal cortex mRNA expression of (a) 5-HT<sub>2A</sub>R, (b) 5-HT<sub>2C</sub>R  
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30 or (c) 5-HT<sub>1A</sub>R. Students t-test. *N* = 8. Data presented as % of saline control ± SEM.  
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Treatment paradigm



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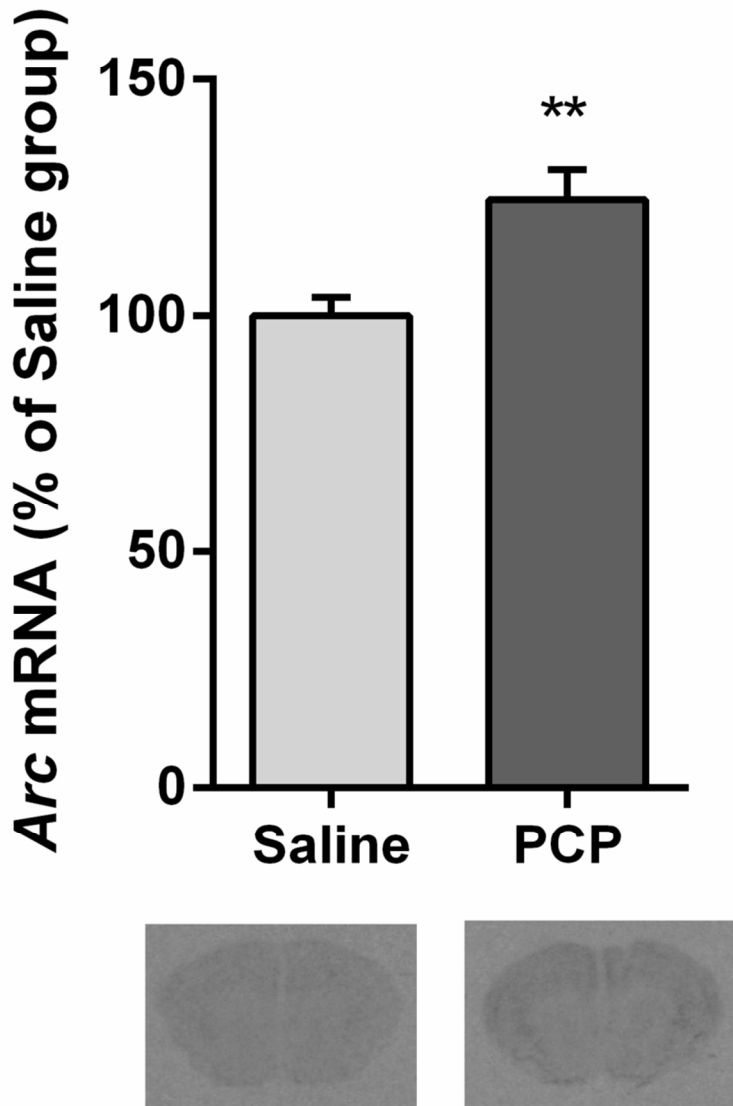


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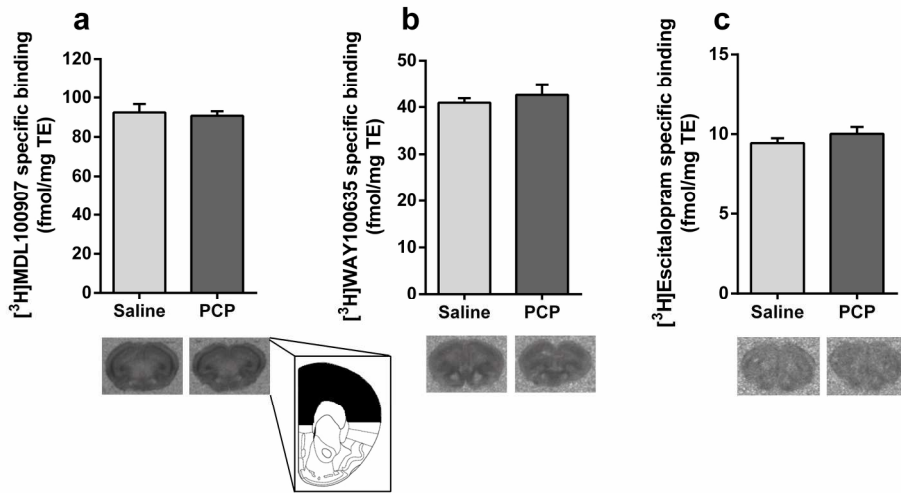




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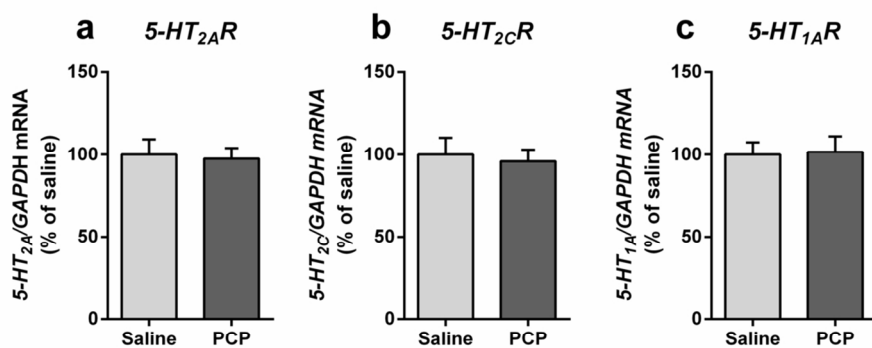
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# *Manuscript III*

Santini MA, Balu DT, Hill-Smith TE, Lucki I, Mikkelsen JD, Coyle JT (2012) Forebrain NMDA receptor hypofunction attenuates pharmacological and behavioral effects induced by the hallucinogenic 5-HT<sub>2A</sub> receptor agonist DOI, *Manuscript*

# **Forebrain NMDA receptor hypofunction attenuates pharmacological and behavioral effects induced by the hallucinogenic 5-HT<sub>2A</sub> receptor agonist DOI**

Martin A. Santini<sup>1, 2, 3</sup>, Darrick T. Balu<sup>2, 3</sup>, Tiffany E. Hill-Smith<sup>5</sup>, Irwin Lucki<sup>4, 5</sup>, Jens D. Mikkelsen<sup>1\*</sup> and Joseph T. Coyle<sup>2, 3</sup>

<sup>1</sup>*Neurobiology Research Unit, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*

<sup>2</sup>*Laboratory for Psychiatric and Molecular Neuroscience, McLean Hospital, Belmont, MA, USA*

<sup>3</sup>*Department of Psychiatry, Harvard Medical School, Boston, MA, USA*

<sup>4</sup>*Department of Psychiatry, University of Pennsylvania, Philadelphia, PA, USA*

<sup>5</sup>*Department of Pharmacology, University of Pennsylvania, Philadelphia, PA, USA*

\* Corresponding Author:

Jens D. Mikkelsen

Neurobiology Research Unit

Blegdamsvej 9

Rigshospitalet, building 9201

DK-2100 Copenhagen, Denmark

Phone (+45) 35456701 / Fax: (+45) 35456713

Email: jens.mikkelsen@nru.dk

## **Abstract**

Both the serotonin and glutamate systems have been implicated in the pathophysiology of schizophrenia, as well as in the mechanism of action of antipsychotic drugs. Psychedelic drugs act through the serotonin 2A receptor (5-HT<sub>2A</sub>R), and elicit a head-twitch response (HTR) in mice, which directly correlates to 5-HT<sub>2A</sub>R activation and is absent in 5-HT<sub>2A</sub>R knockout mice. The precise mechanism of this response remains unclear, but both an intrinsic cortical-cortical pathway and a thalamo-cortical pathway involving glutamate release have been proposed. Here, we used a genetic model of NMDAR hypofunction, a serine racemase knockout (SR<sup>-/-</sup>) mouse, to explore the role of the glutamate system in regulating the 5-HT<sub>2A</sub>R-mediated cellular and the behavioral responses. SR<sup>-/-</sup> mice treated with the 5-HT<sub>2A</sub>R agonist (+/-)-2,5-dimethoxy-4-iodoamphetamine (DOI) showed a clearly diminished HTR and lower induction of *c-fos* mRNA. These altered functional responses in SR<sup>-/-</sup> mice were not associated with changes in cortical 5-HT levels or in 5-HT<sub>2A</sub>R and metabotropic glutamate 2 receptor (mGluR2) mRNA and protein expression in M2/CG1/PrL cortex, S1 cortex or hippocampus. Together, these findings confirm the involvement of NMDARs in mediating the cellular and behavioral effects of 5-HT<sub>2A</sub>R activation.

## Introduction

Activation of serotonin 2A receptors (5-HT<sub>2A</sub>Rs) by psychedelic drugs mediates their psychotomimetic effects, which include alterations of human consciousness, emotion and cognition (Vollenweider et al., 1998). In rodents, administration of hallucinogens results in an induction of immediate-early genes (IEGs), such as *Arc*, *c-fos* and *Egr-2* in cortical brain regions and a characteristic head-twitch response (HTR) (Gonzalez-Maeso et al., 2003; Pei et al., 2004). These cellular and behavioral outputs are eliminated in 5-HT<sub>2A</sub>R<sup>-/-</sup> mice, but can be rescued by genetic restoration of 5-HT<sub>2A</sub>R to cortical pyramidal neurons (Gonzalez-Maeso et al., 2007). Furthermore, only compounds, which cause hallucinations in humans, induces HTR, suggesting that this response is an indicator of hallucinogenic action (Gonzalez-Maeso et al., 2007). Thus, both the induction of cortical IEG expression and HTR can be utilized as a 5-HT<sub>2A</sub>R-specific measure of receptor efficiency.

Several studies suggest an interaction between 5-HT<sub>2A</sub>Rs and the glutamate system. 5-HT<sub>2A</sub>Rs are primarily expressed on apical dendrites of pyramidal neurons, particularly in cortical layer V (Jakab and Goldman-Rakic, 1998). Glutamate serves as the principal neurotransmitter of the pyramidal cells, and it is thought that corticocortical connections, which are mostly comprised of synaptic contacts at apical dendrites (Spratling, 2002), are important in generating and shaping the neural activity that underlies consciousness (Tononi and Edelman, 1998).

Acute administration of the NMDA receptor (NMDAR) antagonists PCP and MK-801 to rodents induce hyperlocomotion and HTR, which are blocked by 5-HT<sub>2A</sub>R antagonists (Kitaichi et al., 1994; O'Neill et al., 1998; Nabeshima et al., 1987). Also, atypical antipsychotics with 5-HT<sub>2A</sub>R antagonistic properties are able to reverse acute PCP-induced cellular disruptions in the prefrontal cortex (PFC) (Kargieman et al., 2007). Together, these studies indicate a potential role of circuits working through the 5-HT<sub>2A</sub>R in mediating the behavioral responses induced by NMDAR antagonists.

Previous studies have proposed that cortical activation by DOI requires the activation of 5-HT<sub>2A</sub>Rs expressed on thalamocortical axon terminals (Marek et al., 2001). However, recent evidence suggests that the circuit involved in this effect is a cortical-cortical circuit and does not require activation of a thalamocortical pathway (Gonzalez-Maeso et al., 2007). In fact, DOI does not induce *Arc* directly in 5-HT<sub>2A</sub>R expressing neurons but in neurons containing AMPA and NMDA receptors (Pei et al., 2004). Instead, activation of 5-HT<sub>2A</sub>Rs in the PFC results in a robust

increase in glutamate receptor activation, which suggests that certain actions of 5-HT<sub>2A</sub>R in this region may be mediated by the release of glutamate (Beique et al., 2007).

To study the involvement of the glutamate system in mediating the cellular and behavioral responses to 5-HT<sub>2A</sub>R activation, we used an animal model of NMDAR hypofunction, the constitutive serine racemase knockout (SR<sup>-/-</sup>) mouse. Activation of NMDARs requires the binding of either glycine or D-serine to the glycine modulatory site (GMS) of the NR1 subunit. SR<sup>-/-</sup> mice, which lack the ability to convert L-serine to D-serine, have a >80% reduction in brain D-serine (Basu et al., 2009). Thus, SR<sup>-/-</sup> mice have impaired NMDAR function in forebrain and consequently cognitive alterations including memory for order, which is a cognitive task requiring the PFC (Devito et al., 2011). In the present study, we tested whether the functional outputs following 5-HT<sub>2A</sub>R activation were altered because of decreased NMDAR activity.



## Methods

### *Animals*

SR<sup>-/-</sup> mice were generated as previously described (Basu et al., 2009). Mice with a serine racemase null mutation resulting from targeted deletion of the first coding exon were backcrossed for over 10 generations onto a C57BL/6J background. SR<sup>+/-</sup> sires and dams were bred to produce wild-type (WT), as well as SR<sup>-/-</sup> offspring. Adult male and female mice were used for all the experiments in this study. Animals were housed in groups of four in polycarbonate cages and maintained on a 12:12 h light/dark cycle in a temperature (22 °C) and humidity controlled vivarium. Animals were given access to food and water ad libitum. All the animal procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee.

### *Drugs*

DOI [(±)-2,5-dimethoxy-4-iodoamphetamine] hydrochloride (synthesized at the Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Copenhagen University) was dissolved in 0.9 % saline.

### *Head-twitch response*

The head-twitch response scoring was performed as previously reported (Darmani, 2001) with minor modifications. In brief, animals ( $n = 6$  per group) were injected with either DOI (2 mg/kg i.p.) or a corresponding volume of saline. After drug administration the mice were immediately moved to a standard cage (15 x 24 cm) without enrichment material and videotaped for 25 min. Subsequently, the total number of head-twitches was scored in the interval between 5 and 25 minutes after drug administration by an observer not aware of the treatment of the animal. One week later, the experiment was repeated so animals receiving DOI on the first test day were given saline and vice versa.

### *Quantitative assessment of mRNA levels using RT-qPCR*

Animals ( $n = 6-9$  per group) were injected with either DOI (2 mg/kg, 10 ml/kg i.p.) or a corresponding volume of saline and returned to their home-cage. After 60 minutes, the mice were killed by cervical dislocation, and their brains quickly removed and frozen in powdered dry ice. Total RNA from cortical samples was isolated with Trizol Reagent (Sigma-Aldrich). cDNA for each RNA sample was generated using the High Capacity cDNA Reverse transcription kit (Applied

Biosystems; Foster City, CA) according to the manufacturer's instructions. The real-time qPCR reactions were performed by adding the sample cDNA to a reaction mixture consisting of 1x Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA) and 15 pmol of each primer. Specific primers were as follows: *GAPDH* primers, Forward: 5'-CATCAAGAAGGTGGTGAAGCA-3', Reverse: 5'-CTGTTGAAGTCACAGGAGACA-3'; *c-fos* primers, Forward: 5'-CAAAGTAGAGCAGCTATCTCC-3', Reverse: 5'-CTCGTCTTCAAGTTGATCTGT-3'; *5-HT<sub>2A</sub>R* primers, Forward: 5'-CCGCTTCAACTCCAGAACCAAAGC-3', Reverse: 5'-CTTCGAATCATCCTGTACCCGAA-3'; *5-mGluR2* primers, Forward: 5'-CCATCTTCTACGTCACCTCC-3', Reverse: 5'-AGGAACAAGCTGGGATCCAG-3'. Data were collected using a 48-well MJ Minioption Personal thermal cycler (BioRad; Hercules, CA). Each sample was assayed in triplicate. For relative quantification of mRNA expression, geometric means were calculated using the comparative  $2^{-\Delta\Delta Ct}$  method, with the housekeeping gene *GAPDH* used as the endogenous reference.

### *Receptor autoradiography*

To determine receptor binding, animals ( $n = 8$  per group) were decapitated and brains were quickly removed and stored at  $\pm 80$  °C until sectioning. Brains were cut in 12  $\mu$ m coronal sections and mounted on Super Frost Plus slides and stored at  $-80$  °C until further processing. *5-HT<sub>2A</sub>R* autoradiography was performed using [<sup>3</sup>H]-MDL100907 [R(+)- $\alpha$ -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)-ethyl]-4-piperidin-methanol] (specific activity; 64 Ci/mmol, a gift from Professor Halldin, Karolinska Institute, Stockholm), and non-specific binding was determined using 10  $\mu$ M ketanserin tartrate (Sigma). For *mGluR2/3* autoradiography we used [<sup>3</sup>H]-LY341495 (GE Healthcare, UK) and measured non-specific binding with 10  $\mu$ M glutamate (Sigma). Briefly, sections were allowed to thaw for 1 h at RT and then pre-incubated with 50 mM Tris-HCl (Sigma), pH 7.4 for 30 min at RT under constant gentle shaking. Sections were then incubated for 60 min at RT using the same buffer containing either 2 nM of [<sup>3</sup>H]-MDL100907 or 2 nM [<sup>3</sup>H]-LY341495 with or without the respective cold ligand. Following incubation, slides were washed for  $2 \times 20$  s in ice-cold 50 mM Tris-HCl, pH 7.4, for 20 s in ice-cold H<sub>2</sub>O, and dried for 1 h under a gentle stream of air.

All sections were placed at 4 °C overnight in a fixator containing paraformaldehyde vapour and then put in an excicator box for 3 h before slides were together with [<sup>3</sup>H]-microscales (GE Healthcare, UK) exposed to a BAS-TR2040 Imaging Plate (Science Imaging Scandinavia AB,

Nacka, Sweden) for 3-14 days at 4 °C. Finally, the imaging plate was scanned on a BAS-2500 phosphoimage scanner (Fujifilm Europe GmbH, Düsseldorf, Germany) and specific and non-specific binding was determined at two brain levels where the following regions were analyzed (i) bregma 2.34 mm: total M2/CG1/PrL cortex, (ii) bregma -2.06 mm: hippocampus and S1 cortex.

#### *Tissue monoamine analysis*

Tissue samples were homogenized in 0.1 N perchloric acid with 100 µM EDTA (15 µl/mg of tissue) using a tissuemizer (Tekmar, Cleveland, OH, USA). Samples were centrifuged at 15,000 rpm (23,143 g) for 15 min at 2–8°C. The supernatants were filtered using Costar Spin- XTM centrifugal filters (Fisher Scientific, Pittsburgh, PA, USA) and then split into two aliquots. Samples (12 µl) were injected by an autosampler (Sample Sentinel, Bioanalytical Systems, West Lafayette, IN, USA) and analyzed in separate assays for tissue content of 5-HT and 5- hydroxyindoleacetic acid (5-HIAA).

The HPLC separation for 5-HT consisted of a PM80 solvent delivery system and a 10 µl sample loop linked in series to a reversed phase microbore column (ODS 3 µm, 100°µ1 mm; Bioanalytical Systems). The mobile phase for the separation of 5-HT consisted of 12.42 mM citric acid (Sigma), 39.85 mM NaPO<sub>4</sub> monobasic (Fluka, Buchs SG, Switzerland), 0.25 mM EDTA (Fluka), 0.737 mM 1-decanesulfonic acid (Sigma), 10 mM NaCl (Fluka), 0.2% triethylamine (Sigma), 16.5% methanol (Fisher Scientific) adjusted to a pH of 4.1. The flow rate through the system was 60 µl/min, and the detector was set at a potential of +0.60 V relative to a Ag/AgCl reference electrode.

A standard concentration of 5-HT was prepared before injection of tissue samples. Tissue concentrations of monoamines were determined using a linear regression analysis of the peak heights obtained from a range of standards and expressed as pg/mg tissue.

#### *Statistical analyses*

All analyses were performed using GraphPad 5. Data are presented as mean ± SEM. The data were analyzed using Student's *t*-test if comparing the two groups, one-way ANOVA with Tukey's post hoc test when comparing multiple groups, and two-way ANOVA with Tukey's post hoc test, when comparing the groups with more than one variable. The level of statistical significance was set to  $P < 0.05$ .

## Results

### *DOI-induced HTR and c-fos mRNA expression is reduced in SR-/- mice*

Head-twitch behavior and *c-fos* mRNA expression in cortical areas are reliably and robustly elicited by hallucinogenic 5-HT<sub>2A</sub>R agonists in rodents. We first assayed the HTR induced by DOI in wild-type and SR-/- mice (Fig. 1). Two-way ANOVA showed a significant interaction between treatment [ $F(1,19) = 131.4$ ;  $p < 0.0001$ ] and genotype [ $F(1,19) = 10.06$ ;  $p < 0.0050$ ]. Further, the *post hoc* analysis revealed that DOI induced significantly fewer head-twitches in SR-/- mice ( $p = 0.0054$ ).

Next we assessed *c-fos* mRNA expression in S1 cortex following DOI treatment. Again a two-way ANOVA showed an interaction between the effects of treatment [Fig. 2;  $F(1,26) = 19.81$ ;  $p = 0.0001$ ] and genotype [ $F(1,26) = 4.651$ ;  $p = 0.0405$ ]. The *post hoc* analysis revealed that DOI induced *c-fos* mRNA to a lesser extent in SR-/- mice compared to wild-type ( $p = 0.0054$ ).

### *5-HT<sub>2A</sub>R and mGluR2 protein and mRNA expression is unaltered in SR-/- mice*

The decreased functional responses in SR-/- mice following administration of DOI led us to examine the level of expression of 5-HT<sub>2A</sub> and mGluR2/3 receptors. The densities of 5-HT<sub>2A</sub> and mGluR2/3 receptors in M2/CG1/PrL cortex, S1 cortex and hippocampus were determined using quantitative in vitro receptor autoradiography. No differences in [<sup>3</sup>H]-MDL100907 (Fig 2a-c) or [<sup>3</sup>H]-LY341495 (Fig. 2d-f) binding were observed between WT and SR-/- mice. Further, S1 cortex and hippocampus mRNA expression of the two receptors was unaltered in SR-/- mice compared to WT controls (Fig. 3 a-d).

### *SR-/- mice have stable 5-HT and 5-HIAA levels*

To assess whether the changes in 5-HT<sub>2A</sub>R-mediated responses were mediated by changes in the serotonin content of the cortex, we measured the levels of 5-HT and its metabolite 5-HIAA. We found no difference in either of these, suggesting that SR-/- mice have normal 5-HT levels and turnover in the frontal cortex (Fig. 4)

## Discussion

In the present study, we show that NMDAR hypofunction leads to attenuated functional responses to 5-HT<sub>2A</sub>R activation. We found that both cortical *c-fos* mRNA induction and HTR after DOI treatment was significantly reduced in SR<sup>-/-</sup>, suggesting that glutamate signalling is necessary for at least some of the cellular and behavioral responses induced by 5-HT<sub>2A</sub>R activation.

This is consistent with previous reports showing that 5-HT<sub>2A</sub>R receptor activation by DOI significantly increases glutamate release and glutamatergic pyramidal neuronal activity in the medial PFC layer V (Celada et al., 2008; Beique et al., 2007; Marek et al., 2001). Results from both *in vitro* and *in vivo* studies in rats show that DOI increases spontaneous and evoked excitatory currents in layer V pyramidal cells (EPSCs) (Aghajanian and Marek, 1997; Aghajanian and Marek, 1999) and increases the firing rate of a majority of neurons (Puig et al., 2003). Further, DOI-mediated induction of *Arc* mRNA in rat PFC can be blocked by the NMDAR antagonist MK-801 (Pei et al., 2004), suggesting that the cellular effects of DOI are glutamate release mediated.

The mechanism behind HTR is more complex, but likely also involves glutamatergic signaling. Both systemic and direct injection of 5-HT<sub>2A</sub>R agonists into the PFC produce HTR (Willins and Meltzer, 1997), which is dependent exclusively on 5-HT<sub>2A</sub>R in the pyramidal neurons in the cerebral cortex, because region-specific rescue of 5-HT<sub>2A</sub>R expression in these particular cells in transgenic animals is sufficient to restore induction of transcriptional factors after systemic administration of DOI (Gonzalez-Maeso et al., 2007). In rodents, hyperlocomotion and HTR induced by acute PCP can be blocked by 5-HT<sub>2A</sub>R antagonists (Kitaichi et al., 1994; O'Neill et al., 1998; Nabeshima et al., 1987), and 5-HT<sub>2A</sub>R antagonists are able to reverse acute PCP-induced cellular disruptions in PFC (Kargieman et al., 2007). These findings indicate that the cellular and behavioral responses induced by acute PCP are mediated through circuits that are modulated by 5-HT<sub>2A</sub>R.

To complicate matters, it was recently demonstrated that the mGluR2 receptor interacts with the 5-HT<sub>2A</sub>R, forming a heteromeric complex, in which mGluR2 activation attenuates both 5-HT<sub>2A</sub>R-mediated *c-fos* mRNA expression and HTR (Gonzalez-Maeso et al., 2008). However, signaling through 5-HT<sub>2A</sub>R is also dependent on mGluR2, as mGluR2-receptor knockout mice are insensitive to HTR-inducing effects of DOI (and LSD) (Moreno et al., 2011). Although the neuropharmacological mechanisms underlying these interactions have not been delineated, these data suggest a prominent role for glutamate receptor systems in the behavioral effects of DOI, and a strong interaction between glutamatergic and 5-HT<sub>2A</sub>R systems.

The decreased *c-fos* mRNA induction and HTR following administration of DOI led us to examine the level of expression of 5-HT<sub>2A</sub> and mGluR2 receptors in the SR<sup>-/-</sup> mice. We found no changes in the binding of [<sup>3</sup>H]-MDL100907 and [<sup>3</sup>H]-LY341495 – measuring 5-HT<sub>2A</sub>R and mGluR2R, respectively, in M2/CG1/PrL cortex, S1 cortex or hippocampus. Further, there was no difference between wild-type and SR<sup>-/-</sup> mice in the mRNA expression of 5-HT<sub>2A</sub>R and mGluR2. These results suggest that the functional deficits observed in SR<sup>-/-</sup> mice are not caused directly by changes in receptor density, but could be due to decreased secondary activation of post-synaptic NMDARs and/or altered G-protein coupling.

SR<sup>-/-</sup> mice have >80% reduction in brain D-serine and exhibit reduced forebrain NMDAR-mediated neurotransmission leading to impaired LTP at the Schaffer collateral-CA1 pyramidal neuron synapse (Basu et al., 2009). It is conceivable that this reduction in NMDAR-mediated signalling is also present in the cortex, the brain region responsible for the cellular and behavioural effects of 5-HT<sub>2A</sub>R activation (Gonzalez-Maeso et al., 2007). Taken together our study suggests a prominent role of NMDARs in regulating the cellular and behavioral responses to 5-HT<sub>2A</sub>R activation, and show that 5-HT<sub>2A</sub>R-mediated signalling can be altered without correlation to receptor binding.

## Reference List

- Aghajanian GK, Marek GJ, 1999. Serotonin, via 5-HT<sub>2A</sub> receptors, increases EPSCs in layer V pyramidal cells of prefrontal cortex by an asynchronous mode of glutamate release. *Brain Res.* 825: 161-171.
- Aghajanian GK, Marek GJ, 1997. Serotonin induces excitatory postsynaptic potentials in apical dendrites of neocortical pyramidal cells. *Neuropharmacology* 36: 589-599.
- Basu AC, Tsai GE, Ma CL, Ehmsen JT, Mustafa AK, Han L, Jiang ZI, Benneyworth MA, Froimowitz MP, Lange N, Snyder SH, Bergeron R, Coyle JT, 2009. Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. *Mol. Psychiatry* 14: 719-727.
- Beique JC, Imad M, Mladenovic L, Gingrich JA, Andrade R, 2007. Mechanism of the 5-hydroxytryptamine 2A receptor-mediated facilitation of synaptic activity in prefrontal cortex. *Proc Natl Acad Sci U S A* 104: 9870-5.
- Celada P, Puig MV, az-Mataix L, Artigas F, 2008. The hallucinogen DOI reduces low-frequency oscillations in rat prefrontal cortex: reversal by antipsychotic drugs. *Biol. Psychiatry* 64: 392-400.
- Darmani NA, 2001. Cannabinoids of diverse structure inhibit two DOI-induced 5-HT(2A) receptor-mediated behaviors in mice. *Pharmacol. Biochem. Behav.* 68: 311-317.
- Devito LM, Balu DT, Kanter BR, Lykken C, Basu AC, Coyle JT, Eichenbaum H, 2011. Serine racemase deletion disrupts memory for order and alters cortical dendritic morphology. *Genes Brain Behav.* 10: 210-222.
- Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, Zhou M, Okawa Y, Callado LF, Milligan G, Gingrich JA, Filizola M, Meana JJ, Sealfon SC, 2008. Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452: 93-7.
- Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC, Gingrich JA, 2007. Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron* 53: 439-452.
- Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, Weisstaub N, Hen R, Gingrich JA, Sealfon SC, 2003. Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. *J. Neurosci.* 23: 8836-8843.
- Jakab RL, Goldman-Rakic PS, 1998. 5-Hydroxytryptamine<sub>2A</sub> serotonin receptors in the primate cerebral cortex: possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites. *Proc. Natl. Acad. Sci. U. S. A* 95: 735-740.
- Kargieman L, Santana N, Mengod G, Celada P, Artigas F, 2007. Antipsychotic drugs reverse the disruption in prefrontal cortex function produced by NMDA receptor blockade with phencyclidine. *Proc. Natl. Acad. Sci. U. S. A* 104: 14843-14848.

- Kitaichi K, Yamada K, Hasegawa T, Furukawa H, Nabeshima T, 1994. Effects of risperidone on phencyclidine-induced behaviors: comparison with haloperidol and ritanserin. *Jpn. J. Pharmacol.* 66: 181-189.
- Marek GJ, Wright RA, Gewirtz JC, Schoepp DD, 2001. A major role for thalamocortical afferents in serotonergic hallucinogen receptor function in the rat neocortex. *Neuroscience* 105: 379-392.
- Moreno JL, Holloway T, Albizu L, Sealfon SC, Gonzalez-Maeso J, 2011. Metabotropic glutamate mGlu2 receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT<sub>2A</sub> receptor agonists. *Neurosci. Lett.* 493: 76-79.
- Nabeshima T, Ishikawa K, Yamaguchi K, Furukawa H, Kameyama T, 1987. Phencyclidine-induced head-twitch responses as 5-HT<sub>2</sub> receptor-mediated behavior in rats. *Neurosci. Lett.* 76: 335-338.
- O'Neill MF, Hicks CA, Shaw G, Parameswaran T, Cardwell GP, O'Neill MJ, 1998. Effects of 5-hydroxytryptamine<sub>2</sub> receptor antagonism on the behavioral activation and immediate early gene expression induced by dizocilpine. *J. Pharmacol. Exp. Ther.* 287: 839-846.
- Pei Q, Tordera R, Sprakes M, Sharp T, 2004. Glutamate receptor activation is involved in 5-HT<sub>2</sub> agonist-induced Arc gene expression in the rat cortex. *Neuropharmacology* 46: 331-339.
- Puig MV, Celada P, az-Mataix L, Artigas F, 2003. In vivo modulation of the activity of pyramidal neurons in the rat medial prefrontal cortex by 5-HT<sub>2A</sub> receptors: relationship to thalamocortical afferents. *Cereb. Cortex* 13: 870-882.
- Spratling MW, 2002. Cortical region interactions and the functional role of apical dendrites. *Behav. Cogn Neurosci. Rev.* 1: 219-228.
- Tononi G, Edelman GM, 1998. Consciousness and the integration of information in the brain. *Adv. Neurol.* 77: 245-279.
- Vollenweider FX, Vollenweider-Scherpenhuyzen MF, Babler A, Vogel H, Hell D, 1998. Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2 agonist action. *Neuroreport* 9: 3897-3902.
- Willins DL, Meltzer HY, 1997. Direct injection of 5-HT<sub>2A</sub> receptor agonists into the medial prefrontal cortex produces a head-twitch response in rats. *J. Pharmacol. Exp. Ther.* 282: 699-706.



## Figure legends

**Fig. 1. DOI-induced HTR and *c-fos* mRNA expression is reduced in SR<sup>-/-</sup> mice.** (a) Two-way ANOVA showed a significant interaction between treatment [ $F(1,19) = 131.4$ ;  $p < 0.0001$ ] and genotype [ $F(1,19) = 10.06$ ;  $p < 0.0050$ ]. For both WT and SR<sup>-/-</sup> animals, 2 mg/kg DOI highly induced head-twitches ( $^{***}P < 0.001$ ). However, DOI, showed a significant lower level of HTR ( $^{\#}P < 0.0054$ )  $N = 6$ . (b) Two-way ANOVA showed an interaction between the effects of treatment [Fig. 2;  $F(1,26) = 19.81$ ;  $p = 0.0001$ ] and genotype [ $F(1,26) = 4.651$ ;  $p = 0.0405$ ]. DOI induced *c-fos* mRNA in both WT and SR<sup>-/-</sup> mice ( $^{***}P < 0.001$  and  $^*P < 0.05$  for WT and SR<sup>-/-</sup> respectively, students t-test), however, the *post hoc* analysis revealed that DOI induced *c-fos* mRNA to a lesser extent in SR<sup>-/-</sup> mice compared to wild-type ( $p = 0.0054$ ).  $N = 6-9$ . Data presented as mean  $\pm$  SEM.

**Fig. 2. Regional protein expression of 5-HT<sub>2A</sub>R and mGluR2/3 receptors.** (a-c) No difference was observed in the 5-HT<sub>2A</sub>R antagonist [<sup>3</sup>H]-MDL100907 binding in M2/CG1/PrL cortex, hippocampus and S1 cortex between WT and SR<sup>-/-</sup> animals. (d-f) No difference was observed in mGluR2/3 antagonist [<sup>3</sup>H]-LY341495 binding in M2/CG1/PrL cortex, hippocampus and S1 cortex. Students t-test.  $N = 8$ . Data presented as mean  $\pm$  SEM.

**Fig. 2. mRNA expression of 5-HT<sub>2A</sub>R and mGluR2/3 receptors in S1 cortex and hippocampus.** (a-b) No difference was observed in the 5-HT<sub>2A</sub>R mRNA expression between WT and SR<sup>-/-</sup> animals. (c-d) No difference in mGluR2/3 mRNA expression binding in hippocampus and S1 cortex. Students t-test.  $N = 8$ . Data presented as mean  $\pm$  SEM.

**Fig. 4. No changes in 5-HT or 5-HIAA levels in SR<sup>-/-</sup> mice.** No difference was observed in the level of 5-HT or 5-HIAA in frontal cortex. Students t-test.  $N = 5-6$ . Data presented as mean  $\pm$  SEM.

## Figures

Figure 1

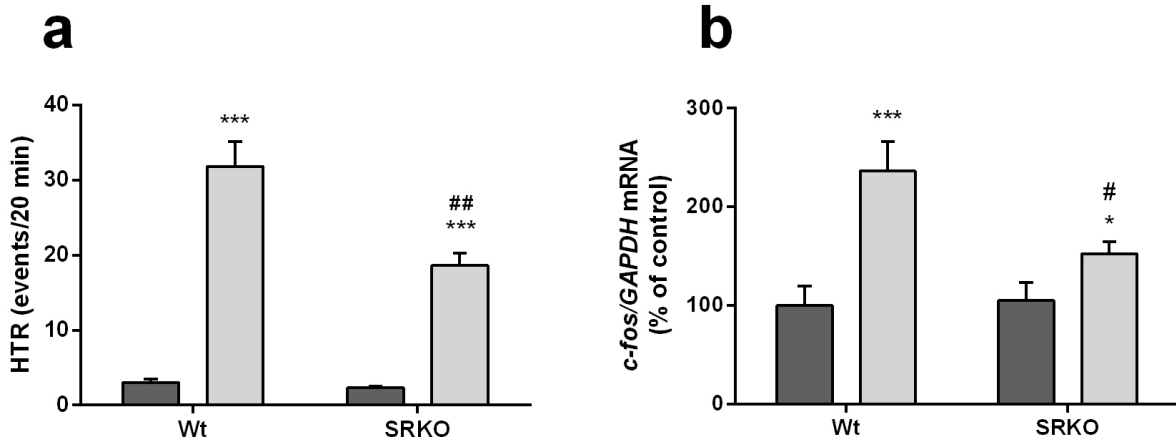


Figure 2

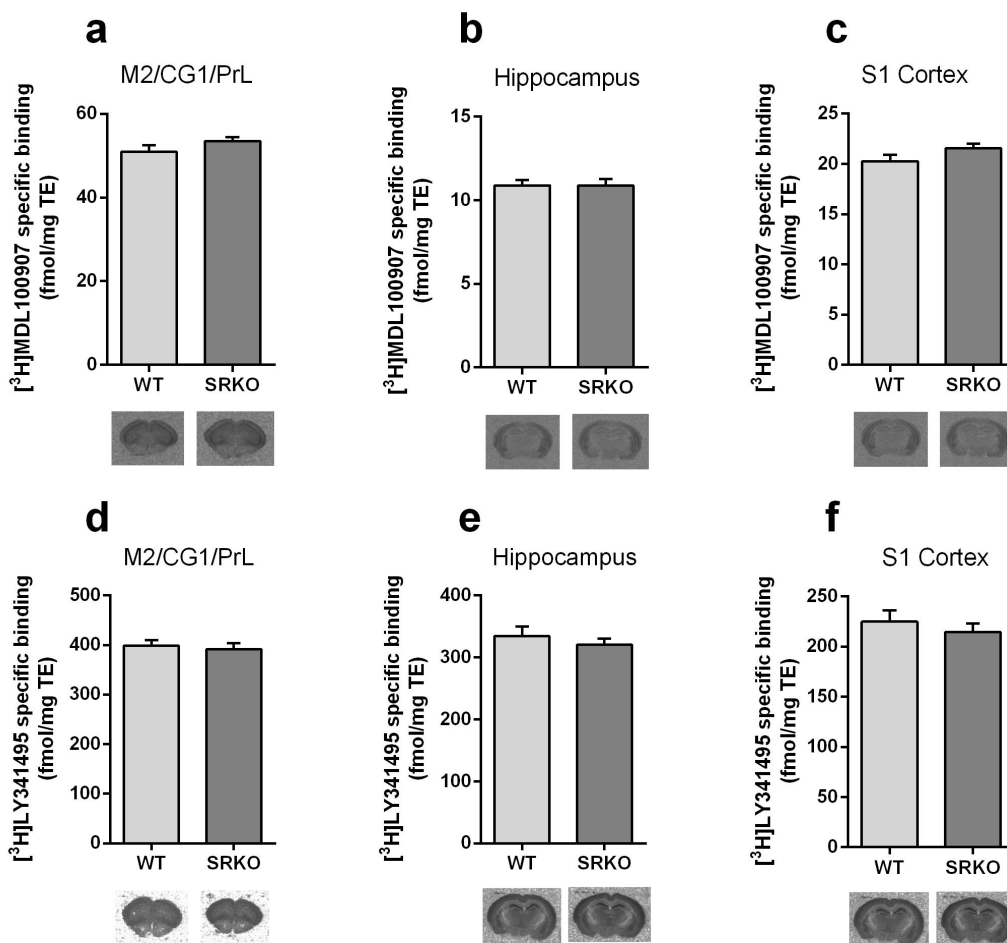


Figure 3

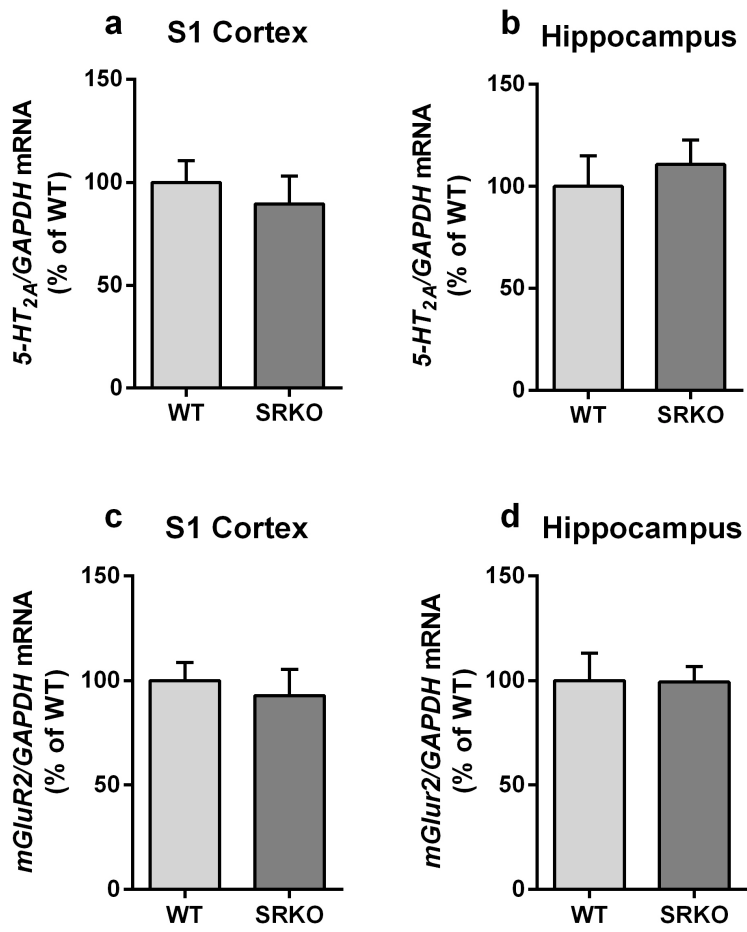
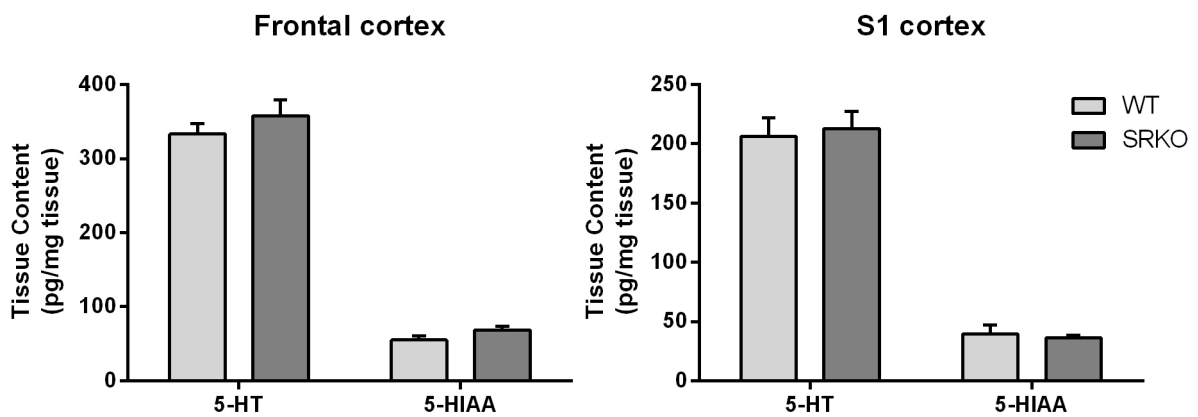


Figure 4



# *Manuscript IV*

Svensson KA, Santini MA, Mikkelsen JD (2012) The mGlu2/3 Receptor Agonists LY354740 and LY379268 Differentially Regulate Restraint Stress-induced Expression of c-Fos in Rat Cerebral Cortex, *Manuscript*

# **The mGlu2/3 Receptor Agonists LY354740 and LY379268 Differentially Regulate Restraint Stress-induced Expression of c-Fos in Rat Cerebral Cortex**

**Svensson KA<sup>1\*</sup>, Santini MA<sup>2</sup> and Mikkelsen JD<sup>2</sup>**

<sup>1</sup>Neuroscience Discovery, Eli Lilly & Company, Indianapolis, IN 46285, USA

<sup>2</sup>Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark.

\* Corresponding Author:

Kjell A. Svensson, PhD

Neuroscience Discovery

Eli Lilly and Company

Indianapolis, IN 46285, USA

Phone +1 317 433 1889/ Fax: +1 317 276 5546

Email: [svenssonka@lilly.com](mailto:svenssonka@lilly.com)

## **Abstract**

Metabotropic glutamate 2/3 (mGlu2/3) receptors have emerged as potential therapeutic targets due to the ability of mGlu2/3 receptor agonists to modulate excitatory transmission at specific synapses. LY354740 and LY379268 are selective and potent mGlu2/3 receptor agonists that show both anxiolytic- and antipsychotic-like effects in animal models. We compared the efficacy of LY354740 and LY379268 in attenuating restraint stress-induced expression of the immediate early gene c-Fos in the rat prelimbic (PrL) and infralimbic (IL) cortex. LY354740 (10 and 30 mg/kg, i.p.) showed statistically significant and dose-related attenuation of stress-induced increase in c-Fos expression, in the rat cortex. By contrast, LY379268 had no effect on restraint-stress induced c-Fos upregulation (0.3-10 mg/kg, i.p.). Because both compounds inhibit serotonin 2A receptor (5-HT<sub>2A</sub>R)-induced c-Fos expression, we hypothesize that LY354740 and LY379268 have different *in vivo* properties and that 5-HT<sub>2A</sub>R activation and restraint stress induces c-Fos through distinct mechanisms.

## Introduction

Preclinical and clinical studies indicate that modulation of glutamatergic activity in the brain may have therapeutic value for the treatment of schizophrenia and anxiety-related disorders (Marek, 2010; Swanson et al., 2005). Glutamate acts through ligand-gated ion channels and G-protein coupled metabotropic glutamate (mGlu) receptors. The mGlu receptors can be subdivided into three groups (Group I: mGlu1, 5; Group II: mGlu2, 3; Group III: mGlu4, 6, 7, 8) based on sequence homology, signal transduction pathways and pharmacology (Conn and Pin, 1997; Schoepp et al., 1999). Activation of presynaptic mGlu2 receptors with mGlu2/3 agonists negatively modulates the release of glutamate providing feedback that prevents excessive glutamate release (Anwyl, 1999; Scanziani et al., 1997). Presynaptic mGlu2/3 receptors also regulate the release of other neurotransmitters (Cartmell and Schoepp, 2000) and postsynaptic mGlu2/3 receptors can regulate neuronal excitability via modulation of ion channel functions (Anwyl, 1999).

The actions of multiple mGlu2/3 agonists and mGlu2 PAMs have been explored in animal models predictive of antipsychotic and anxiolytic activity. Of these, the two orthosteric mGlu2/3 agonists LY354740, and the structurally related LY379268, have been mostly studied. LY354740 and LY379268 block PCP- and amphetamine-induced hyperlocomotion (Cartmell et al., 1999), two commonly used models of the positive symptoms of schizophrenia. Both compounds also show efficacy in alleviating cognitive deficits induced by PCP. For example, LY354740 improved the detrimental effects of PCP on performance in a T-maze task (Moghaddam and Adams, 1998), while LY379268 attenuated a PCP-induced cognitive deficit in the 5-choice serial reaction task (Greco et al., 2005).

In anxiety models however, the properties of LY354740 and LY379268 seems to differ. LY354740 show activity in a wide variety of anxiety models (such as fear-induced potentiated startle (Helton et al., 1998), elevated plus maze (Helton et al., 1998; Monn et al., 1997) and stress-induced hyperthermia) and improves the symptoms of patients with generalized anxiety disorder with similar efficacy as a benzodiazepine comparator (Dunayevich et al., 2008). However, although only a few reports have evaluated the anxiolytic properties of LY379268, the compound does not have identical effects. While LY379268 reduces stress-induced hyperthermia (Satow et al., 2008) and inhibits immobilization-induced hyperprolactinemia (Johnson and

Chamberlain, 2002) it has no effect in the elevated plus maze (Satow et al., 2008) and might even have anxiogenic properties, as it increases startle reflex magnitude (Imre et al., 2006).

The aim of the present study was to evaluate further the effect of LY354740 and LY379268 in animal models relevant to both antipsychotic and anxiolytic activity. The first part of the study evaluated the efficacy of LY354740 and LY379268 by assessing modulation of restraint stress-induced neuronal activation as measured by c-Fos protein. Extensive characterization of restraint-induced c-Fos expression in rat forebrain has previously been reported (Cullinan et al., 1995; Girotti et al., 2006; Imaki et al., 1993; Weinberg et al., 2007) and the suppression of stress-induced c-Fos expression in the rat brain has been associated with the anxiolytic effects of benzodiazepines in the fear conditioning model of anxiety (Beck and Fibiger, 1995).

One mechanism through which this class of compounds is considered to act is via negative modulation of serotonin 2A receptor (5-HT<sub>2A</sub>R)-dependent signaling. 5-HT<sub>2A</sub>R and mGlu2 forms a functional heteromeric complex, where activation of mGlu2 attenuates signaling through 5-HT<sub>2A</sub>R (Gonzalez-Maeso et al., 2008). In vivo electrophysiological studies have demonstrated that the 5-HT<sub>2A/2C</sub> receptor agonist, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) enhances glutamatergic synaptic transmission in the prefrontal cortex of rats (Gewirtz and Marek, 2000), a response which can be attenuated by mGlu2 activation with LY354740 (Marek et al., 2001). Administration of DOI also result in an increase in the expression of c-Fos in several regions of the cortex, including the prefrontal cortex (Leslie et al., 1993). LY379268 has previously been shown to reverse DOI-induced c-Fos upregulation in dmPFC (Wischhof and Koch, 2012). In the second part of the study, we tested the effect of LY354740 on DOI-induced increase in c-Fos expression in the rat prelimbic (PrL) and infralimbic (IL) cortex.



## Results

### *The effect of LY354740 and LY379268 pretreatment on restraint stress-induced c-Fos expression*

The effect of LY354740 and LY379268 was examined in two different, but comparable experiments. Restraint stress significantly increased the number of positive cells containing c-Fos immunoreactivity relative to vehicle in both the PrL and IL cortex ( $P < 0.001$ ; Figs. 1 and 2). The magnitude of induction was similar between the two areas, and between the two experiments. Prior administration of LY354740 (3 and 10 mg/kg) significantly reduced the effect of restraint stress induced c-Fos protein expression ( $P < 0.01$  in both PrL and IL cortex). Administration of LY354740 alone (10 and 30 mg/kg) had no significant effect on c-Fos expression in any area examined.

Pre-treatment with the LY379268 (0.3, 1 and 3 mg/kg) had no effect on restraint stress induced c-Fos protein expression in any of the two areas. Alprazolam (3 mg/kg, i.p.) significantly reduced the effect of restraint stress induced c-Fos protein expression in both PrL and IL ( $P < 0.01$ ). While the low dose of LY379268 alone had no effect on basal c-Fos levels, a higher dose (10 mg/kg) produced a significant up-regulation of c-Fos expression to a similar extent as restraint stress ( $P < 0.01$ ).

### *LY354740 pretreatment attenuates DOI-induced c-Fos expression*

DOI (3 mg/kg, i.p.) produced a significant increase in c-Fos protein expression in the PrL and IL cortex relative to vehicle-treated animals ( $P < 0.001$ ; Fig. 3). The magnitude of increase was much higher than seen for restraint stress. LY354740 pretreatment at 3 mg/kg, i.p. ( $P < 0.01$  in both PrL and IL cortex) and 10 mg/kg, i.p. ( $p < 0.001$  in both PrL and IL cortex) significantly reduced the effect of DOI induced c-Fos protein expression.

## Discussion

In the present study we examined the effect of the mGluR2/3 agonists LY354740 and LY379268 on immediate early gene (IEG) expression induced by restraint stress. c-Fos is considered a marker of neuronal activity and may have a common mechanism of induction by acute restraint stress and DOI/PCP treatment (Beique et al., 2007; Moghaddam, 1993). Both anxiolytics and antipsychotics have been shown to attenuate IEG expression in cortex induced by stress and the NMDA receptor antagonist phencyclidine (PCP), which are used to model anxiety and schizophrenia respectively (de Medeiros et al., 2005; Izumi et al., 2006; Kargieman et al., 2007).

We used restraint stress to induce an anxiety-like state, as this type of stressor has been shown to mediate fear potentiation in the elevated plus-maze (Heinrichs et al., 1994) and robustly increase circulating levels of ACTH and corticosterone (Mikkelsen and Larsen, 2006). Consistent with previous reports we observed an increase in c-Fos (Cullinan et al., 1995; Girotti et al., 2006; Trneckova et al., 2006; Weinberg et al., 2007) expression in the forebrain after restraint stress.

It was revealed that while LY354740 had a strong and dose-dependent inhibitory effect on stress-induced gene expression, LY379268 had not. These results are in line with behavioral experiments. In the fear-potentiated startle response paradigms, LY354740 demonstrated efficacy in rodents (Helton et al., 1998), to decrease stress-induced hyperthermia (Spooren et al., 2002) and has even been reported to possess anxiolytic activity in one clinical trial (Dunayevich et al., 2008). It is striking that LY379268 had no effects in the same model as LY379268 did not attenuate restraint stress induced c-Fos expression in wide ranged doses. By contrast, LY379268 alone caused c-Fos upregulation in PFC to a comparable degree as restraint stress at high dose. These results clearly show that LY354740 and LY379268 - although highly structurally related - have different properties.

The reason for the difference in *in vivo* efficacy between the two compounds is still speculative. However, LY354740 and LY379268 differ somewhat in their *in vitro* potencies at mGlu2 vs mGlu3 receptors (Monn et al., 1999). While LY354740 is approximately equipotent at mGlu2 and mGlu3, LY379268 is overall more potent at both receptor subtypes, showing a 5x higher potency at mGlu2 and a 16x higher potency at mGlu3 compared to LY354740 (Monn et al., 1999). This difference in *in vitro* profile may account for differences *in vivo*, including brain

2-deoxyglucose utilization studies in the rat, where the LY354740 shows a more general suppression of glucose use across different brain areas (Lam et al., 1999). In this study the authors also noted differences in the overt behavioral responses of the two mGlu2/3 agonists. These results may contribute to the understanding of why LY354740 and LY379268 have different effects in behavioral models of anxiety, such as fear-induced startle (Helton et al., 1998; Imre et al., 2006) and elevated plus maze (Helton et al., 1998; Monn et al., 1997; Satow et al., 2008).

Finally, we investigated the interaction between 5-HT<sub>2A</sub>R and mGlu2/3 receptors, because recent studies suggest that the effects of mGlu2/3 agonists are partly mediated through 5-HT<sub>2A</sub> receptors (Gonzalez-Maeso et al., 2008; Fribourg et al., 2011). Previously, LY379268 has been shown to decrease DOI-induced c-Fos expression in dmPFC (Wischhof and Koch, 2012). We demonstrated the ability of LY354740 to attenuate the DOI-induced c-Fos expression in the rat PrL and IL cortex. Because increased excitation of the PFC has been implicated in the pathophysiology of schizophrenia, the ability of LY354740 and LY379268 to reduce the hallucinogenic drug action in this region could be directly related to its antipsychotic-like efficacy (Benneyworth et al., 2007). Antidepressants with anxiolytic properties act in part by blocking the activation of the serotonin 5-HT<sub>2A</sub> receptors (Croom et al., 2009), and mice lacking functional 5-HT<sub>2A</sub> receptors have reduced anxiety-like behaviors (Weisstaub et al., 2006). Further, the ability of both LY354740 and LY379268 to attenuate DOI-induced c-Fos suggests that this c-Fos response is caused by a different mechanism than for restraint stress.

In perspective, recent evidence suggests that hyperactivity of the glutamatergic systems in the limbic cortex may contribute to the symptoms of schizophrenia and anxiety (Harvey and Shahid, 2011; Krystal et al., 1999). The selective modulation of restraint stress- and DOI-induced c-Fos expression in the limbic cortex provides additional evidence that mGlu2/3 agonists may serve as an effective therapeutic strategy for preferentially targeting the glutamatergic dysfunction in schizophrenia and anxiety. Thus, the results from our studies provide further insight in the utility of mGlu2/3 orthosteric agonists in the treatment of a variety of psychiatric conditions including schizophrenia and anxiety.

## **Experimental Procedures**

### *Animals*

All experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Eli Lilly Institutional Animal Care and Use Committee. Male Sprague Dawley rats, from Harlan, Indianapolis, IN, were housed in groups of four per cage in standard conditions, free food and water and maintained on a 12-h light/dark cycle (lights on at 06:00, lights off at 18:00). Animals were acclimatized to the vivarium for at least 6 days prior to the initiation of the study. Studies were performed according to the guidelines of the Animal Care and Use Committee of Eli Lilly and Company.

### *Drug Treatment*

For the DOI studies, rats were pre-handled 3-4 days prior to experimentation to minimize stress. On the day of the study, animals were pretreated with vehicle or LY354740 (i.p.) and returned to their home cages. Thirty minutes later, animals were treated with DOI (3 mg/kg, i.p.) or vehicle for DOI. Animals (n = 7 to 8 per group) were euthanized by decapitation 2 hours after vehicle or DOI. Whole brains were rapidly removed and immediately immersed in isopentane over dry ice and then stored at -80°C until sectioned.

For the restraint stress studies, rats were pre-handled 4-5 days prior to experimentation to minimize stress. On the day of the study animals were pretreated with vehicle, LY354740 (1, 3, 10, 30 mg/kg, i.p.), LY379268 (0.3, 1, 3, 10 mg/kg, i.p.) or Alprazolam (3 mg/kg, i.p.) and returned to their home cages. After 30 minutes, animals were restrained in an acrylic flat-bottomed restrainer for 20 minutes. All animals subjected to the restraint stress were housed individually in a quiet room after the removal from the restrainer. Animals (n = 7-8 per group) were euthanized by decapitation 2 hours after onset of restraint. Whole brains were rapidly removed and immediately immersed in isopentane over dry ice and then stored at -80°C until sectioned.

### *Fos Immunohistochemistry*

Coronal sections through the rat prelimbic (PrL, bregma +2.70 mm) and infralimbic (IL, bregma +2.70 mm) were cut at 14 µm in a cryostat and thaw-mounted onto Superfrost Plus slides. Sections were allowed to air dry at room temperature until completely dry and were stored

at -20°C until processed. c-Fos single label immunohistochemistry was performed on 14 µm fresh-frozen brain sections.

Slides were brought to room temperature. Slides were immersed in a freshly-prepared solution of 4% paraformaldehyde in phosphate-buffered saline for 10 minutes. After being fixed and washed, slides were placed in methanol containing 0.3% hydrogen peroxide for 15 minutes to quench endogenous peroxidases followed by several washes in Tris-buffered saline solution containing 0.05% Tween 20 (TBS-T). Endogenous proteins were blocked by 5 minute incubation in a solution Innogenex Power Block reagent. Excess reagent was carefully suctioned off, and sections were then incubated in a goat anti-Fos IgG (1:750; SC52G, Santa Cruz Biotechnology) in antibody diluent for 90 minutes followed by several washes in TBS-T. Sections were then incubated in a biotinylated horse anti-goat IgG (1:200; Vector Laboratories) in antibody diluent for 30 minutes, followed by several washes in TBS-T. Sections were reacted with avidin-biotin peroxidase complex (Vectastain Elite Kit; Vector Laboratories) for 30 minutes and washed in TBS-T. Fos immunoreactive nuclei were visualized using the Vector VIP substrate kit for peroxidase followed by several washes in water. The slides were then dehydrated and cover slipped.

#### *4.1. Quantification and data analysis*

A numbered key identified all slides, and quantification was carried out blinded to experimental treatment group. For c-Fos experiments quantification was done using a Sony XC-77 monochrome video camera mounted on a Leica DMR fluorescence microscope. Images were counted live using the image analysis software MCID Elite 6.0. Fos positive nuclei within 129,000 µm<sup>2</sup> in the PrL, 100,000 µm<sup>2</sup> in the IL, were counted relative to a threshold based on staining density, target size, and target shape. Counts were made on the left and right sides of 2 sections per animal.

The data was analyzed using a one-way ANOVA followed by Newman-Keuls post-hoc test (Graph Pad Prism 4.03). The level of significance was set at \*  $P < 0.05$  (compared to Vehicle for LY354740 + Vehicle for DOI), #  $P < 0.05$  (compared to Vehicle for LY354740 + DOI).

## Figure Legends

Figure 1: Restraint stress upregulated c-Fos in the PrL (1a), and IL (1b) cortex. Pretreatment with the mGlu2/3 agonist LY354740 (10 mg/kg, i.p.) attenuated the restraint-induced increase in c-Fos expression in both the PrL and IL cortex. \* indicates significantly different from Vehicle + Home Cage; \*\*\*P < 0.001. # indicates significantly different from Vehicle + Restraint stress; ##P < 0.01. One-way ANOVA with Newman-Keuls post-hoc test. Each bar represents the mean ( $\pm$  SEM), N = 7-8.

Figure 2: Restraint stress induced c-Fos in the PrL cortex (\*\*\*P < 0.001). Pretreatment with LY354740 (10 and 30 mg/kg) attenuated the c-Fos response (##P < 0.01). Pretreatment with LY379268 (10 mg/kg, i.p.) had no effect on restraint-induced c-Fos expression and in itself induced c-Fos in PrL cortex (\*P < 0.01). One-way ANOVA with Newman-Keuls post-hoc test. Data presented as mean  $\pm$  SEM.

Figure 3: Restraint stress upregulated c-Fos in the PrL (3a), and IL (3b) cortex. Pretreatment with the mGlu2/3 agonist LY379268 (0.3-3 mg/kg, i.p.) had no effect on the restraint-induced increase in c-Fos expression in both the PrL and IL cortex. Pretreatment with Alprazolam (3 mg/kg, i.p.) attenuated the c-Fos response in both the PrL and IL cortex. \* indicates significantly different from Vehicle + Home Cage; \*\*\*P < 0.001. # indicates significantly different from Vehicle + Restraint stress; ##P < 0.01, ###P < 0.001. One-way ANOVA with Newman-Keuls post-hoc test. Each bar represents the mean ( $\pm$  SEM), N = 7-8.

Figure 4: DOI produced an increase in c-Fos in the PrL (3a) and IL (3b) cortex. Pretreatment with LY354740 (3 and 10 mg/kg) attenuated the DOI-induced increase in the PrL and IL cortex. \*Significantly different from Vehicle; \*\*\*P < 0.001, #significantly different from DOI; ##P < 0.01, ###P < 0.001. One-way ANOVA with Newman-Keuls post-hoc test. Each bar represents the mean ( $\pm$  SEM), N = 7-8.

**Figures**

Figure 1

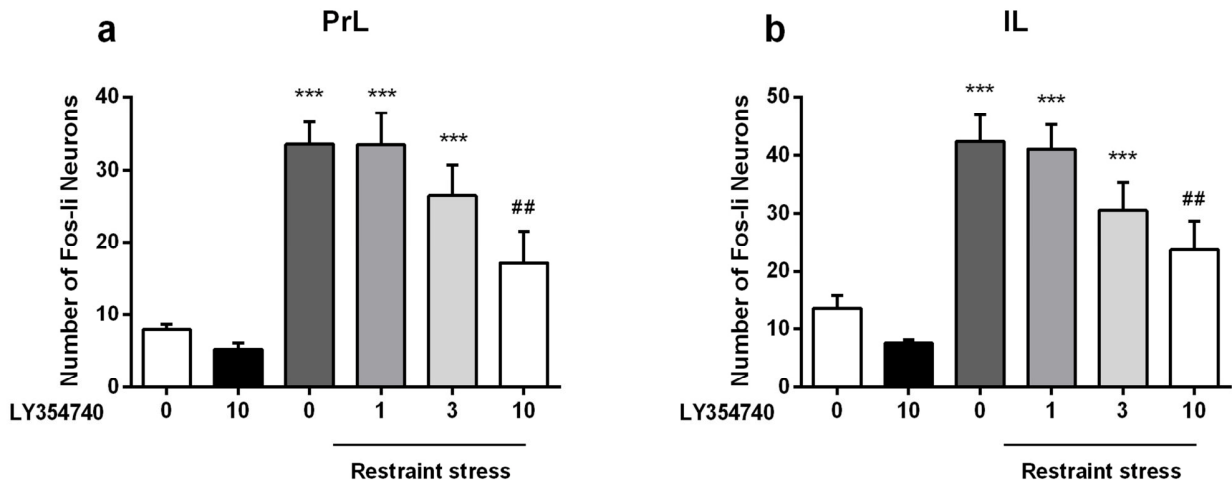


Figure 2

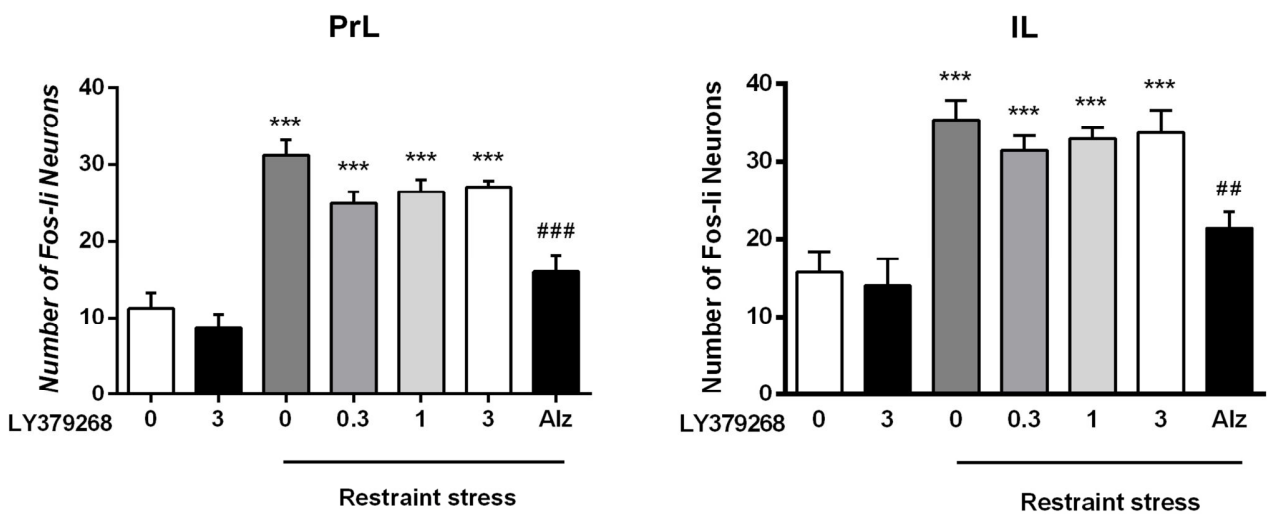


Figure 3

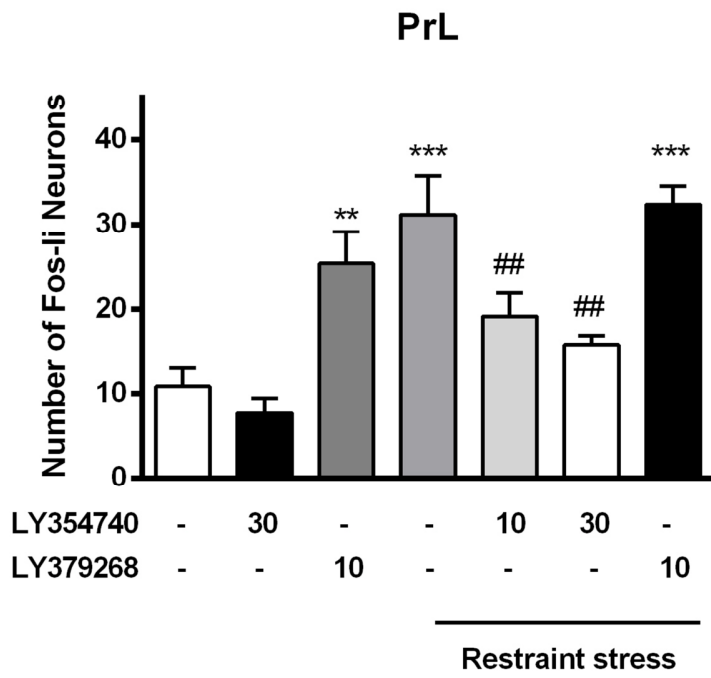
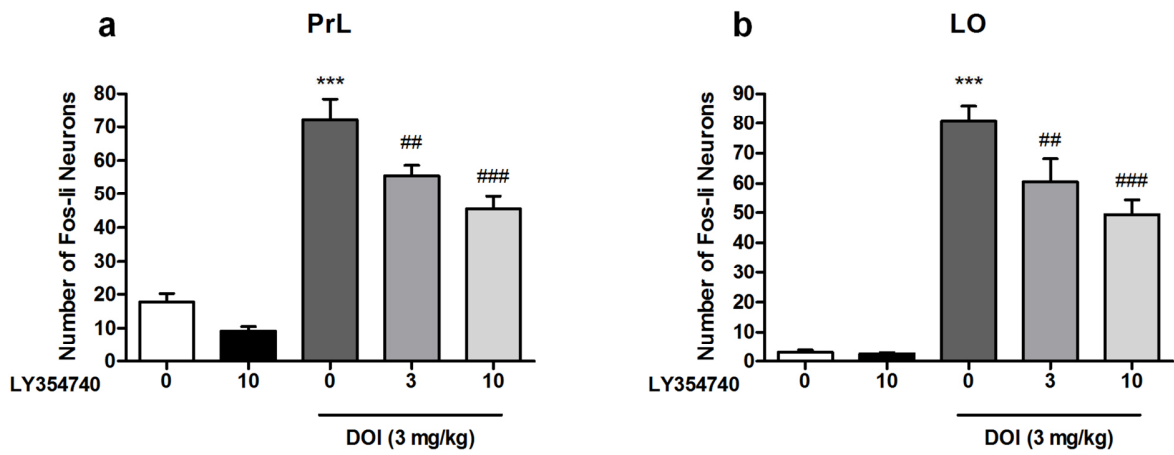


Figure 4





## Reference List

Anwyl R, 1999. Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. *Brain Res. Brain Res. Rev.* 29: 83-120.

Beck CH, Fibiger HC, 1995. Conditioned fear-induced changes in behavior and in the expression of the immediate early gene c-fos: with and without diazepam pretreatment. *J. Neurosci.* 15: 709-720.

Beique JC, Imad M, Mladenovic L, Gingrich JA, Andrade R, 2007. Mechanism of the 5-hydroxytryptamine 2A receptor-mediated facilitation of synaptic activity in prefrontal cortex. *Proc Natl Acad Sci U S A* 104: 9870-5.

Benneyworth MA, Xiang Z, Smith RL, Garcia EE, Conn PJ, Sanders-Bush E, 2007. A selective positive allosteric modulator of metabotropic glutamate receptor subtype 2 blocks a hallucinogenic drug model of psychosis. *Mol. Pharmacol.* 72: 477-484.

Cartmell J, Monn JA, Schoepp DD, 1999. The metabotropic glutamate 2/3 receptor agonists LY354740 and LY379268 selectively attenuate phencyclidine versus d-amphetamine motor behaviors in rats. *J. Pharmacol. Exp. Ther.* 291: 161-170.

Conn PJ, Pin JP, 1997. Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.* 37: 205-237.

Croom KF, Perry CM, Plosker GL, 2009. Mirtazapine: a review of its use in major depression and other psychiatric disorders. *CNS. Drugs* 23: 427-452.

Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ, 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64: 477-505.

de Medeiros MA, Carlos RL, Eugenio ML, 2005. Stress-induced c-Fos expression is differentially modulated by dexamethasone, diazepam and imipramine. *Neuropsychopharmacology* 30: 1246-1256.

Dunayevich E, Erickson J, Levine L, Landbloom R, Schoepp DD, Tollefson GD, 2008. Efficacy and tolerability of an mGlu2/3 agonist in the treatment of generalized anxiety disorder. *Neuropsychopharmacology* 33: 1603-1610.

Fribourg M, Moreno JL, Holloway T, Provasi D, Baki L, Mahajan R, Park G, Adney SK, Hatcher C, Eltit JM, Ruta JD, Albizu L, Li Z, Umali A, Shim J, Fabiato A, MacKerell AD, Jr., Brezina V, Sealfon SC, Filizola M, Gonzalez-Maeso J, Logothetis DE, 2011. Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell* 147: 1011-1023.

Gewirtz JC, Marek GJ, 2000. Behavioral evidence for interactions between a hallucinogenic drug and group II metabotropic glutamate receptors. *Neuropsychopharmacology* 23: 569-576.

Girotti M, Pace TW, Gaylord RI, Rubin BA, Herman JP, Spencer RL, 2006. Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. *Neuroscience* 138: 1067-1081.

Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, Zhou M, Okawa Y, Callado LF, Milligan G, Gingrich JA, Filizola M, Meana JJ, Sealfon SC, 2008. Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452: 93-7.

Greco B, Invernizzi RW, Carli M, 2005. Phencyclidine-induced impairment in attention and response control depends on the background genotype of mice: reversal by the mGlu(2/3) receptor agonist LY379268. *Psychopharmacology (Berl)* 179: 68-76.

Harvey BH, Shahid M, 2011. Metabotropic and ionotropic glutamate receptors as neurobiological targets in anxiety and stress-related disorders: Focus on pharmacology and preclinical translational models. *Pharmacol. Biochem. Behav.*

Heinrichs SC, Menzaghi F, Pich EM, Baldwin HA, Rassnick S, Britton KT, Koob GF, 1994. Anti-stress action of a corticotropin-releasing factor antagonist on behavioral reactivity to stressors of varying type and intensity. *Neuropsychopharmacology* 11: 179-186.

Helton DR, Tizzano JP, Monn JA, Schoepp DD, Kallman MJ, 1998. Anxiolytic and side-effect profile of LY354740: a potent, highly selective, orally active agonist for group II metabotropic glutamate receptors. *J. Pharmacol. Exp. Ther.* 284: 651-660.

Imaki T, Shibasaki T, Hotta M, Demura H, 1993. Intracerebroventricular administration of corticotropin-releasing factor induces c-fos mRNA expression in brain regions related to stress responses: comparison with pattern of c-fos mRNA induction after stress. *Brain Res.* 616: 114-125.

Imre G, Salomons A, Jongasma M, Fokkema DS, Den Boer JA, Ter Horst GJ, 2006. Effects of the mGluR2/3 agonist LY379268 on ketamine-evoked behaviours and neurochemical changes in the dentate gyrus of the rat. *Pharmacol. Biochem. Behav.* 84: 392-399.

Izumi T, Inoue T, Kitaichi Y, Nakagawa S, Koyama T, 2006. Target brain sites of the anxiolytic effect of citalopram, a selective serotonin reuptake inhibitor. *Eur. J. Pharmacol.* 534: 129-132.

Johnson MP, Chamberlain M, 2002. Modulation of stress-induced and stimulated hyperprolactinemia with the group II metabotropic glutamate receptor selective agonist, LY379268. *Neuropharmacology* 43: 799-808.

Kargieman L, Santana N, Mengod G, Celada P, Artigas F, 2007. Antipsychotic drugs reverse the disruption in prefrontal cortex function produced by NMDA receptor blockade with phencyclidine. *Proc. Natl. Acad. Sci. U. S. A* 104: 14843-14848.

Krystal JH, D'Souza DC, Petrakis IL, Belger A, Berman RM, Charney DS, bi-Saab W, Madonick S, 1999. NMDA agonists and antagonists as probes of glutamatergic dysfunction and pharmacotherapies in neuropsychiatric disorders. *Harv. Rev. Psychiatry* 7: 125-143.

Lam AG, Monn JA, Schoepp DD, Lodge D, McCulloch J, 1999. Group II selective metabotropic glutamate receptor agonists and local cerebral glucose use in the rat. *J. Cereb. Blood Flow Metab* 19: 1083-1091.

Leslie RA, Moorman JM, Grahame-Smith DG, 1993. Lithium enhances 5-HT<sub>2A</sub> receptor-mediated c-fos expression in rat cerebral cortex. *Neuroreport* 5: 241-244.

Marek GJ, 2010. Metabotropic glutamate<sub>2/3</sub> (mGlu<sub>2/3</sub>) receptors, schizophrenia and cognition. *Eur. J. Pharmacol.* 639: 81-90.

Marek GJ, Wright RA, Gewirtz JC, Schoepp DD, 2001. A major role for thalamocortical afferents in serotonergic hallucinogen receptor function in the rat neocortex. *Neuroscience* 105: 379-392.

Mikkelsen JD, Larsen MH, 2006. Effects of stress and adrenalectomy on activity-regulated cytoskeleton protein (Arc) gene expression. *Neurosci. Lett.* 403: 239-243.

Moghaddam B, 1993. Stress preferentially increases extraneuronal levels of excitatory amino acids in the prefrontal cortex: comparison to hippocampus and basal ganglia. *J. Neurochem.* 60: 1650-1657.

Moghaddam B, Adams BW, 1998. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. *Science* 281: 1349-1352.

Monn JA, Valli MJ, Massey SM, Hansen MM, Kress TJ, Wepsiec JP, Harkness AR, Grutsch JL, Jr., Wright RA, Johnson BG, Andis SL, Kingston A, Tomlinson R, Lewis R, Griffey KR, Tizzano JP, Schoepp DD, 1999. Synthesis, pharmacological characterization, and molecular modeling of heterobicyclic amino acids related to (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740): identification of two new potent, selective, and systemically active agonists for group II metabotropic glutamate receptors. *J. Med. Chem.* 42: 1027-1040.

Monn JA, Valli MJ, Massey SM, Wright RA, Salhoff CR, Johnson BG, Howe T, Alt CA, Rhodes GA, Robey RL, Griffey KR, Tizzano JP, Kallman MJ, Helton DR, Schoepp DD, 1997. Design, synthesis, and pharmacological characterization of (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740): a potent, selective, and orally active group 2 metabotropic glutamate receptor agonist possessing anticonvulsant and anxiolytic properties. *J. Med. Chem.* 40: 528-537.

Satow A, Maehara S, Ise S, Hikichi H, Fukushima M, Suzuki G, Kimura T, Tanak T, Ito S, Kawamoto H, Ohta H, 2008. Pharmacological effects of the metabotropic glutamate receptor 1 antagonist compared with those of the metabotropic glutamate receptor 5 antagonist and metabotropic glutamate receptor 2/3 agonist in rodents: detailed investigations with a selective allosteric metabotropic glutamate receptor 1 antagonist, FTIDC [4-[1-(2-fluoropyridine-3-yl)-5-methyl-1H-1,2,3-triazol-4-yl]-N-isopropyl-N-methyl-3,6-dihydropyridine-1(2H)-carboxamide]. *J. Pharmacol. Exp. Ther.* 326: 577-586.

Scanziani M, Salin PA, Vogt KE, Malenka RC, Nicoll RA, 1997. Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. *Nature* 385: 630-634.

Schoepp DD, Jane DE, Monn JA, 1999. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* 38: 1431-1476.

Spooren WP, Schoeffer P, Gasparini F, Kuhn R, Gentsch C, 2002. Pharmacological and endocrinological characterisation of stress-induced hyperthermia in singly housed mice using classical and candidate anxiolytics (LY314582, MPEP and NKP608). *Eur. J. Pharmacol.* 435: 161-170.

Swanson CJ, Bures M, Johnson MP, Linden AM, Monn JA, Schoepp DD, 2005. Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. *Nat. Rev. Drug Discov.* 4: 131-144.

Trneckova L, Armario A, Hynie S, Sida P, Klenerova V, 2006. Differences in the brain expression of c-fos mRNA after restraint stress in Lewis compared to Sprague-Dawley rats. *Brain Res.* 1077: 7-15.

Weinberg MS, Girotti M, Spencer RL, 2007. Restraint-induced fra-2 and c-fos expression in the rat forebrain: relationship to stress duration. *Neuroscience* 150: 478-486.

Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP, Sibille E, Underwood M, Itohara S, Dauer WT, Ansorge MS, Morelli E, Mann JJ, Toth M, Aghajanian G, Sealfon SC, Hen R, Gingrich JA, 2006. Cortical 5-HT<sub>2A</sub> receptor signaling modulates anxiety-like behaviors in mice. *Science* 313: 536-540.

Wisshof L, Koch M, 2012. Pre-treatment with the mGlu<sub>2/3</sub> receptor agonist LY379268 attenuates DOI-induced impulsive responding and regional c-Fos protein expression. *Psychopharmacology (Berl)* 219: 387-400.

# *Co-author statements*



## Declaration of co-authorship

This form must be filled in on the screen, printed, signed and sent to the PhD administration

- You can use the TAB-button to jump between the grey boxes.

Information on PhD student:	
Name of PhD student	Martin Andreas Santini
E-mail	santini@nru.dk
Date of birth	11/09-1980
Work place	Neurobiology Research Unit, Rigshospitalet
Faculty supervisor	Jens Damsgaard Mikkelsen

Title of PhD thesis:
The role of serotonin 2A receptors in prefrontal cortex function - Implications in schizophrenia

This declaration concerns the following article:
Novelty-induced activity-regulated cytoskeletal-associated protein (Arc) expression in frontal cortex requires serotonin 2A receptor activation

The PhD student's contribution to the article: (please use the scale (A,B,C) below as benchmark*)	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

*Benchmark scale of the PhD student's contribution to the article		
A. refers to:	Has contributed to the co-operation	0-33 %
B. refers to:	Has contributed considerably to the co-operation	34-66 %
C. refers to:	Has predominantly executed the work independently	67-100 %

Signature of the co-authors:			
Date:	Name:	Title:	Signature:
10.12.12	Anders B. Klein	MSc, PhD	<i>Anders Klein</i>
05.12.12	Mona El-Sayed	M.Sc.	<i>Mona El-Sayed</i>
4.12.12	Cecilia Ratner	M.sc.	<i>Cecilia Ratner</i>

Signature of the PhD student and the Faculty supervisor:	
Date: 27/11-12 PhD student: <i>Martin A. Santini</i>	Date: 29/11/12 Faculty supervisor: <i>Jens D. Mikkelsen</i>



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*This form must be filled in on the screen, printed, signed and sent to the PhD administration*

*- You can use the TAB-button to jump between the grey boxes.*

### Information on PhD student:

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E-mail	santini@nru.dk
Date of birth	11/09-1980
Work place	Neurobiology Research Unit, Rigshospitalet
Faculty supervisor	Jens Damsgaard Mikkelsen

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### Signature of the co-authors:

Date:	Name:	Title:	Signature:
13/12-12	Gitte M. Knudsen	PROF	
27 Nov 12	Jens D. Mikkelsen	Ass. Prof. Ext	
19 dec.	Susana Aznar	Head of section	

### Signature of the PhD student and the Faculty supervisor:

Date: 27/11-12		Date: 27 Nov 2012	
PhD student:	Martin A. Santini	Faculty supervisor:	Jens D. Mikkelsen



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The PhD student's contribution to the article: (please use the scale (A,B,C) below as benchmark*)	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

*Benchmark scale of the PhD student's contribution to the article		
A. refers to:	Has contributed to the co-operation	0-33 %
B. refers to:	Has contributed considerably to the co-operation	34-66 %
C. refers to:	Has predominantly executed the work independently	67-100 %

Signature of the co-authors:			
Date:	Name:	Title:	Signature:
4.12.12	Cecilia Ratner	M. sc.	
19.12.12	Susana Aznar	Head of Section	
11.12.12	Anders B. Klein	MSc, PhD	

Signature of the PhD student and the Faculty supervisor:	
Date: 27/11-12 PhD student:	Date: 29/11.12 Faculty supervisor:





## Declaration of co-authorship

*This form must be filled in on the screen, printed, signed and sent to the PhD administration*

*- You can use the TAB-button to jump between the grey boxes.*

### Information on PhD student:

Name of PhD student	Martin Andreas Santini
E-mail	santini@nru.dk
Date of birth	11/09-1980
Work place	Neurobiology Research Unit, Rigshospitalet
Faculty supervisor	Jens Damsgaard Mikkelsen

### Title of PhD thesis:

The role of serotonin 2A receptors in prefrontal cortex function - Implications in schizophrenia

### This declaration concerns the following article:

Enhanced prefrontal serotonin 2A receptor signaling in the subchronic PCP mouse model of schizophrenia

### The PhD student's contribution to the article:

*(please use the scale (A,B,C) below as benchmark\*)*

(A,B,C)

1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
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### Signature of the co-authors:

Date:	Name:	Title:	Signature:
13/12-12	Gitte M. Knudsen	PROF	
27 Nov 12	Jens D. Mikkelsen	Ass Prof. Ext	

### Signature of the PhD student and the Faculty supervisor:

Date: 27/11-12		Date: 27 Nov 2012	
PhD student:		Faculty supervisor:	



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Date of birth	11/09-1980
Work place	Neurobiology Research Unit, Rigshospitalet
Faculty supervisor	Jens Damsgaard Mikkelsen

Title of PhD thesis
The role of serotonin 2A receptors in prefrontal cortex function - implications in schizophrenia

This declaration concerns the following article:
Forebrain NMDA receptor hypofunction attenuates pharmacological and behavioral effects induced by the hallucinogenic 5-HT <sub>2A</sub> receptor agonist DOI

The PhD student's contribution to the article: (please use the scale (A,B,C) below as benchmark)	(A,B,C)
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Signature of the co-authors:			
Date:	Name:	Title:	Signature:
12/9/12	Darrick Balu	Instructor	
12/10/12	Tiffany E. Hill-Smith	Research specialist	
12/10/12	Irwin Luckl	Professor	

Signature of the PhD student and the Faculty supervisor:	
Date: 29/11-12 PhD student:	Date: 29/11-12 Faculty supervisor:



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E-mail	santini@nru.dk
Date of birth	11/09-1980
Work place	Neurobiology Research Unit, Rigshospitalet
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Signature of the co-authors:			
Date:	Name:	Title:	Signature:
29/11/12	Jens D. Mikkelsen	Ass. Prof. Ext	<i>Jens D. Mikkelsen</i>
12/9/12	Joseph T. Coyle	Prof.	<i>Joseph T. Coyle</i>

Signature of the PhD student and the Faculty supervisor:	
Date: 29/11-12 PhD student: <i>Martin A Santini</i>	Date: 29/11/12 Faculty supervisor: <i>Jens D. Mikkelsen</i>



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Name of PhD student	Martin Andreas Santini
E-mail	santini@nru.dk
Date of birth	11/09-1980
Work place	Neurobiology Research Unit, Rigshospitalet
Faculty supervisor	Jens Damsgaard Mikkelsen

The role of serotonin 2A receptors in prefrontal cortex function - implications in schizophrenia

This declaration concerns the following article:

The mGlu2/3 Receptor Agonists LY354740 and LY379268 Differentially Regulate Restraint Stress-induced Expression of c-Fos in Rat Cerebral Cortex

The PhD student's contribution to the article (please use numbers 1-4 below as landmarks)	Level
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	A
2. Planning of the experiments and methodology design, including selection of methods and method development	A
3. Involvement in the experimental work	B
4. Presentation, interpretation and discussion in a journal article format of obtained data	B

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Signatures of the PhD student and the faculty supervisor		
Date:	Name:	Signature:
27 Nov 12	Jens D. Mikkelsen	MD PhD, Ass Prof Jens D. Mikkelsen
22 Dec 12	Kjell A. Svensson	PhD Kjell A. Svensson

Signatures of the PhD student and the faculty supervisor	
Date: 27/11-12 PhD student: Martin A Santini	Date: 27 Nov 12 Faculty supervisor: Jens D. Mikkelsen