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**Intranasal administration of neuropeptides as a new therapeutic strategy to treat social and cognitive alterations relevant to schizophrenia**

Giuliano Gentili

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**Internal Supervisor: Ch.mo Prof. Pietro Giusti**

**External Supervisor: Dott Francesco Papaleo**



Thesis supervisor:

**Dr. Francesco Papaleo**

Genetics of Schizophrenia and Cognition - Team Leader, Senior Researcher

Istituto Italiano di Tecnologia (IIT)

Genova



# Contents

GENERAL ABSTRACT .....	1
AIM OF THE THESIS .....	5
GENERAL INTRODUCTION.....	7
1.1 SCHIZOPHRENIA.....	7
1.2 INTRANASAL ADMINISTRATION OF NEUROPEPTIDES.....	9
INTRANASAL OXYTOCIN AMELIORATES SOCIAL DEFICITS IN THE SCHIZOPHRENIA RELEVANT DYSBINDIN-1 KNOCKOUT MICE.....	11
2.1 INTRODUCTION.....	11
2.2 MATERIALS AND METHODS.....	15
2.2.1 Subjects.....	15
2.2.2 Intranasal OXT Administration.....	16
2.2.3 Behavioural Testing.....	18
2.2.4 Brain autoradiography.....	19
2.2.5 Automated scoring for familiar mice.....	20
2.2.6 Statistical analyses.....	20
2.3 RESULTS .....	20
2.3.1 Social deficits in Dysbindin-1 mutant mice.....	20
2.3.2 Social deficits in Dys mutant mice are ameliorated by chronic intranasal oxytocin.....	21
2.3.3 Disbindin mutations reduce interaction times in female mice.....	25
2.3.4 Social deficits in female-female interaction in Dys mutant mice are ameliorated by acute intranasal oxytocin.....	26
2.3.5 Chronic intranasal oxytocin ameliorates social deficits in familiar mice.....	28
2.3.6 Oxytocin doesn't reduce receptor expression in dys mutant mice.....	30
2.4 DISCUSSION.....	31
INTRANASAL ADMINISTRATION OF THE CRF-BP INHIBITOR CRF(6-33) SELECTIVELY MODULATES IMPULSIVE BEHAVIOR .....	33
3.1 INTRODUCTION .....	34
3.1.1 Cognitive deficits in schizophrenia.....	34
3.1.2 CRF system as a pharmacological target for cognitive modulation.....	35
3.1.3 5-Choice serial reaction time task, a test for assessment of cognitive performance in rodents.....	36
3.1.4 Disbindin 1 and Dopamine D2 receptor interaction as a mouse model relevant to schizophrenia.....	39
3.2 MATERIALS AND METHODS.....	40
3.2.1 Subjects.....	40
3.2.2 Intranasal CRF(6-33) administration.....	40
3.2.3 CSRTT.....	41
3.2.4 Molecular analyses.....	49
3.2.5 Statistical analyses.....	50
3.3 RESULTS .....	51
3.3.1 CRF (6-33) improves accuracy acutely and reduces impulsive response both acutely and chronically.....	51
3.3.2 During SARAT manipulation CRF(6-33) improves accuracy and reduces impulsive responses.....	53
3.3.3 During DISTRACTOR manipulation CRF(6-33) still reduces impulsive responses.....	56

3.3.4 <i>IMPULSIVITY manipulation results:</i> .....	59
3.3.5 <i>Real time PCR analysis confirms the ability of intranasal CRF(6-33) to modify expression of key molecules for the CRF system.</i> .....	59
3.3.6 <i>Acute lower dose of CRF(6-33) is still able to reduce impulsive behaviour.</i> .....	62
3.3.7 <i>Administration of acute intranasal CRF(6-33) may interact in opposite way with schizophrenia relevant mutations Dys+/- and D2L+/-</i> .....	63
3.4 DISCUSSION .....	66
<b>CONCLUSION AND FUTURE DIRECTIONS</b> .....	<b>69</b>
4.1 CONCLUSION .....	69
4.2 FUTURE DIRECTIONS .....	73
<b>BIBLIOGRAPHY</b> .....	<b>75</b>

## General Abstract

Schizophrenia is a chronic enduring disorder ranked among the most debilitating mental illnesses (Mueser & McGurk, 2004; Tandon, Keshavan, & Nasrallah, 2008). Although it has been vigorously studied over the past century, the etiology and pathophysiology of schizophrenia remains largely unknown and currently available treatments, in the form of antipsychotics, are mainly unsatisfactory (Insel, 2010; Tandon et al., 2008).

Schizophrenia is characterized by three broad types of symptoms: positive symptoms, negative symptoms, and cognitive deficits. While drugs currently available for the treatment of this disorder are effective for positive symptoms, negative symptoms (including social impairments) and cognitive deficits still remain mainly untreatable (Keefe et al., 2007; Neill et al., 2010). Negative and cognitive symptoms are more pervasive, fluctuate less over time than psychotic symptoms and are strongly associated with poor psychosocial functioning in community living and work (Kasper & Resinger, 2003; Mueser & McGurk, 2004; Tandon, Nasrallah, & Keshavan, 2009).

Although there have been rapid progress in the development of non-invasive technologies to study human brain structure and function in the last two decades, there are still substantial limitations in our ability to investigate details of the physiology and molecular biology of the human brain (Nestler & Hyman, 2010a). To that end, it is imperative to have carefully validated animal models for continued progress in our understanding of pathophysiology and in the development and screening of novel therapeutic agents in order to enhance functional recovery of patients (Davis et al., 2013; Neill et al., 2010).

Neuropeptides have an important role in intracerebral signaling and might have the potential to be used as therapeutic agents in many psychiatric and neurological diseases (Bedse, Di Domenico, Serviddio, & Cassano, 2015; Erbaş, Çınar, Solmaz, Çavuşoğlu, & Ateş, 2015; Nishimura, Murayama, & Takahashi, 2015; Reglodi et al., 2015, 2015). Unfortunately, they usually can't be administered systemically due to the elevated hydrophilicity and molecular weight that prevent them to overcome the blood brain barrier.

Intranasal administration might be a promising and non-invasive way of administration of neuropeptides: this administration route enables highly hydrophilic and high molecular weight molecules to bypass the blood-brain barrier permitting them to reach the brain in a non-invasive way. This way has been demonstrated in humans that permits the delivery of biologically effective concentrations of many peptides to the brain without eliciting significantly eventual systemic hormone-like side effects (Born et al., 2002); This route of administration was tested also in mice and rats (Born et al., 2002; Neumann, Maloumy, Beiderbeck, Lukas, & Landgraf, 2013) and, recently, was successfully used specifically in the context of behavioural studies in mice (Huang et al., 2014).

The aim of the studies described in this thesis is to investigate, through the use of clinically-relevant animal models of schizophrenia, the pharmacogenetics behavioral effects of intranasal administration of two different neuropeptides:

- oxytocin (OXT), a neurohypophyseal peptide suggested to have beneficial effects in social behaviors (Meyer-Lindenberg, Domes, Kirsch, & Heinrichs, 2011; Striepens, Kendrick, Maier, & Hurlmann, 2011) and currently in clinical studies for mental disorders characterized by social behavioral alterations such as autism (Anagnostou et al., 2012; Guastella et al., 2010) and schizophrenia (Feifel et al., 2010).
- CRF(6-33), a synthetic peptide designed to be an effective competitive antagonist for the binding between the endogenous CRF neuropeptide and its binding protein (CRFbp)(Sutton et al., 1995) suggested in preclinical ICV studies to be an effective pro cognitive agent with potential therapeutic applications (Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003; Koob & Bloom, 1985)

In previous work from our laboratory (Huang et al., 2014), we already implemented, for the first time, the use of intranasal oxytocin in C57BL/6J mice, checking different behavioral effects. Thus, we now tested the effects of intranasal OXT in the schizophrenia-relevant dysbindin-1 knockout mouse model.

Genetic variations of the dysbindin-1 gene (DTNBP 1) has been associated with susceptibility to schizophrenia (O'Tuathaigh et al., 2007; Ross, Margolis, Reading, Pletnikov, & Coyle, 2006; Straub et al., 2002) and severity of negative symptoms and cognitive dysfunction in schizophrenic patients (Burdick et al., 2007; DeRosse et al., 2006; Fanous et al., 2005; Straub et al., 2002). Thereafter, using a Dys knockout mutant mouse model, we demonstrated that both the heterozygous and homozygous knockout mice manifested a reduction in social interaction compared to wildtype mice. Similar social deficits in Dys mutant mice have been reported by other groups (Feng et al., 2008; Hattori et al., 2008).

Interestingly, both chronic and acute intranasal OXT treatments were able to ameliorate the social deficits observed in the Dys knockout mice. These data suggest that intranasal oxytocin might be beneficial to subjects with genetic modifications relevant to schizophrenia, while administration to healthy subjects has not significant behavioral effect or may eventually be detrimental (Huang et al., 2014).

This opens new ways of exploration in relationship to the beneficial effects of OXT treatment and its utility in the clinical setting.

The data (aforementioned) correlate well with the molecular data available. As a matter of fact, OXT receptors are downregulated in WT subjects that are chronically IN -OXT treated, and even mutant mice, though treated with the same therapeutic protocol, show that the receptors are not subject to



variation , compared to the control subjects treated with the vehicle substance only. Indeed, after being chronically treated, WT mice showed a decrease in social behaviour, while mutant mice (under an equivalent OXT treatment) showed an increase in social behaviour.

For the CRF(6-33) part, in order to set the ground for future studies with genetically modified mice, disease-related mouse models and facilitate inter-laboratory comparisons, we first tested the effects of both chronic and acute intranasal CRF treatments in C57BL/6J mice. Considered that OXT did not show significant improvement in cognitive performance, we chose to target these functions with a different peptide that in previous intracerebroventricular studies had shown to improve cognitive functions (Behan et al., 1995; Eckart et al., 1999; S C Heinrichs et al., 1997; Stephen C Heinrichs & Koob, 2004; Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003; Koob & Bloom, 1985; Lee, Lee, Wang, & Lin, 1993; Lee & Sung, 1989; Thompson, Erickson, Schulkin, & Rosen, 2004) had shown to improve cognitive functions . To asses cognitive functions we used a modified version of the 5-Choice Serial Reaction Time Task (5-csrtt), a rodent test designed as analog of the Continuous Performance Test (cpt) used to asses quantitatively attentional control in humans (Amitai & Markou, 2011). The modifications are intended to reduce the time needed to train the animals , reduce stressful manipulations as food restriction or single housing. Additionally, several new manipulations have been implemented to investigate various specific cognitive functions such as attention, broad monitoring / compulsivity, response disinhibition/ impulsivity, distractibility and processing speed.

We were able to demonstrate that the intranasal administration of CRF(6-33) can produce selective behavioral effects in mice. In particular, acute administration was able to improve accuracy of responses and reduce impulsivity, while chronic administrations produced a delay in correct responses. Gene expression studies with real time PCR are starting to suggest that the CRF(6-33) is able to reach the brain, as both CRFr1 and CRFbp were altered following intranasal CRF(6-33) in different and specific brain areas (i.e. Hippocampus and Prefrontal Cortex ).

Subsequent dose-response tests confirmed the ability of evocate behavioral effects with a much lower dose of CRF(6-33). Moreover, we discovered a rebound effect in impulsive behavior the day after administration of higher doses.. This detrimental effect was absent with the lowest doses that was still able to significantly reduce impulsive behavior.

Lastly we tested ability of CRF(6-33) to ameliorate an impulsive phenotype in a genetic modified mouse model of schizophrenia. The chosen model was a double mutant for Dysbindin and for the receptor D2. The D2 receptor that had proven in previous test to have an increased impulsive behaviour.

The dopamine D2 receptor (D2) gene is another important risk gene identified for schizophrenia. Functional genetic variants in the D2 gene have been found to be differently expressed in patients

with schizophrenia (Kaalund et al., 2013) and might modulate schizophrenia-related phenotypes by modifying the ratio of the short isoform (D2S) to the long isoform (D2L) (Bertolino et al., 2009). Heterozygote mutant for D2L has an increased D2S (receptor D2 short form)/ D2L (receptor D2 long form) ratio.

From preliminary data CRF(6-33) was not able to significantly affect impulsive behaviour in double heterozygote Dys +/- D2L +/- . Interestingly, in single heterozygote Dys +/- was observed a trend of increased impulsivity in CRF(6-33) group suggesting a detrimental interaction with Dys deficient genotype.

From this studies we can conclude that both OXT than CRF(6-33) have the potential to be used for treatment, respectively of social and impulsivity deficits. For OXT so far we observed a positive interaction with Dys deficient genotype , that needs to be confirmed in other schizophrenia relevant mouse models of social deficits. CRF(6-33) so far has demonstrated only to improve social performance in WT mice. The preliminary study on mutated mouse,if confirmed, seems to suggest that it might worsen the phenotype in presence of certain genetic mutations. It should be important to define mechanisms of interaction of CRF(6-33) with genetics as to define when it could be positively used for therapy and when it shouldn't in a view of a genetic driven personalized schizophrenia therapy

## **Aim of the thesis**

The general aim of the studies described in this thesis is to investigate behavioral effects produced by intranasal administrations of two different peptides in mice. In particular, we focused on oxytocin (OXT) and CRF(6-33) as potential therapeutic agents for social and cognitive impairments relevant to schizophrenia, respectively. Similarly to autism, the intranasal administration of OXT is attracting an increasing world-wide interest as an appealing treatment for, at least, some social/negative symptoms in schizophrenia. However, our own work (Huang et al., 2014) and other preclinical and clinical studies (K L Bales et al., 2014; Horta de Macedo, Zuardi, Machado-de-Sousa, Chagas, & Hallak, 2014) are starting to alert that OXT treatment might not be beneficial to the overall population. Thus here we aimed to better investigate the use of intranasal OXT based on different genetic backgrounds. Furthermore, while preliminary pre-clinical studies showed promising procognitive improvements following of CRF(6-33) administrations, (Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003; Koob & Bloom, 1985), these studies were only done by intracerebroventricular administration. This route of administration is suitable only for research use, just to test potential efficacy of the molecule or to study general functions of the system involved. However, ICV administration has really poor translational valence to human studies, as it is a really invasive procedure. Thus, here we tested it intranasally; being first time this molecule was tested by this route we chose to assay effects in the 5-CSRTT as it is a quite sensible test for cognitive functions that permits to perform many different manipulations and to evaluate many aspects of cognition. A better implementation of intranasal OXT administration in mice will help clarify the mechanism of action of OXT and dissect therapeutic potentials in relation to psychiatric disease characterized by social impairment. So far there is no therapeutic agents for schizophrenia-associated cognitive impairment, implementation of intranasal administrations for CRF(6-33) might provide us a new and valuable therapeutic tool for targeting such kind of impairment, define drug genetic interaction in genetic mutated murine models and, moreover, easily translate results to humans, as intranasal administration is suitable for human treatment too.



# Chapter 1

## General Introduction

### 1.1 Schizophrenia

Schizophrenia is a chronic, debilitating neuropsychiatric disorder typified by positive symptoms (hallucinations, delusions, disordered thoughts and behaviours), negative symptoms (blunted affect, alogia, anhedonia, asociality, avolition) and cognitive deficits (Kvajo, McKellar, & Gogos, 2012; Lewis & Gonzalez-Burgos, 2006). Affecting about 1% of the world's population, it is a mental illness which, globally speaking, represents one of the ten most important causes of long-term disability (Mueser & McGurk, 2004).

The etiology of this devastating disorder is complex and still largely un-known. Stemming from the evidence found within the findings of family and twin studies, the correlation between schizophrenia and a strong genetic component is undoubtedly clear: having an affected family member substantially increases the risk of developing schizophrenia and this risk increases in turn as the degree of genetic affinity with the affected family member increases (Tandon et al., 2008). However, it has been difficult to find the 'culprit' gene, so to speak, as several common risk alleles of small effect and/or rare but penetrant copy number variations are involved and have been found to confer only a slight increase in risk (odds ratio < 2) (M. Burmeister, 1999; O'Tuathaigh, Desbonnet, Moran, & Waddington, 2012; Tandon et al., 2008). Despite the results obtained from numerous genome-wide association studies (GWAS), even the common risk alleles for schizophrenia proved to be inconsistent due to the impossibility of replication in multiple populations (Margit Burmeister, McClinnis, & Zöllner, 2008). A further complexity is shown by the interaction of epigenetic and environmental factors with the multiple susceptibility genes, which ostensibly may lead involved also interact with to increase the risk for the disorder development (Mueser & McGurk, 2004; O'Tuathaigh et al., 2012).

Animal models that recapitulate the full phenotypic spectrum of neuropsychiatric disorders, such as schizophrenia, are impossible; nevertheless these models are indispensable to our understanding of the predisposing causal factors underlying these disorders and to permit to generate novel, mechanism-based and personalised treatments and the study of the biological role of the genetic variants implicated in Genome-Wide Association Studies (GWAS) of schizophrenia (Arguello & Gogos, 2006).

Moreover, data from animal models are much more sensible in detection as they simplify the system in a neutral genetic background. Animal models permits to isolate effects of single gene mutation or

to provide information of interaction of specific susceptibility genes. Thus, data from animal studies, if taken together with human data, can help in the identification and prioritisation of the candidate genes. (Ayalew et al., 2012).

Genetically modified mouse models for genes relevant to schizophrenia could validate the research construct and try to give support to the comprehension of the function of genes and their contribution to the pathophysiology of cognitive/schizophrenia-related abnormalities (Nestler & Hyman, 2010b; Papaleo, Lipska, & Weinberger, 2012)

In particular, being deficits in social functioning representative of a core negative symptom in schizophrenia (Tandon et al., 2008), social interaction in transgenic mouse models may constitute an easily-accessible index of negative symptomatology (Kirby, Waddington, & O'Tuathaigh, 2010). Moreover, cognitive symptoms can be effectively modelled in transgenic mice and these mouse models will allow for both elucidation and in-vivo characterisation of the relationship between the specific genes and cognitive dysfunction.

On top of that, due to the lack of effective pharmacotherapies for social and cognitive dysfunction in schizophrenia, the need for better animal models is heightened: while existing typical and atypical anti-psychotic drugs proved effective in reducing severity of positive symptoms, they show little or no effect against negative symptoms or social and cognitive impairments (Arguello & Gogos, 2006; Kvaajo et al., 2012).

The main mechanism concerning the therapeutic efficacy of most first-generation anti-psychotic drugs (a.k.a. typical) is an attenuation in dopamine-mediated neurotransmission through antagonism (or weak partial atomism) of dopamine D2 receptors. Un-fortunately, this dopaminergic blockade could lead to severe motor or extrapyramidal Parkinson-like adverse effects (tardive dyskinesia, bradykinesia, tremors, muscle rigidity, and postural instability) (Gobira, Ropke, Aguiar, Crippa, & Moreira, 2013; Nestler & Hyman, 2010b).

Second-generation (atypical) antipsychotics (clozapine, risperidone, olanzapine, quetiapine, etc) prove to less likely cause neurological side effects but are more liable to have different side effects and contribute to both an increase in weight and adverse metabolic effects, that is insulin resistance and dyslipidemia (Kane & Correll, 2010).

Provided that we are currently limited in pharmacological treatments for negative symptoms and the urgency of finding some effective treatments for these deficits prevents us from taking many other further clues into consideration for the result-oriented treatment of schizophrenia-related social and cognitive impairments, the availability of carefully-validated animal models, through which developing and screening of novel therapeutic agents, could greatly enhance functional recovery of patients(Davis et al., 2013; Neill et al., 2010).

Growing evidence indicates that risk genes associate with specific features of schizophrenia pathology (O'Connell, Lawrie, McIntosh, & Hall, 2011). For instance, studies suggest that variations in the dystrobrevin-binding protein 1 gene (DTNBP 1) are associated with severity of negative symptoms and cognitive dysfunction in schizophrenic patients (Burdick et al., 2007; DeRosse et al., 2006; Fanous et al., 2005; Straub et al., 2002). In this context, risk genes have the potential to serve as biomarkers by providing an index of an individual's pathologic profile, and then lead to increased specificity in clinical testing and the development of more personalised therapies (O'Connell et al., 2011).

## **1.2 Intranasal administration of neuropeptides**

A steadily increasing amount of attention has been paid to the intranasal route of administering substances recently. Stemming from a mere search among the academic articles, one could certainly notice that by now almost 13,000 scientific papers dealing with intranasal-administration of a large variety of substances have been published (Veening & Olivier, 2013). Peculiarly, promising potential for the treatment of brain diseases is held by intranasal administration of neuropeptides, since it might deliver concentrations to the brain which have proven to be effective at a biological level without causing potent systemic hormone-like side effects (Born et al., 2002).

In rats and mice, intranasal administration of vasopressin (AVP) and of OXT have been reported to be the starting point leading to the increase (both in the cerebrospinal fluid and selected brain regions) of the intracerebral concentration of these peptides, (Born et al., 2002; Neumann et al., 2013). Nevertheless, AVP/OXT endogenous release mediated by peripheral mechanisms are a likely to cause central effects (Ludwig et al., 2013); without a doubt, central effects are observed in humans after intranasal administration, regardless of the mechanism(s) involved (Veening & Olivier, 2013).

Basically, substances applied on the mucosal wall of the nasal cavity do have three plausible paths to reach the cranial cavity and sections of the brain itself. Since these routes-of-access do not exclude each other, they are worth to be described in brief as the real measure of efficacy of neuropeptides exploiting selectively one or a combination of these possible mechanisms. These possibilities are: (1) intra-axonal and transneuronal transport mechanisms via the olfactory pathways; (2) via the peripheral blood stream and the Blood-Brain-Barrier (BBB) after crossing the mucosal walls of the nasal cavity; (3) via perineuronal and other spaces along the olfactory fibers and other cranial nerves to enter the arachnoid space and Cerebrospinal fluid (CSF) surrounding the brain.

Neuroanatomical tracers can be conveyed from the olfactory sensory neurons (OSN), as stated within several studies in the rat, in the epithelium covering both the nasal cavities to the olfactory bulb and all second order olfactory regions after transneuronal transport, . Ostensibly, transport occurs in both anterograde and retrograde directions, which brought out all central olfactory connections (Shipley, 1985). these findings were confirmed in other species both such as rat at the electronmicroscopical level (Baker & Spencer, 1986) and in mice at a genic level through the transfer of a plant lectin as a

transneuronal tracer in transgenic mice to target the olfactory system and study its connectivity (Horowitz, Montmayeur, Echelard, & Buck, 1999).

Being it is painfully slow and without justifying the fastness of observed effects by intranasal administration, this way is rather not likely to be the main way dealing with intranasal brain administration of neuropeptides, and the distribution to brain not exclusively limited to olfactory areas (Veening & Olivier, 2013)

Concerning a peripheral transport via the blood stream after crossing the nasal mucosa an already existing functional pathway between the nose and the brain must be taken into consideration, in that it appears to be a chemical exchange between venous and arterial vessels. Substances such as neuropeptides, progesterone, tritiated water, tyrosine, propanol and diazepam, and alike are transferred from the nasal cavity into these cranial arteries, sprouting from the internal carotid artery. (Veening & Olivier, 2013). Notwithstanding the molecules flow into the cerebral blood circle, thanks to the good functionality of the BBB, which blocks the entrance of 98% of small and nearly 100% of large molecules (and the elevated levels of substances in specific brain vessels), it does not mean that more of the substance involved enters either the brain or the extracellular spaces, (Dhuria, Hanson, & Frey, 2010; Pardridge, 2005). As a general rule, this makes it highly improbable that 'nasal delivery', occurs via the general circulation. Nevertheless, it has to be kept in mind that under certain conditions and for certain substances, the systemic- route contributions may play some role in the effects of intranasal -administration observed, especially if the substances cross the BBB rather easily.

Various studies agree that additional transport pathways, extra-axonal and extra-systemic and therefore called 'direct', must exist between nasal and cranial cavities and/or between olfactory epithelia and olfactory and possibly other brain areas and/or the CSF-containing arachnoid space (Dhuria et al., 2010; Sakka, Coll, & Chazal, 2011). A great many findings provide evidence for the existence of such additional pathways.

'Rapid access': several substances arrive at the olfactory brain regions considerably faster than expected on the basis of systemic or intra-axonal transport mechanisms. Arrival times of less than a minute have been observed for the olfactory bulbs (Born et al., 2002; Charlton et al., 2008; Illum, 2004; Thorne, Pronk, Padmanabhan, & Frey, 2004) while the highest brain levels after systemic administration as well as after intra-axonal transport are reached considerably later, up to hours in the case of intra-axonal transport (Dhuria et al., 2010).

While the data in favour of 'direct pathways' from the nasal to the cranial cavity may be rather convincing, many questions remain unanswered questions, both at the electronmicroscopical and at functional anatomical levels. Many details about these pathways or 'entrance- routes' are not fully elucidated yet.



# Chapter 2

## Intranasal oxytocin ameliorates social deficits in the schizophrenia relevant dysbindin-1 knockout mice

Huang, H.<sup>1</sup>, Gentili, G.<sup>2</sup>, Michetti, C.<sup>3</sup>, Gigliucci, V.<sup>4,5</sup>, Busnelli, M.<sup>4,5</sup>, Giancardo, L.<sup>6</sup>, Hilario, C.<sup>6</sup>, Sona, D.<sup>6</sup>, Murino, V.<sup>6</sup>, Scattoni, M.L.<sup>3</sup>, Chini, B.<sup>4</sup>, Papaleo, F.<sup>1</sup>

<sup>1</sup>Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, via Morego, 30, 16163 Genova, Italy.

<sup>2</sup>Dipartimento di Scienze del Farmaco, Università degli Studi di Padova, Largo Meneghetti, 2, 35131 Padova, Italy.

<sup>3</sup>Behavioural Neurosciences Section, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome, Italy.

<sup>4</sup>Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, Milan, Italy.

<sup>5</sup>CNR, Institute of Neuroscience, Milan, Italy.

<sup>6</sup>Pattern Analysis and Computer Vision, Istituto Italiano di Tecnologia, Genova, Italy.

### Abstract

The dysbindin-1 gene (*dtncp 1*) has been identified as one of the leading susceptibility genes for schizophrenia. In the clinic, this risk gene has been associated with the severity of negative symptoms and cognitive deficits in schizophrenia. Using a dysbindin-1 (*Dys*) knockout mutant mouse model, we demonstrated that both the heterozygous and homozygous knockout mice manifested a reduction in social interaction compared to *Dys* wildtype mice. This is in agreement with other groups' observations of reduced social contacts in the Sandy (*sdv*) mouse, which harbours a spontaneously occurring deletion in the *dtncp 1* gene and expresses no *Dys* protein. However, in contrast to its effects in C57 wildtype mice, chronic intranasal administration of oxytocin (OXT) was able to ameliorate the social deficits observed in heterozygous but not in homozygous *Dys* knockout mice. Furthermore we demonstrated that acute administration of oxytocin was able to improve social deficits both in hetero and in homo *Dys* KO mice. These results provide initial indication that, conversely to subjects with a healthy OXTergic system, intranasal OXT produce prosocial effects in socially and OXTR altered systems with relevance to schizophrenia.

### 2.1 Introduction

The neuropeptide oxytocin (OXT) has been strongly implicated in social behaviours including social recognition, social approach, pair bonding, paternal care and maternal behaviour (Lim, Bielsky, & Young, 2005; Mariaelvina Sala et al., 2011b; Winslow & Insel, 2002). This has led to the proposal of its use as an adjunctive therapeutic treatment in neuropsychiatric diseases characterized by impaired social behaviours such as social anxiety disorder, borderline personality disorder, autism spectrum disorders and schizophrenia (Meyer-Lindenberg et al., 2011; Striepens et al., 2011). This would be

particularly important as social alterations are considered among the most debilitating core features of these mental illnesses and currently available treatments are not effective in ameliorating these symptoms. Intranasal administration of neuropeptides holds promising potential for the treatment of brain diseases as it might deliver biologically effective concentrations to the brain without eliciting potent systemic hormone-like side effects (Born et al., 2002). Intranasal administration of vasopressin (avp) and of OXT have been reported, in rats and mice, to increase the intracerebral concentration of these peptides, not only in the cerebrospinal fluid but also in selected brain regions (Born et al., 2002; Neumann et al., 2013). However, central effects might be also due to avp/OXT endogenous release mediated by peripheral mechanisms (Ludwig et al., 2013). Whatever the mechanism(s) involved, there is no doubt that central effects are observed in humans after intranasal administration (Veening & Olivier, 2013). Acute intranasal OXT seems to produce no negative side effects in both healthy volunteers and subjects with developmental or mental health difficulties when delivered in doses of 18-40 IU (MacDonald et al., 2011). Moreover, in subjects diagnosed with autism, a single intranasal administration of OXT might be able to improve, at least temporarily, emotion recognition abilities in the Reading the Mind in the Eyes test and the patients' ability to process socially relevant cues in a computer-based game (Andari et al., 2010; Green & Hollander, 2010; Guastella, Mitchell, & Dadds, 2008). Furthermore, in patients with schizophrenia, an adjunctive 3-week treatment with intranasal OXT has been found to improve Positive and Negative Syndrome Scale (PANSS) total scores significantly (Feifel et al., 2010) and verbal memory measures of the California Verbal Learning Test (Feifel, Macdonald, Cobb, & Minassian, 2012). Despite these promising initial results, it is not yet clear if chronic intranasal OXT might affect psychiatric-relevant behavioural measures (e.g. in social and memory domains), and if chronic intranasal OXT has different effects compared with single administration. This is critical for the aforementioned chronic mental disorders that might potentially involve life-long treatments. Unfortunately, ethical issues, genetic, environmental and clinical heterogeneity in human studies might dampen the resolution of these questions and thus, slow down the progress towards this new promising therapeutic treatment. In this context, preclinical studies in rodents might be critical to dissect OXT-dependent behavioural effects and hold the potential to better understand the impact of intranasal OXT in brain functioning. Just recently was demonstrated in our lab possibility to use intranasal administrations in mice so now we have Recently introduced the possibility to test intranasal OXT treatments (Huang et al., 2014) for large genetic and pharmacological screenings aiming at developing more selective and personalized therapeutic strategies for human pathologies such as autism and schizophrenia. The role of the OXT system has been investigated in rodent models mainly using OXT-related mutant mice and giving OXT through intracerebroventricular (ICV), intraperitoneal (ip) and subcutaneous (sc) injections (Bielsky & Young, 2004; Jin et al., 2007; Popik, Vetulani, & Van Ree, 1996; Mariaelvina Sala et al., 2011b, 2013; Takayanagi et al., 2005; Winslow & Insel, 2002). Before our previous work (Huang et al., 2014) only one study in prairie voles (*Microtus ochrogaster*) (Karen L Bales et al., 2013) has initiated to investigate the behavioural effects of intranasal OXT administration before adulthood (i.e. from weaning through sexual maturity); after that study, intranasal oxytocin was still used just in other two

studies in mice (K L Bales et al., 2014; Gigliucci et al., 2014) even if both times just in models for autism. Thus, the present study aimed at elucidating the behavioural effects of intranasal administration of OXT in adult mouse model relevant for schizophrenia, and the Dysbindin-1 deficient mouse model was chosen for this test .

Dysbindin-1 (Dys) is encoded by the Dystrobrevin-binding protein 1 gene (DTNBP 1) and is expressed widely in human and mouse brains (Burdick et al., 2006). Dysbindin-1 has been implicated in the regulation of vesicle formation and synaptic release (Chen et al., 2008; Dickman & Davis, 2009) and is a component of the biogenesis of lysosome-related organelles complex (BLOC-1) (Iizuka, Sei, Weinberger, & Straub, 2007). Recent studies in mice show that Dysbindin-1 is more highly expressed during embryonic and early postnatal development compared to adulthood and BLOC-1 is involved in neurite outgrowth (Ghiani et al., 2010). These findings point to a potential role of Dysbindin-1 in normal development of brain structure and function. In addition, abnormalities in Dysbindin-1 have been found to also affect cognition in humans as well as in mice, and variations in the DTNBP 1 gene are associated with alterations in general cognitive abilities (Burdick et al., 2006; Fallgatter et al., 2006; Papaleo & Weinberger, 2011; Papaleo, Yang, et al., 2012) such as; selective attention, general, verbal, sometimes visual memory measured on the revised Wechsler Memory Scales (Hashimoto et al., 2009, 2010), working memory, verbal declarative memory, mental processing speed, executive function measured with multiple tests (Luciano et al., 2009; Markov et al., 2010), and semantic verbal fluency (Markov et al., 2009). Genetic variations (single nucleotide polymorphisms; SNPs and/or combinations of them; haplotypes) in DTNBP 1 is also associated with psychiatric conditions. (Straub et al., 2002) first identified an association between schizophrenia and several SNPs within the DTNBP 1 gene, in a linkage disequilibrium study across the 6p24-21 susceptibility interval. Subsequent studies have since confirmed this association in multiple, independent populations (Schwab et al., 2003; Van Den Bogaert et al., 2003) and DTNBP 1 remains among the leading candidate genes in meta-analyses (Allen et al., 2008). Schizophrenic patients have been reported to have reduced expression of Dysbindin-1 gene and protein in the prefrontal cortex (PFC) and hippocampus, regions associated with dysfunction of executive control and working memory functions in the disorder (Talbot et al., 2004; Tang et al., 2009; Weickert et al., 2004; Weickert, Rothmond, Hyde, Kleinman, & Straub, 2008) but the molecular mechanisms by which this contributes to pathogenesis and symptomatology are as yet unknown (Iizuka et al., 2007). Association studies have also implicated DTNBP 1 variation in the severity of negative symptoms and cognitive impairments in schizophrenia (Burdick et al., 2007; DeRosse et al., 2006; Fanous et al., 2005; Straub et al., 2002). The *Sandy* (*sd*) mouse (Figure 1), which has a naturally occurring deletion mutation of exons 6 and 7 in the gene (DTNBP 1) encoding the mouse protein, provides an animal model of Dysbindin-1. The mutation results in loss of Dys in homozygous animals. Dysbindin-1 knock-out (*Dys*<sup>-/-</sup>) mice show selective alterations in internal trafficking of specific components of dopamine and glutamate signalling, including D2 receptors and NR2A receptor subunits (Karlskold et al., 2011; Papaleo, Yang, et al., 2012). Cortical neuronal cultures from *Dys*<sup>-/-</sup> mice show signalling alterations that may underlie the deficits in cognitive performance.

In particular, Dysbindin-1 has been associated with various aspects of synaptic function (Cox et al., 2009; Dickman & Davis, 2009), and the regulation of both dopamine and glutamate signalling in the brain (Papaleo & Weinberger, 2011). Results from in-vitro experiments suggest that Dysbindin-1 suppresses dopamine release (Kumamoto et al., 2006) and cultured neurons from Dys<sup>-/-</sup> mice have increased cell surface expression of the D2 receptor due to an increased membranal insertion rate (Ji et al., 2009). Layer II/III pyramidal neurons from the PFC of Dys<sup>-/-</sup> mice show increased activity at baseline, but decreased activity after D2 stimulation compared to wild-type mice and these effects may be due to D2-mediated alterations in the excitability of fast-spiking GABAergic interneurons (Ji et al., 2009; Papaleo, Yang, et al., 2012). Reduced Dysbindin-1 function also impairs glutamate signalling in neuronal culture (Numakawa et al., 2004). In agreement with these in-vitro studies, sdy mice display decreased excitability of glutamatergic neurons in the PFC and a decrease in the release of glutamate in the hippocampus (Chen et al., 2008; Jentsch et al., 2009). Furthermore, recent work demonstrates that Dys<sup>-/-</sup> mice exhibit reduced NMDA-evoked currents and reduced NR1 mRNA expression in the PFC (Karlsgodt et al., 2011). Dys<sup>-/-</sup> mice also show cognitive impairments, for example; spatial memory deficits in the Morris water maze (Cox et al., 2009), working memory deficits in a discrete paired-trial T-maze task (Papaleo, Yang, et al., 2012) and in a delayed non-match-to-position operant task (Karlsgodt et al., 2011). Taken together, these data implicate a reduction of Dysbindin-1 function thought to play a role in the development of cognitive abnormalities found in schizophrenia. Therefore, the Dys null mutant mice represent an important model of Dysbindin-1 down-regulation and D2 up-regulation that will help unravel the molecular mechanism for the association of Dysbindin-1 with psychosis, social and cognitive deficits (Papaleo, Yang, et al., 2012). In a previous work (Huang et al., 2014), it was shown that chronic intranasal oxytocin (OXT) treatment in wild-type C57BL/6J adult mice produced a selective reduction of social behaviours concomitant to a reduction of the OXT receptors throughout the brain. These results indicate that a prolonged over-stimulation of a "healthy" oxytocinergic brain system, with no inherent deficits in social interaction and normal endogenous levels of OXT caused specific detrimental effects in social behaviours. In view of the literature that shows sdy mutant mice with a deletion in DTNBP1, exhibit deficits in social interaction, the purpose of the following experiments was to test whether acute and chronic intranasal OXT treatment could ameliorate these deficits.

The genetic background strain chosen for the study C57BL/6j is a classical background strain for genetically modified mice and animal models of neurodegenerative and neurodevelopmental disorders. In line with clinical settings where intranasal OXT treatments would be mainly administered to address chronic symptoms in psychiatric disorders, we first performed behavioural and molecular studies in mice undergoing chronic intranasal treatment and thereafter, also separately tested the effects of acute intranasal OXT treatment.

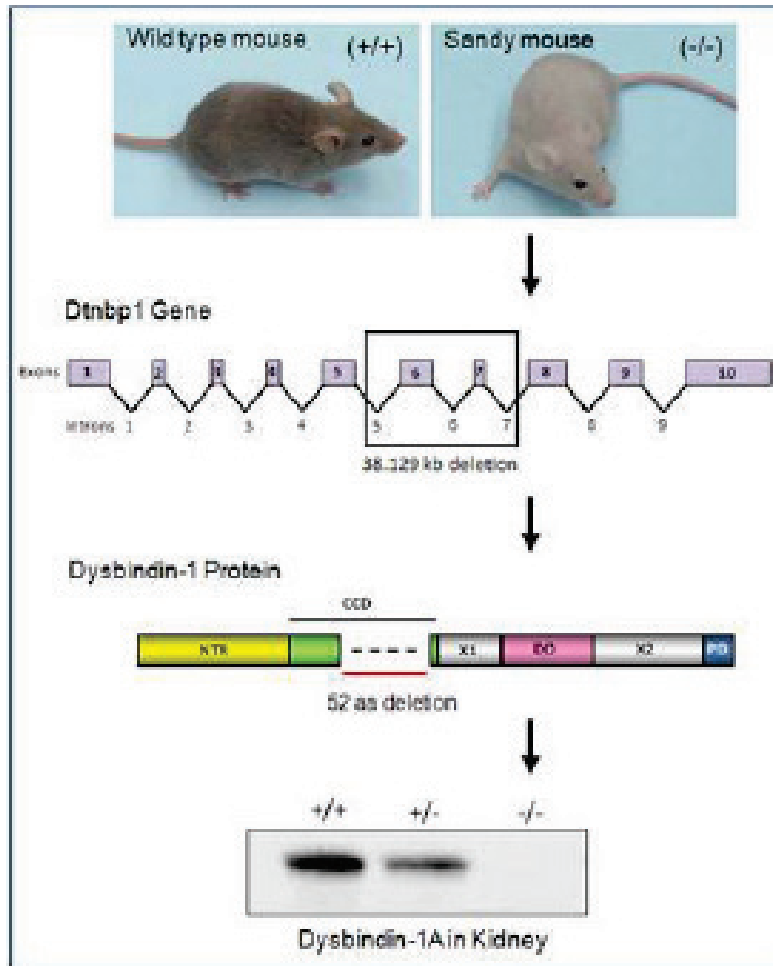


Figure 1: From top: First 2 pictures show the coat-color difference between wild type and *sd*y homozygous mice due to defective melanosome formation. Second picture shows the basic exonic-intronic structure of mouse DTNBP 1 gene showing large deletion mutation of exons 6 and 7 in *sd*y mice reported by (Li et al., 2003). Third picture shows a schematic of dysbindin-1 lacking 52 amino acids in the coiled coil domain due to mutation-induced truncation of transcript. The last picture shows dysbindin-1A decrease and loss in kidney tissue of heterozygous and homozygous animals, respectively (Li et al., 2003), probably due to rapid degradation as a result of failure to form stable interactions with *bloc-1* binding partners in the absence of an intact coiled coil domain. [Modified from (Talbot, 2009).]

## 2.2 Materials and Methods

### 2.2.1 Subjects

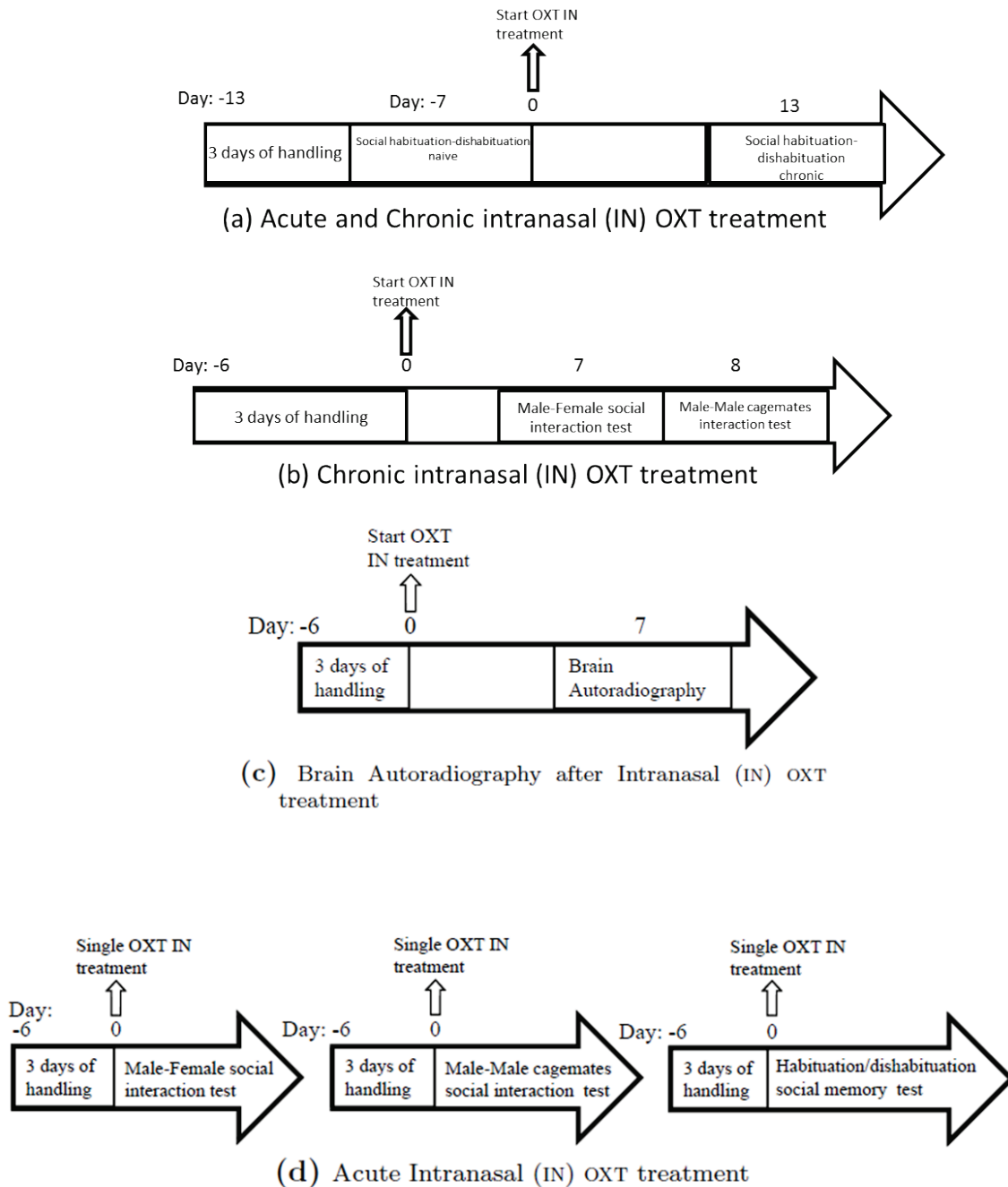
All procedures were approved by the Italian Ministry of Health (permit n. 230/2009-B) and local Animal Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives. Sandy (*sd*y) is an autosomal recessive coat mutation that occurred spontaneously in the inbred DBA/2J strain in 1983 at The Jackson Laboratory. The mutation present in our mice was

transferred to the C57BL/6J (B6) genetic background by 11 generations of backcrossing into B6 at The Jackson Laboratory and National Institute of Mental Health. The purpose of this backcrossing procedure was to remove the effects on behaviour and dopamine function of the native *sdv* mouse background strain (DBA/2J), which has been shown to have abnormal behavioural phenotypes referable to the dopamine system (D'Este, Casini, Puglisi-Allegra, Cabib, & Renda, 2007). In particular, previous reports have shown that on the DBA/2J genetic background, *sdv* mice exhibit strong locomotor activity and coordination deficits, as demonstrated by poor performance in the rotarod test and even death during a forced swimming test (Takao et al., 2008). Also, DBA/2J mice are impaired in  $\theta$  burst long-term potentiation, in aspects of learning and memory, have higher dopaminergic activity in the forebrain, and are homozygous for four other mutations (cadherin, glycoprotein, tyrosinase-related protein 1 and hemolytic complement) compared with B6 mice (Cox et al., 2009; D'Este et al., 2007; Nguyen, 2000). Therefore, the role of the *dysbindin-1* mutation is potentially confounded when studied on the DBA/2J genetic background (Talbot, 2009). In these experiments, we used male littermates that were *dysbindin-1* null mutant (*Dys*<sup>-/-</sup>), heterozygous (*Dys*<sup>+/-</sup>), and wild-type (*Dys*<sup>+/+</sup>) bred by a heterozygous (*Dys*<sup>+/-</sup> × *Dys*<sup>+/-</sup>) mating strategy. Genotypes were identified by pcr analysis of tail dna. Animals were housed two to four per cage, in a climate-controlled animal facility (22°C ± 2°C) and maintained on a 12-hour light/dark cycle, with food and water available ad libitum throughout the experiments. All behavioural testing and procedures were conducted during the light phase of the cycle. The experimenter handled the mice on alternate days during the week preceding the first behavioural test. Experimenters were blind to the mouse treatments during testing and behavioural scoring.

### 2.2.2 Intranasal OXT Administration

Oxytocin (Novartis Pharma AG, Switzerland) was dissolved in saline (0.9% NaCl) and administered intranasally in a volume of 5  $\mu$ l to each mouse in dose of 0.3 IU/5  $\mu$ l (OXT 0.3 IU). In our solution 1 IU of oxytocin is equal to 1.667  $\mu$ g of synthetic OXT. Thus, 0.3 IU corresponded 0.5001  $\mu$ g ( $\approx 4.96 \times 10^{-7}$  mol) of oxytocin, for each administration of 5  $\mu$ l (OXT 0.3 IU  $\approx$  19  $\mu$ g/kg or 11 IU/kg). This dose was chosen in order to be much lower than subcutaneous oxytocin doses (i.e. 250  $\mu$ g/kg) used in mice that could have produced peripheral effects (Mariaelvina Sala et al., 2011b). The dose we used was also similar to the higher range of intranasal oxytocin doses recently given to adolescent prairie voles (Karen L Bales et al., 2013) and furthermore resulted to be effective in previous works. (Huang et al., 2014). A 20  $\mu$ l Eppendorf pipette with gel-loading tips (Costar no. 4853) were used for administration. Drops of the 5  $\mu$ l solution were gently placed equally on both nostrils of each mouse, and whose were inhaled reflexively. Control mice received the same volume of saline (veh). Administration was rapid (less than 30 seconds) and handling was consistent across treatment groups. The detailed timelines of each procedural manipulation and oxytocin treatments are reported in Figure 2 Briefly, for the chronic intranasal treatments, mice were administered two times a day, once in the morning and once in the evening, for 7-21 consecutive days and tested as described below, one hour after the last

administration. For the acute intranasal treatments, mice were administered with OXT only once, 5 minutes before the test. The delay of only 5 minutes was chosen based on evidence indicating that intranasal administration of OXT has very rapid pharmacokinetics with effects expected to appear already within a few minutes (Veening & Olivier, 2013).



**Figure 2 Experimental timelines.** (a) Naive and Chronic 7 days of IN VEH or OXT in treatment. (b) Chronic male-female 7 days of IN VEH or OXT in treatment. (c) Brain autoradiography after 7 days of 7 days of IN VEH or OXT in treatment. (d) Single acute IN dose VEH

### 2.2.3 Behavioural Testing

#### *Male-Female social interaction test*

The test was conducted in 2150E Tecniplast cages (35.5×23.5×19 cm) lightly illuminated (5±1 lux) and video-recorded using a Unibrain Fire-iTM Digital Camera. The video camera was mounted facing the front of the cage to record the session for subsequent scoring of social investigation parameters as previously described (M L Scattoni, Ricceri, & Crawley, 2011). Unfamiliar female stimulus mice in oestrus were matched to the subject male mice by age and maintained in social groups of four per home cage. In a subgroup of animals, ultrasonic vocalisations (usvs) were also recorded during the test.

All the equipment was kept in a sound-attenuating chamber (TSE Multi Conditioning Systems).

Each male mouse was placed in a test cage and left to habituate for one hour before the test. Then, an unfamiliar female in oestrus (stimulus mouse) was placed into the testing cage of each isolated male for a 5-min test session. In addition to the resident mouse, the cage contained litter (1.5-cm deep) while the lid was removed during the test. Female stimulus mice were matched to the subject male mice by age and maintained in social groups of four per home cage.

On the day of testing, the vaginal oestrus condition of each female was assessed as previously described (Rugh, 1990). Only females in oestrus were selected for the test. Behavioural responses were analysed by NOLDUS OB-SERVER XT 10 software (Noldus Information Technology, Leesburg, VA, USA).

Social interaction was scored from the recorded videos measuring frequencies and durations of the following behavioural responses performed by the male mouse:

1. Social behaviours: anogenital sniffing (direct contact with the anogenital area), body sniffing (sniffing or snout contact with the flank area), head sniffing (sniffing or snout contact with the head/neck/mouth area) and following (time spent in following the female mice).
2. Non-social behaviours: stand/walk alone, digging in the bedding, grooming (self-cleaning and licking any part of its own body), rearing up in the centre of the cage and rearing up against the wall of the home cage.
3. Total social behaviour and total non-social behaviour were obtained by summing up frequency or duration of all social behaviours and all non-social behaviours as listed above, respectively.

No observations of wrestling behaviours were observed during the male-female interaction test.



### *Habituation/Dishabituation social interaction test*

Naive mice were tested as similarly reported previously (Ferguson et al., 2000; Huang et al., 2014; Scearce-Levie et al., 2008) in 2150E Tecniplast cages (35.5x23.5x19 cm) lightly illuminated (5±1 lux) and video-recorded using a Unibrain Fire-I TM Digital Camera. Male or female subject mice were individually placed in the testing cage 1 hour before the testing. No previous single housing manipulation was adopted to avoid any instauration of home-cage territory and aggressive behaviors. Testing began 5 minutes after the intranasal treatment when a stimulus same sex mouse was introduced into the testing cage for a 1-min interaction. At the end of the 1-min trial, we removed the stimulus animal and returned it to an individual holding cage. We repeated this sequence for three trials with 3-min inter-trial intervals introducing the same stimulus mouse in all four trials. In a fifth 'dishabituation' trial, we introduced a new unfamiliar stimulus mouse (as previously of the same sex of the subject) in the testing cage. Videos of behaviors were recorded and subsequently manually scored offline.

### **2.2.4 Brain autoradiography**

A separate cohort of naive mice was handled and intranasally treated with vehicle or oxytocin as described above and, at the same time-point when the social interaction test would have been performed, their brains were rapidly dissected and frozen in isopentane at -25°C and then stored at -80 °C. Brains were cut in coronal sections of 14-µm thickness and mounted on chrome-alum-gelatin coated microscope slides. All slides were stored at -80 °C until used in receptor autoradiography. The binding procedure was performed as previously described (Tribollet, Barberis, & Arsenijevic, 1997). Brain sections were lightly fixed by dipping the slides for 5 min in a solution of 0.2% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4), then rinsed by two 5-min washes in 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.1% bovine serum albumin. OXT binding sites were labelled with the radioiodinated OXT-receptor antagonist d(CH<sub>2</sub>)<sub>5</sub>[Tyr(Me)<sub>2</sub>,Thr<sub>4</sub>,Tyr<sub>9</sub>-NH<sub>2</sub>]OVT (125I-OVTA, Perkin Elmer, MA, USA) at 0.02 nM (Elands et al., 1988). AVP binding sites were labelled with the radioiodinated linear vasopressin, V1aR antagonist HO-Phaa<sub>1</sub>-D-Tyr(Me)<sub>2</sub>-Phe<sub>3</sub>-Gln<sub>4</sub>-Asn<sub>5</sub>-Arg<sub>6</sub>-Pro<sub>7</sub>-Arg<sub>8</sub>-NH<sub>2</sub> (125ILVA, Perkin Elmer, MA, USA) at 0.02 nM. The specific activity of radioligands was 2200 Ci/mmol; 1Ci=37 GBq). Non-specific binding was determined in the presence of 2 µM OXT or avp. Binding was carried out in a humid chamber, at room temperature, by covering each slide with 400 µl of incubation medium (50 mM Tris-HCl, 0.025% bacitracin, 5 mM MgCl<sub>2</sub>, 0.1% bovine serum albumin) containing 125I-OVTA or 125I-LVA, either alone or in the presence of OXT or avp. Incubation lasted 1 hour at room temperature under gentle agitation and was followed by two 5-min washes in ice-cold incubation medium and a quick rinse in distilled water. The slides were dried under a stream of cool air. Once dry, the slides were exposed to Biomax MR Films (Kodak) for 72 hours. The quantification of optical binding density was conducted on the resulting autoradiograms images as previously described (Rubino, Forlani, Viganò, Zippel, & Parolaro, 2005). In particular, each

cerebral area was traced with the mouse cursor and its densitometric gray level was calculated by subtracting the background of the film using NIH ImageJ software. Optical densities were then converted to nCi/mg tissue equivalent with the standard curve generated by calibrated iodinated microscales (0.72-52.13 nCi/mg tissue equivalent, Amersham). The number of slides for each area varied, but averaged approximately ten sections per area.

### 2.2.5 Automated scoring for familiar mice

The test was conducted in an empty open arena with opaque grey walls (40×40×40 cm) and dimly illuminated by overhead red lightning (mainly to facilitate mice handling by the experimenter). Mice were monitored by an infrared camera mounted 1.5 m above the arena. The camera was a FLIR A315 capable of a spatial resolution of 320×240 px at 30fps, with a thermal sensitivity of <0.05°C at +30°C (according to the producer) that allows full video recording even in full darkness. All the animals were recorded without any external artificial tagging (such as coloured paintings).

Analyses of social behaviour was performed by an automated tracking and social behaviour analysis system previously described in (Giancardo et al., 2013).

### 2.2.6 Statistical analyses

Results are expressed as mean ± standard error of the mean (sem) throughout. One- or two-way analyses of variance (anovas) were used for each single parameter measured. Newman-Keul's post-hoc test was used for making comparisons between groups when the overall anova showed statistical significant differences for the main factors. The accepted value for significance was  $p \leq 0.05$ . All statistical analyses were performed using the Statistica version 11 software (Statistica, StatSoft, Inc.).

## 2.3 Results

### 2.3.1 Social deficits in Dysbindin-1 mutant mice

A significant genotype effect was evident in anogenital sniffing (frequency:  $F_{2,25}=4$ ,  $p<0.05$ ; duration:  $F_{2,25}=7.85$ ,  $p<0.005$ ). In particular, compared with the Dys+/+ group, Dys+/- male mice showed decreased duration of anogenital sniffing events ( $p<0.05$ ; Fig. 3.2b). Additionally, for the Dys-/- group, there was a significant decrease in the number of anogenital sniffing events ( $p<0.05$ ; Fig. 3.2a) and in their duration ( $p<0.005$ ; Fig. 3.2b) relative to the Dys+/+ group.

No significant effect of genotype was observed in other measures of social interaction such as head sniffing (frequency:  $F_{2,25}=1.09$ ,  $p=0.35$ ; duration:  $F_{2,25}=1$ ,  $p=0.55$ ), body sniffing (frequency:  $F_{2,25}=1$ ,  $p=0.35$ ; duration:  $F_{2,25}=1.74$ ,  $p=0.20$ ), following (frequency:  $F_{2,25}=2$ ,  $p=0.16$ ; duration:  $F_{2,25}=1.99$ ,  $p=0.16$ ). (**Figure 3a, 4b**).

Similarly, in comparison with the Dys+/+ group, both Dys+/- and Dys-/- groups showed no significant differences in the non-social behaviours such as digging (frequency:  $F_{2,25}=0.31$ ,  $p=0.73$ ; duration:  $F_{2,25}=0.36$ ,  $p=0.70$ ), and wall rearing (frequency:  $F_{2,25}=2$ ,  $p=0.23$ ; duration:  $F_{2,25}=1.21$ ,  $p=0.31$ ). (Figure 3c, Figure 3d).

Overall, there was a significant effect of genotype in the duration of social behaviours ( $F_{2,25}=8.63$ ,  $p<0.005$ ), but not in the number of social behaviour events (frequency:  $F_{2,25}=3$ ,  $p=0.08$ ; Fig. 3.2e), nor in non-social behaviours (frequency:  $F_{2,25}=1$ ,  $p=0.34$ ; duration:  $F_{2,25}=0.37$ ,  $p=0.70$ ). In particular, post-hoc tests showed a decrease in the duration of social behaviours in both the Dys+/- ( $p<0.05$ ) and Dys-/- groups ( $p<0.005$ ) compared with Dys+/+ mice (Figure 3f).

During the male-female social interaction test, it is also possible to record usvs emitted by the male mice that are considered facets of social communication among rodents (M L Scattoni et al., 2011; Maria Luisa Scattoni, Crawley, & Ricceri, 2009). There was a significant effect of genotype in the total number of vocalisations emitted ( $F_{2,25}=5.62$ ,  $p<0.05$ ). In particular, both the Dys+/- and Dys-/- groups of mice showed a decrease in the total number ( $p<0.05$ ; Fig. 3.3) of vocalisations emitted compared to the Dys+/+ group.

Overall, these findings indicate that a relative reduction or complete deletion of dysbindin-1 function produces a decrease in social interaction behaviours towards opposite-sex female mice.

### 2.3.2 Social deficits in Dys mutant mice are ameliorated by chronic intranasal oxytocin

As discussed in chapter 2.1, the OXT system has been extensively involved in the modulation of social-related behaviours (Bielsky & Young, 2004; M Sala et al., 2013; Mariaelvina Sala et al., 2011a; Winslow & Insel, 2002). Thus, in order to assess possible rescue effects of chronic intranasal OXT treatment in the social deficits that has been found in Dys knockout mutant mice (both heterozygous and homozygous knockouts), we tested mice chronically treated with intranasal OXT or veh in the male-female social interaction test (M L Scattoni et al., 2011). A significant genotype effect was apparent for body sniffing (frequency:  $F_{2,28}=8.15$ ,  $p<0.005$ ; duration:  $F_{2,28}=9.23$ ,  $p<0.005$ ). Specifically, compared with the Dys+/+ veh-treated group, Dys+/- and Dys-/- veh-treated male mice showed decreased frequency and duration of body sniffing events ( $p<0.005$ ; Figure 3 a. Figure 3 b). These results provides further confirmation of the social deficits that we have observed in the previous experiment (see above Section 2.3.3).

In addition, a significant OXT-treatment effect is observed in the Dys+/-veh and OXT-treated groups for body sniffing (frequency:  $F_{2,28}=3.81$ ,  $p<0.05$ ; Fig 3.4a) and anogenital sniffing (duration:  $F_{2,29}=4.25$ ,  $p<0.05$ ; Fig 3.4b). In particular, both the Dys+/- 0.15 IU OXT and 0.3 IU OXT groups exhibited an increase in the number of body sniffing events ( $p<0.05$ ; Fig 3.4a) and the Dys+/- 0.3 IU OXT group, an increase in the duration of anogenital sniffing events ( $p<0.05$ ; Fig 3.4b) compared with the Dys+/- veh group.

No significant genotype effect has been found in the non-social behaviours, except for wall rearing (duration:  $F_{2,28}=4.45$ ,  $p<0.05$ ; Fig 3.5b) where the Dys $^{+/-}$  veh group displayed an increase in the duration of wall rearing events ( $p<0.05$ ) compared to the Dys $^{+/+}$  veh group.

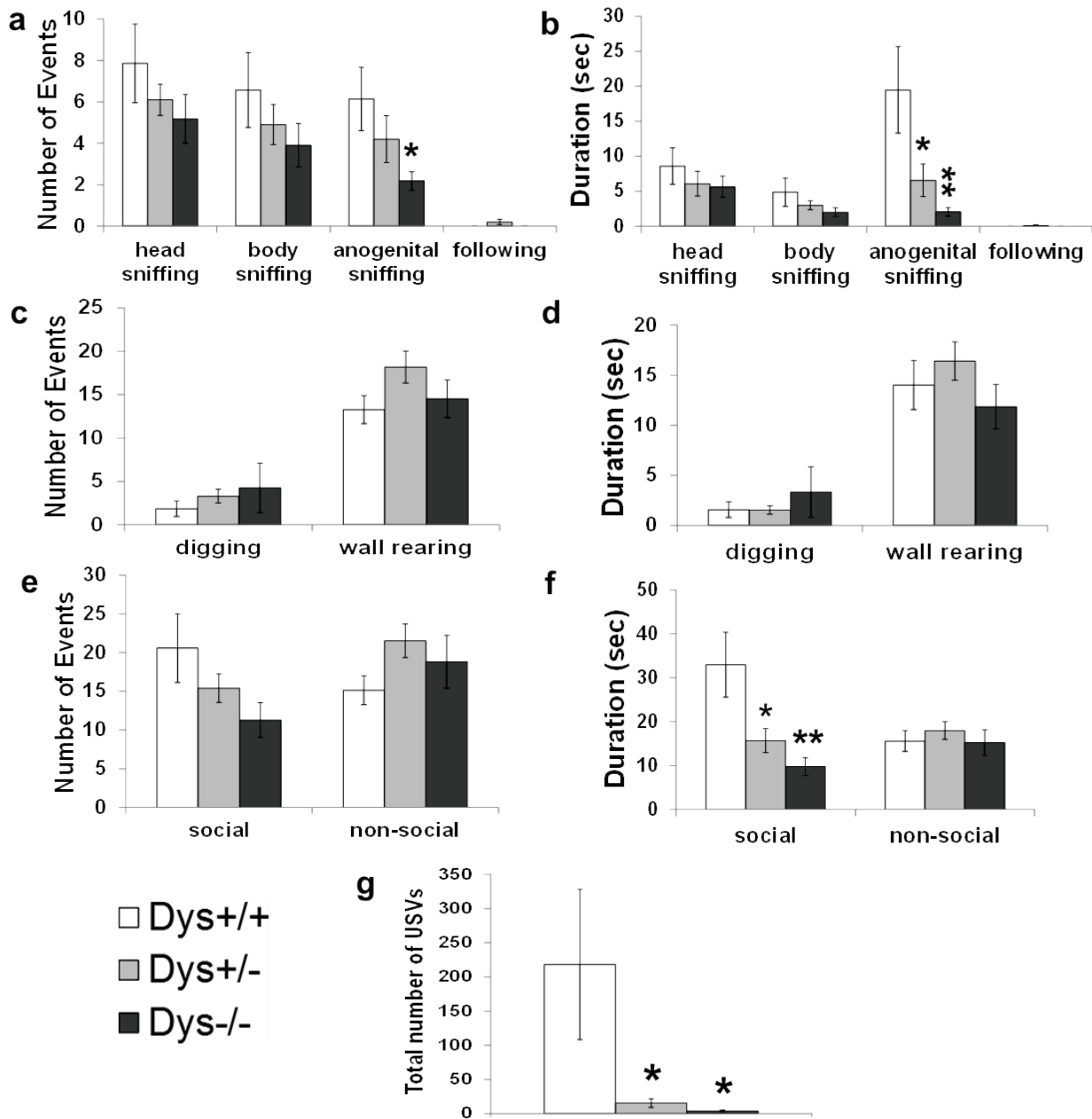
However, a significant effect of OXT-treatment is observed in the Dys $^{+/-}$ -veh and OXT-treated groups for rearing (duration:  $F_{2,29}=6.94$ ,  $p<0.005$ ; Fig 3.5b) and wall rearing (frequency:  $F_{2,29}=4.12$ ,  $p<0.05$ ; duration:  $F_{2,29}=4.87$ ,  $p<0.05$ ; Fig 3.5a, 3.5b). Chiefly, the Dys $^{+/-}$  0.15 IU OXT-treated mice showed an increase in the duration of rearing events ( $p<0.05$ ) and a decrease in the number ( $p<0.05$ ) and duration ( $p<0.05$ ) of wall rearing events compared with Dys $^{+/-}$  veh-treated mice. Furthermore, Dys $^{+/-}$  0.3 IU OXT-treated mice also displayed a reduced duration of wall rearing events ( $p<0.05$ ) in comparison to Dys $^{+/-}$  veh-treated mice.

In summary, there was a significant genotype effect in the frequency of total social behaviours ( $F_{2,28}=4.50$ ,  $p<0.05$ ; Fig 3.6a) but none in the duration of total social behaviours ( $F_{2,28}=2.87$ ,  $p=0.07$ ) nor in total non-social behaviours (frequency:  $F_{2,28}=0.43$ ,  $p=0.66$ ; duration:  $F_{2,28}=0.88$ ,  $p=0.42$ ; Fig 3.6a, 3.6b). Post-hoc tests revealed a decrease in the number of total social behaviours in Dys $^{+/-}$  and Dys $^{-/-}$  veh-treated mice with respect to Dys $^{+/+}$  veh-treated mice ( $p<0.05$ ). Furthermore, OXT treatment showed a significant effect in total social behaviours of Dys $^{+/-}$  OXT 0.15 IU and 0.3 IU groups (frequency:  $F_{2,29}=3.16$ ,  $p=0.05$ ; duration:  $F_{2,29}=6.71$ ,  $p<0.005$ ) and in the duration of total non-social behaviours ( $F_{2,29}=3.78$ ,  $p<0.05$ ) but not in the frequency ( $F_{2,29}=1.98$ ,  $p=0.16$ ). Specifically, the Dys $^{+/-}$  0.15 IU OXT group exhibited an increase in the number of total social behaviours ( $p=0.05$ ) and both Dys $^{+/-}$  0.15 IU and 0.3 IU OXT groups showed an increase in the duration of total social behaviours ( $p<0.05$ ) with respect to the Dys $^{+/-}$  veh group. On top of that, the Dys $^{+/-}$  0.3 IU OXT group also displayed a decrease in the duration of total non-social behaviours ( $p<0.05$ ) compared with the Dys $^{+/-}$  veh group.

Statistical comparisons between the Dys $^{+/-}$  and Dys $^{-/-}$  OXT-treated groups with the Dys $^{+/+}$  veh group revealed that there are no differences in all of the measures of social behaviours between the groups, except for anogenital sniffing (Dys $^{+/+}$  veh vs Dys $^{+/-}$  and Dys $^{-/-}$  0.3 IU OXT groups: frequency:  $F_{2,29}=4.32$ ,  $p<0.05$ ; duration:  $F_{2,29}=3.96$ ,  $p<0.05$ ). Notably, post-hoc tests showed that the number of anogenital sniffing events of the Dys $^{-/-}$  0.3 IU OXT group is reduced ( $p<0.05$ ; Fig 3.4a) as compared to that of the Dys $^{+/+}$  veh

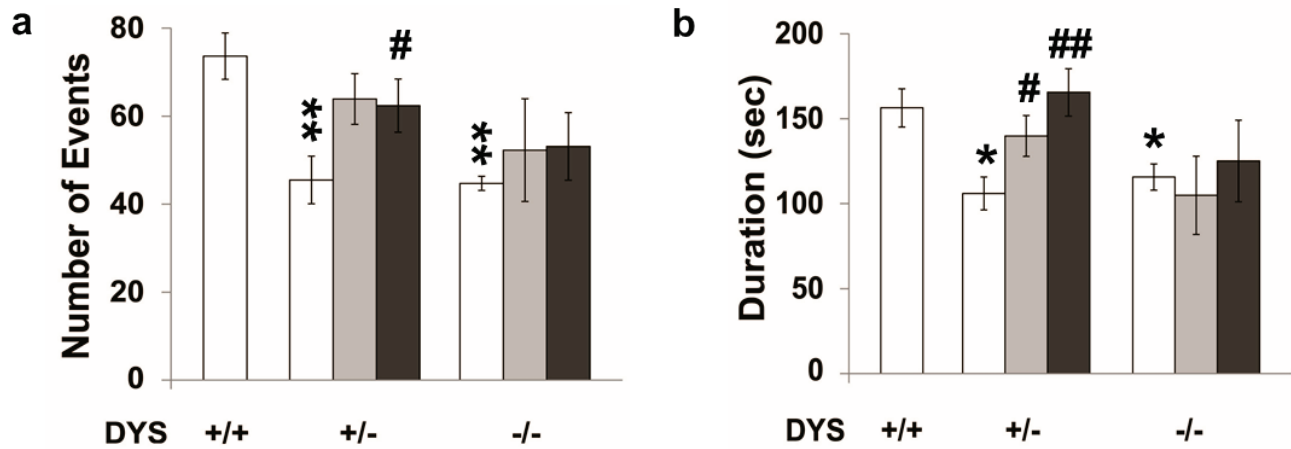
group, whereas the duration of anogenital sniffing events of the Dys $^{+/-}$  0.3 IU OXT group is longer ( $p<0.05$ ; Fig 3.4b) than that of the Dys $^{+/+}$  veh group. These results indicate that chronic intranasal OXT treatment can potentially rescue the social deficits that have been observed in Dys mutant mice.

### Social deficits in Dysbindin-1 knockout mice: male-female interaction

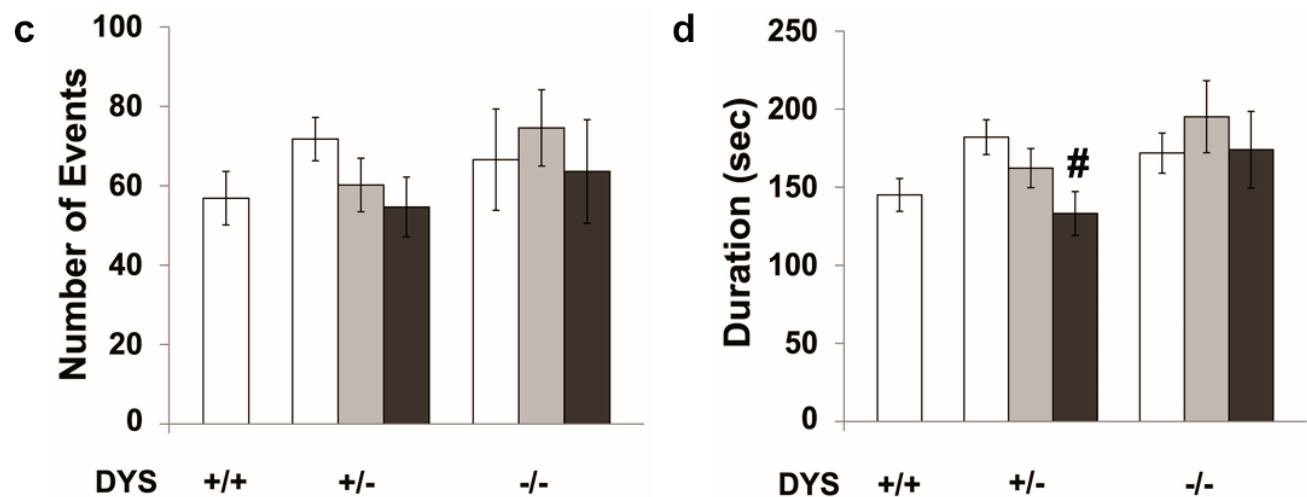


**Figure 3** Dys mice show a reduction in male-female social interaction. (a) Frequency of occurrence of various social behaviours such as head sniffing, body sniffing, anogenital sniffing and following. (b) Duration of events of above-mentioned social behaviours. (c) Frequency of occurrence of various non-social behaviours such as digging and wall rearing. (d) Duration of events of above-mentioned non-social behaviours. (e) Sum of the frequency of all events of social and non-social behaviours. (f) Sum of the duration of all events of social and non-social behaviours. (g) Total number of USV calls in 5 minutes. Ns= 7 Dys+/+, 10 Dys+/-, 10 Dys-/- . (\*) p<0.05, (\*\*) p<0.005 vs. Dys+/+ group. Values represent mean ± SEM throughout all Figures.

## Social Behaviours



## Non-Social Behaviours



**Figure 4 Chronic intranasal OXT treatment reverted reduction in total social behaviours in Dys heterozygous knockout mice.** (a) Sum of the frequency of all events of social and non-social behaviours. (b) Sum of the duration of all events of social and non-social behaviours. Ns= 14 Dys+/+ VEH, 12 Dys+/- VEH, 7 Dys-/- VEH, 10 Dys+/- OXT 0.15 IU/5  $\mu$ l, 7 Dys-/- OXT 0.15 IU /5  $\mu$ l, 12 Dys+/- OXT 0.3 IU/5  $\mu$ l, 8 Dys-/- OXT 0.3 IU/5  $\mu$ l.

(\*)  $p < 0.05$ , (\*\*)  $p < 0.005$  vs. Dys+/+ VEH group. (#)  $p < 0.05$ , (##)  $p < 0.005$  vs. Dys+/- VEH group. Values represent mean  $\pm$  SEM throughout all Figures.

### 2.3.3 Disbindin mutations reduce interaction times in female mice

In social habituation-dishabituation test on naive untreated mice was observed a significant reduction of social interaction time for both Dys +/- and Dys -/- groups. Particularly for Dys -/- groups reduction was observed in the first trial of interaction with the unfamiliar female ( interaction time 11.51 s, p=0.0028 vs DYS +/+), in the second trial ( interaction time 6.67 s, p=0.025 vs DYS +/+) and in the last trial after introduction of a new unfamiliar female ( interaction time 11.89 s, p=0.0025 vs DYS +/+), and for Dys +/- a significant interaction time reduction was observed in the same trials (respectively interaction time 29.64 s, p=0.035 vs DYS +/+ , interaction time 14.90 s, p=0.0036 vs DYS +/+ , interaction time 20.70 s, p=0.00018 vs DYS +/+) ( Figure 5).

Same test in males revealed no significant difference between the 3 groups (Figure 6)

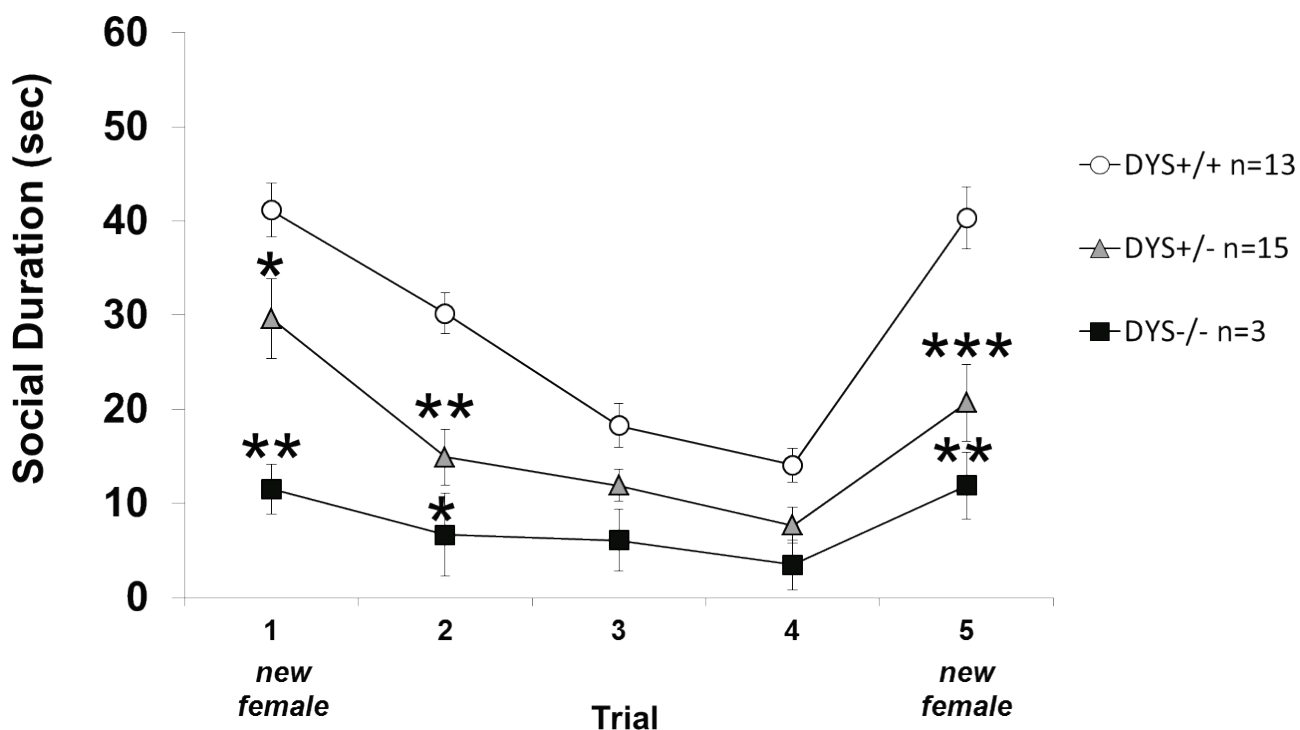
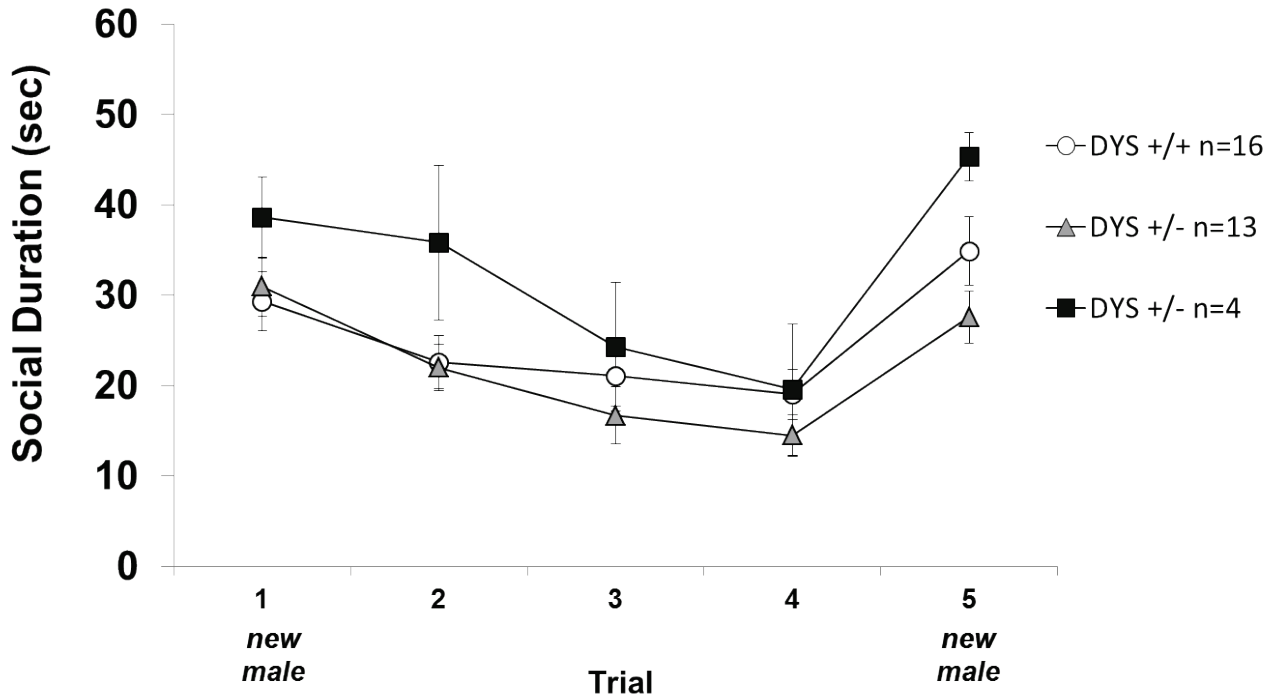


Figure 5 **Dys female mice show reduced social interaction between unfamiliar females.** Data depict the amount of time in seconds allocated to investigation of the same unfamiliar female during each of four successive 1-min trials. A fifth 'dishabituation' trial depicts the response of the subject females to the presentation of a new unfamiliar female in a 1-min pairing 3 min after the fourth trial. Ns= 13 Dys+/, 15 Dys+/- , 3 Dys-/-.

(\*) p<0.05, (\*\*) p<0.005 and (\*\*\*) p<0.0005 vs. respective trial of DYS+/>

Values represent mean ± SEM throughout all Figures.



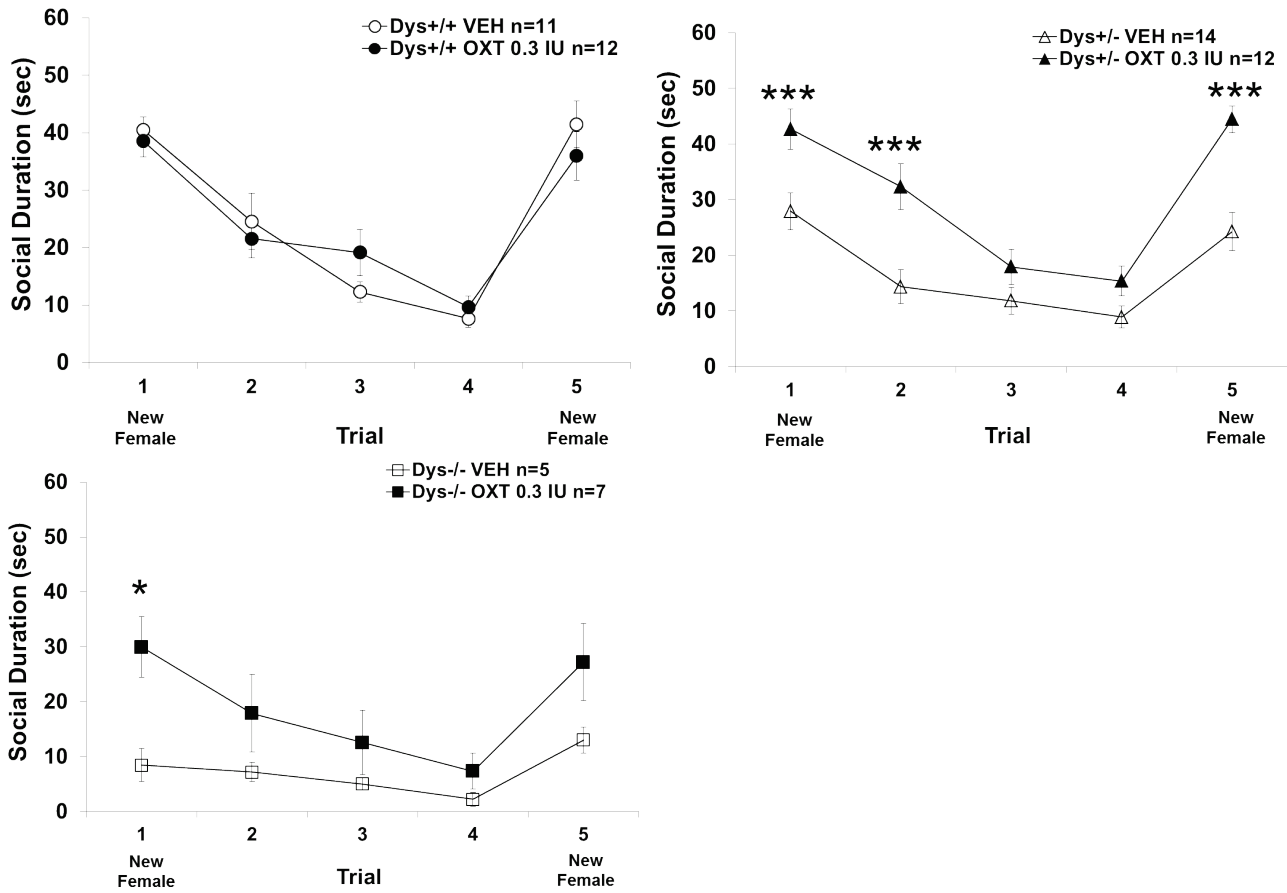
**Figure 6 Dys male mice don't show reduced social interaction between unfamiliar males.** Data depict the amount of time in seconds allocated to investigation of the same unfamiliar male during each of four successive 1-min trials. A fifth 'dishabituation' trial depicts the response of the subject females to the presentation of a new unfamiliar female in a 1-min pairing 3 min after the fourth trial. Ns= 16 Dys+/, 13 Dys+/-, 4 Dys-/. No significant difference was observed. Values represent mean  $\pm$  SEM throughout all Figures.

### 2.3.4 Social deficits in female-female interaction in Dys mutant mice are ameliorated by acute intranasal oxytocin

As oxytocin has extensively involved in the modulation of social-related behaviors (Bielsky & Young, 2004; Mariaelvina Sala et al., 2011b, 2013; Winslow & Insel, 2002). Thus, in order to assess possible rescue effects of chronic intranasal oxytocin treatment in the social deficits that has been found both in Dys +/- and Dys -/- mutant mice, we tested mice acutely and chronically treated with intranasal oxytocin or vehicle in the social habituation-dishabituation test (Huang et al., 2014).

In female-female social habituation –dishabituation test, acute administration of oxytocin ameliorated social deficit observed in both Dys +/- and Dys -/-. Particularly for Dys -/- was observed a significant increase of social interaction time vs control for trial 1 ( $p=0.004$ ), 2 ( $p=0.002$ ), 5 ( $p=0.0003$ ), while for dys -/- was observed a significant improvement of sociability for trial 1 ( $p=0.02$ ); while for trial 5 there was still a trend of improvement ( $p=0.08$ ). No effect was observed in Dys -/- group treated with oxytocin.





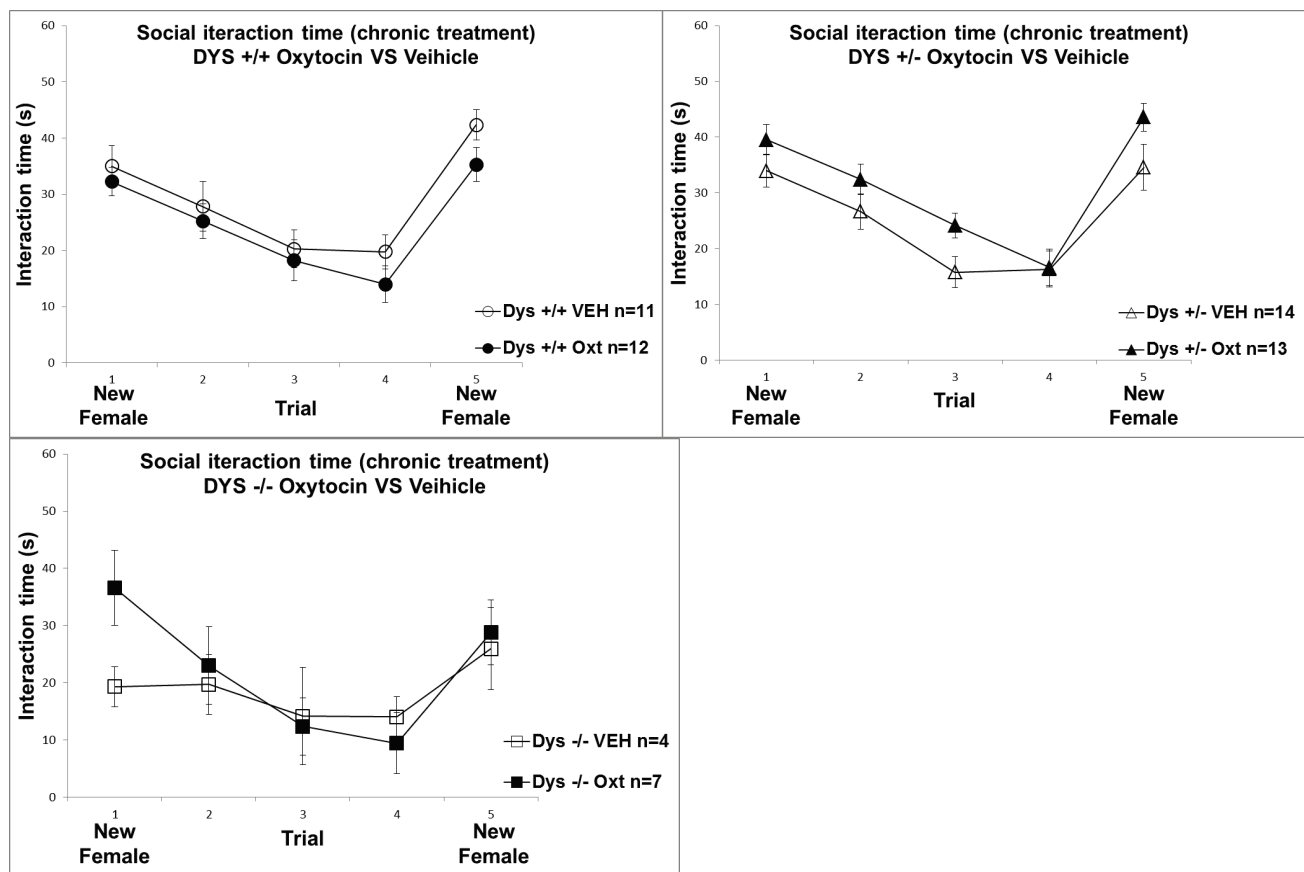
**Figure 7 Acute intranasal OXT treatment reverted decrease in social interaction between unfamiliar females.**

Data depict the amount of time in seconds allocated to investigation of the same unfamiliar female during each of four successive 1-min trials. A fifth 'dishabituation' trial depicts the response of the subject females to the presentation of a new unfamiliar female in a 1-min pairing 3 min after the fourth trial. Ns= 11 Dys+/+ VEH, 14 Dys+/- VEH, 5 Dys-/- VEH, 12 Dys+/+ OXT 0.3 IU/5  $\mu$ l, 12 Dys+/- OXT 0.3 IU/5  $\mu$ l, 7 Dys-/- OXT 0.3 IU/5  $\mu$ l.

(\*)  $p < 0.05$ , (\*\*)  $p < 0.005$ , (\*\*\*)  $p < 0.0005$  OXT 0.3 IU/5  $\mu$ l vs VEH.

Values represent mean  $\pm$  SEM throughout all Figures.

After chronic treatment was not observed anymore any significant difference in interaction times .

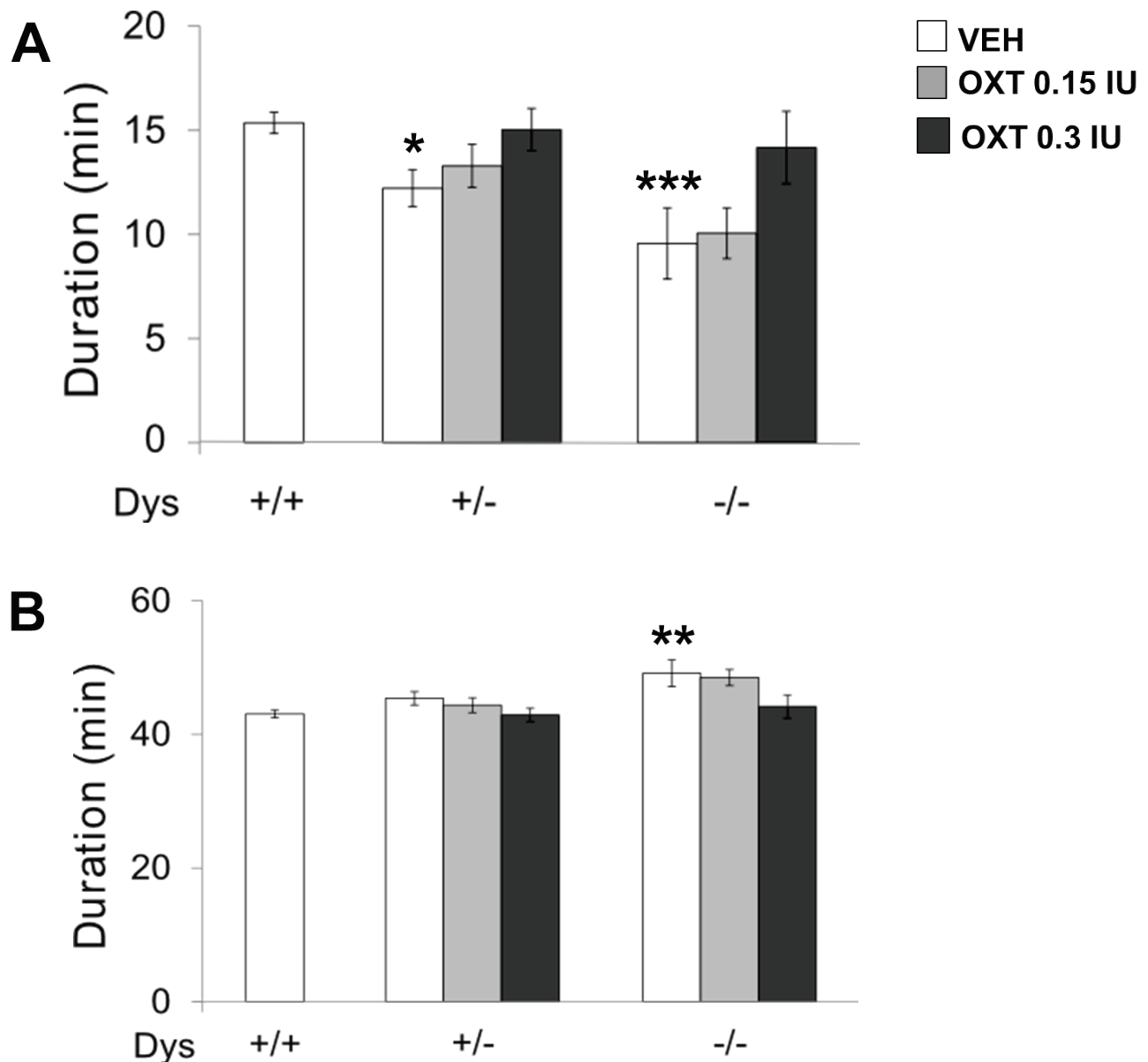


**Figure 8 Chronic intranasal OXT treatment in social interaction between unfamiliar females.** Data depict the amount of time in seconds allocated to investigation of the same unfamiliar female during each of four successive 1-min trials. A fifth `dishabituation' trial depicts the response of the subject females to the presentation of a new unfamiliar female in a 1-min pairing 3 min after the fourth trial. Ns= 11 Dys+/+ VEH, 14 Dys+/- VEH, 5 Dys-/- VEH, 12 Dys+/+ OXT 0.3 IU/5  $\mu$ l, 12 Dys+/- OXT 0.3 IU/5  $\mu$ l, 7 Dys-/- OXT 0.3 IU/5  $\mu$ l. Values represent mean  $\pm$  SEM throughout all Figures.

### 2.3.5 Chronic intranasal oxytocin ameliorates social deficits in familiar mice

Unfamiliar Dys mutant males do not show deficits of sociability VS same sex stimulus mice, as observed in social habituation-dishabituation test **Figure 6**. This is probably because the meeting between unfamiliar males involves different aspects from the commonly-defined meaning of "sociability". In fact in this context, an intruder-response might be involved that we cannot distinguish from common sociability. Moreover, as observed in social habituation-dishabituation test, we could highlight easily a treatment-induced decrease in sociability, while stress of manipulation for intranasal chronic treatment could increase social interaction covering possible deficits. This effect was observed also in literature too in a 3 chamber paradigm (K L Bales et al., 2014). For these reasons, we decided to study effects of chronic treatment in familiar males that should possibly be less stressed and prone to a "normal" sociability between individuals. In this context we observed a significant decrease in

duration of events of total social behaviours VS vehicle WT both for vehicle Dys +/- (p<0.05) and for vehicle Dys -/- (p<0.0005). Moreover, we observed an increase in non social behaviour in Dys(-/-) (p<0.05). For all these groups, deficit was ameliorated after administration of chronic intranasal oxytocin, as all mutant treated individuals (both Dys +/- than -/-) do not significantly differ from WT Vehicle group..

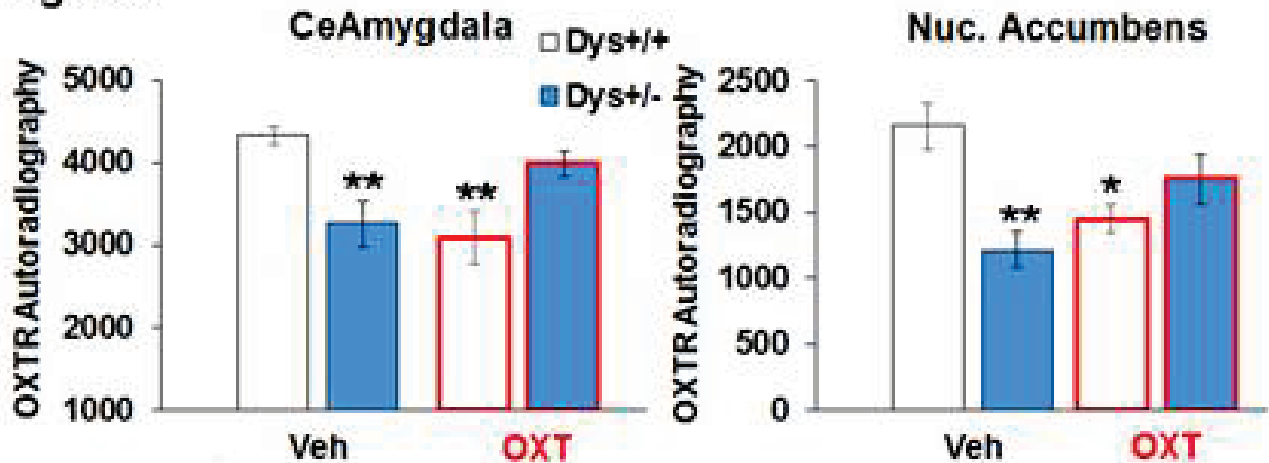


**Figure 9 Chronic intranasal OXT treatment reverted decrease in male-male social behaviours in Dys heterozygous knockout mice.** (A) Duration of events of total social behaviours. (B) Duration of events of total non-social behaviours. Ns= 14 Dys+/+ VEH, 12 Dys+/- VEH, 7 Dys-/- VEH, 10 Dys+/- OXT 0.15 IU/5 µl, 7 Dys-/- OXT 0.15 IU /5 µl, 12 Dys+/- OXT 0.3 IU/5 µl, 8 Dys-/- OXT 0.3 IU/5 µl. (\*) p<0.05, (\*\*) p<0.005, (\*\*\*) p<0.0005 vs. Dys+/+ VEH group.

### 2.3.6 Oxytocin doesn't reduce receptor expression in dys mutant mice

Matching behavioral findings, we are finding that, conversely to wild-type mice (Huang et al., 2014), intranasal OXT did not affect or even rescued the constitutive decrease of OXTR in selective brain areas of dysbindin-1 knockouts (some preliminary in **Figure 10**).

**Figure 7**



**Figure 10** Quantification of OXT-receptor (OXTR) binding in various brain areas. OXT-R ligand binding in Dysbindin-1<sup>125</sup> mouse forebrain. Quantitation of the autoradiographic <sup>125</sup>I-labeled OXT-R antagonist was obtained using NIH ImageJ- Software. Data is expressed as nCi/mg tissue equivalent (1 way and 2-way ANOVAs. N = 5 for WT groups, N = 4 for all Dys groups. (^), (\*) p < 0.05; (\*\*) p < 0.01 vs WT VEH. (^) p < 0.05 vs Dys+/- VEH. (A) Lateral Septum media region; (B) Nucleus Accumbens; (C) Amygdala; (D) Prefrontal cortex.

## 2.4 Discussion

Our experiments have confirmed that the *Dys* knockout mutant mice show reduced social interaction both in the male-female interaction test and in the female-female social habituation dishabituation test. This phenotype has been suggested as rodents' correlate of negative symptoms (social withdrawal) in schizophrenia and was observed in literature in different social paradigms (Feng et al., 2008; Hattori et al., 2008). The demonstration of social deficits in 2 different paradigms indicates that *dysbindin 1* mutant mice are a good model for the study of negative symptoms associated to schizophrenia. Thus, the *Dys* mouse model presents an invaluable tool in the investigation of the aetiology of schizophrenia and in the search and test of novel and more effective therapies for schizophrenia-associated social deficits. With social deficits in mind for which as yet there are no effective therapies concerning human schizophrenia, we tested whether acute or chronic intranasal administration of oxytocin (OXT), a neuropeptide that has been shown to mediate various social behaviours (Lim et al., 2005; Mariaelvina Sala et al., 2011a; Winslow & Insel, 2002), with promising initial results in patients, could ameliorate these deficits. The encouraging results from our male-female interaction test, in chronically treated *Dys* mutant mice, and in female-female social habituation-dishabituation in acutely treated *Dys* mutant mice, provides evidence that both chronic and acute intranasal OXT could rescue the social deficits exhibited by these mice. Curiously, chronic administration in females did not lead to a significant variation of cognitive performance from treated vs untreated mutant mice but just a trend of improvement. However, watching accurately the data is possible to observe a general increase of sociability in both treated and untreated group after one week of chronic administration that suggests certain effects of stress derived from prolonged handling for daily intranasal administration that raises the baseline sociability of the saline control mice masking any possible drug improvement effect. The same effect was observed by Bales (K L Bales et al., 2014) after a chronic protocol of intranasal administrations, though in a different social paradigm (the tree chamber test). This statement suggests that, within the context of an intranasal administration protocol, social habituation-dishabituation test can probably highlight only detrimental effects as a consequence of the treatment (Huang et al., 2014) while it could be unreliable for possible positive effects.

This leads us to the question of how OXT goes about mediating these social behavioural effects. It is not clear whether OXT is exerting its prosocial effects by influencing relatively broad states and orientations (e.g., general anxiety, affiliative motivation, global saliency of social cues) or by interacting with specialised higher-order social cognitive processes (e.g., trust, generosity, suspiciousness, mentalising) (Churchland and Winkielman, 2012).

According to molecular results (autoradiography) is noticeably observable that *DYS* mutant mice chronically administered with intranasal oxytocin do not show a downregulation in oxytocin receptor expression. That result correlates with our results where, in fact, improvement of social performance

is observed just in mutant mice and with behavioural results already observed (Huang et al., 2014) on C57BL/6j where a chronic administration of intranasal oxytocin in males showed a detrimental effect in males. Noticeably in this study chronic administration in females did not show the decrease of sociability previously observed in the same test in males suggesting some sex differences in oxytocin action in male and in female. This data is coherent with different roles of oxytocin in males and in females and with literature that observed different oxytocin and oxytocin receptor expression in males and females in pre-natal period (Tamborski, Mintz, & Caldwell, 2016), and sex specific behavioural effects in adult mice (Steinman et al., 2015).

Finally, involvement of dysbindin-1 reduction seems to trigger some molecular mechanism that conditions responsivity to oxytocin. Indeed while pro-social effects were really effective both in acute and in chronic in our female and male DYS mutant mice, in wild type either no effect or a detrimental effect was observed (Huang et al., 2014) while other groups in different model of social withdrawal both genetic (K L Bales et al., 2014) or stress induced (Steinman et al., 2015) failed to rescue the phenotype. This oxytocin intranasal treatment has a great therapeutic potential for social deficits triggered by specific causes as dysbindin reduction, and probably other that still need to be explored. This molecule should be collocated in a paradigm of personalized-therapy, and the limits of applicability of this therapy may take advantage of the mouse models relevant for psychiatric disease. Moreover, its inefficacy or detrimental effects in unaltered system prevents the abuse of this molecule in a recreational context.

# Chapter 3

## Intranasal administration of the CRF-BP inhibitor CRF(6-33) selectively modulates impulsive behavior

Giuliano Gentili<sup>1</sup>, Simone Guadagna<sup>1</sup>, Francesco Papaleo<sup>2</sup>

<sup>1</sup> Dipartimento di Scienze del Farmaco, Università degli Studi di Padova, Largo Meneghetti, 2, 35131 Padova, Italy.

<sup>2</sup> Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Via Morego, 30, 16163 Genova, Italy.

### Abstract

CRF is a 41 amino acid neuropeptide involved in the stress response. In patients with Alzheimer's disease, a greater cognitive impairment was associated with lower CRF concentrations in the CSF. Moreover, preliminary evidence suggests that ICV administration of CRF might improve cognitive functions in rats. This might suggest a potential use of CRF as a cognitive enhancer. This positive effect, however, could be prevented by the remarkable anxiogenic effects driven by the CRF.

To overcome these limitations, a possible strategy could be to avoid the alteration of CRF levels in brain areas responsible for the anxiogenous-related effects. CRF brain levels are regulated by the CRF binding protein (CRFbp), which shows a region-specific pattern of expression. CRFbp is expressed in the cortex and hippocampus of both humans and rodents. Interestingly, rats administrated ICV with the CRF(6-33), a peptide inhibiting CRF and CRFbp binding, showed improved cognitive functions without exhibiting anxiogenic effects.

To demonstrate the effectiveness of CRF(6-33) by intranasal administration we first tested C57BL/J naïve mice in a modified five Choice Serial Reaction Time Task (5-CSRTT) paradigm. Our results show selective behavioral effects after intranasal administration of CRF(6-33), particularly a reduction of impulsive behaviour. We also found that while acute administration produces some cognitive improvements, chronic administration might be detrimental. Furthermore, intranasal CRF BP was able to change the expression of key players of the CRF-system such as CRF1 receptor in hippocampus and CRFbp in PFC.

Finally, we tested the effects of intranasal CRFBP in genetically modified mice relevant to schizophrenia. We selected mice mutated for dysbindin-1 an D2L as they are two leading susceptibility genes in schizophrenia both involved in dopamine signalling and we had initial evidence that these genetic mutations might increase impulsive behaviours. We confirmed that these mice have an increased impulsive behaviour. However acute administration of CRF(6-33) was not able to

rescue it. Moreover, these preliminary data are showing an interesting trend of exacerbation of the impulsive phenotype in Dys +/- mice suggesting a negative interaction of CRF(6-33) with the genotype.

These results indicate that intranasal CRF(6-33) is able to modulate impulsive behaviours and that these effects might change depending on the genetic background.

### **3.1 Introduction**

#### **3.1.1 Cognitive deficits in schizophrenia**

Cognitive deficits are a core and enduring feature of schizophrenia. These deficits are relatively stable over time across changes in clinical state (Harvey & Bowie, 2003; Nielsen, 2011; Sharma & Antonova, 2003), tend to persist throughout the course of the illness (Heaton et al., 2001) and have high correlation with the functional outcome of patients (Sharma and Antonova, 2003; Carter et al., 2008; Green et al., 2004). Cognitive impairments are wide-spread and severe, affecting various cognitive domains of speed of processing, attention/ vigilance, working memory, verbal learning, visual learning, reasoning, problem solving and social cognition (Keith H Nuechterlein et al., 2008). Among these affected domains, abnormalities in attention have been considered to be fundamental because the lack of selection of relevant information may be a cause of impairment in the other domains (Gold & Thaker, 2002; Zvyagintsev, Parisi, Checkko, Nikolaev, & Mathiak, 2013). Broadly speaking, attention is the ability to select a subset of the available information for preferential processing, while ignoring competing information (Smid, Martens, de Witte, & Bruggeman, 2013). There are different forms of attentional processes that can be measured with different tasks. In general, tasks in which attention is directed to the same stimulus on all stimulus presentations (i.e. on the basis of instructions) engage sustained selective attention, while tasks in which attention is directed to different stimuli on different stimulus presentations (i.e. on the basis of pre-cues and sometimes referred to as 'transient attention') enlist selective attention switching (Smid, de Witte, Homminga, & van den Bosch, 2006; Smid et al., 2013). Sustaining and switching of attention can operate in the visual-spatial and the object domain of selection (Smid et al., 2013). For example, if during a number of sequential stimulus presentations in a task, one is required to focus one's attention always on the same location in the visual field while ignoring other stimulated locations, the task is a sustained attention task in the visual-spatial domain (Smid et al., 2013). On the other hand, if between stimulus presentations in a task, one's focus of attention has to be switched from one location to another (on the basis of cues), it is a spatial attention switching task (Smid et al., 2013). Mounting evidence suggests that patients with schizophrenia have an intact implementation of attention (i.e. sustained attention) while being impaired in the control of selection (i.e. switching the focus of attention) (Smid et al., 2013) and broad monitoring (Hahn et al., 2012).



### 3.1.2 CRF system as a pharmacological target for cognitive modulation

Corticotropin Releasing Factor (CRF) is a 41 amino acid neuropeptide involved in stress responses (Mitchell, 1998).

Involvement of this molecule in cognitive functions was initially studied in patients affected by Alzheimer's disease where a greater cognitive impairment was associated with lower CRF concentrations in CSF (De Souza, Whitehouse, Kuhar, Price, & Vale, 1986; Pomara et al., 1989).

Moreover this peptide has been strongly investigated as a neuromodulator able to improve cognitive functions in rodent models by ICV administrations (Behan et al., 1995; Eckart et al., 1999; S C Heinrichs et al., 1997; Stephen C Heinrichs & Koob, 2004; Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003; Koob & Bloom, 1985; Lee et al., 1993; Lee & Sung, 1989; Thompson et al., 2004)

These evidences might suggest a potential use of CRF as a cognitive enhancer. However, this would be prevented by its remarkable anxiogenic effects (Arborelius, Owens, Plotsky, & Nemeroff, 1999; Behan et al., 1995; Contarino, Heinrichs, & Gold, 1999; Mitchell, 1998; Takahashi, 2001).

To overcome these limitations, a possible strategy could be to avoid the manipulation of CRF levels in brain areas responsible for the anxiogenous-related effects.

In this context, our attention moved to another molecule belonging to the CRF system, CRF binding protein (CRFbp). CRFbp regulates CRF brain levels acting as a sponge for CRF and limiting the quantity of free CRF (Jahn et al., 2005). This protein shows a region-specific pattern of expression and is specifically expressed in the cortex and hippocampus of both humans and rodents (Timofeeva, Deshaies, Picard, & Richard, 1999).

To target this protein we decided to use the synthetic peptide CRF(6-33), being it a subpart of the original CRF molecule that keeps the ability to bind CRFbp with same strength of CRF, but which lost its ability to bind CRF receptors (Sutton et al., 1995). Administration of this molecule aims in fact to dissociate part of CRF from its binding protein, thus increasing the free CRF available just in the areas of brain where CRFbp is expressed.

Interestingly, rats administrated ICV with CRF(6-33) showed improved cognitive functions, without showing anxiogenic effects (Stephen C. Heinrichs & Joppa, 2001).

The initial interest for CRF(6-33) was focused on the development of possible treatments for cognitive impairments associated with Alzheimer's disease (Behan et al., 1995). However, the combination of administration route (ICV) and presence of neurodegeneration in CRFergic neurons (Bissette, Reynolds, Kiltz, Widerlöv, & Nemeroff, 1985) makes this kind of therapeutic strategy not suitable for the treatment of Alzheimer's disease.

Nevertheless, there are many psychiatric diseases that are associated with cognitive impairment, included schizophrenia (O'Carroll, 2000), where this impairment is not concomitant with neurodegeneration.

The aim of this study is to test the potential of CRF(6-33) intranasal administration as a treatment for cognitive deficits associated with psychiatric disorders. In a clinically-relevant perspective, we aimed at investigating whether the intranasal route of administration could be an effective way to apply these drugs. To try to achieve this purpose, we firstly tested the activity of CRF(6-33) in C57BL/6j mice that are a classical standard genetic background strain for genetically-modified mice and animal models in neuroscience. Secondly we tested this intranasal CRF(6-33) in a genetically-modified mouse model relevant for schizophrenia.

The test used for assessing cognitive performance is a modified version of the 5-choice serial reaction time task. This test allowed us to perform many manipulations and to asses different aspects of cognitive performance.

### **3.1.3 5-Choice serial reaction time task, a test for assessment of cognitive performance in rodents**

In the clinic, the Continuous Performance Test (CPT), a visual vigilance task, has been used extensively as a measure of sustained/selective attention deficits in schizophrenic patients (Cornblatt, Risch, Faris, Friedman, & Erlenmeyer-Kimling, 1988; Gold & Thaker, 2002; Keith H Nuechterlein & Dawson, 1984). Briefly, in CPT tasks, subjects are presented with series of stimuli (e.g. letters or auditory tones) and are required to respond only to an infrequent target stimulus (typically 10 to 20% of the total stimulus presentations) over a period of 3 - 10 minutes. Hence, subjects are required to sustain an attentive state over a period of time. During this period, subjects are monitoring for a single target letter (e.g. C) or a target sequence of letters (e.g. A, B, C) while variants of this task may present other types of targets (Gold & Thaker, 2002; K H Nuechterlein & Dawson, 1984). The visuospatial selective attention domain has been studied extensively in schizophrenic patients. The Spatial Attentional Resource Allocation Task (SARAT) has been described and validated as a tool for manipulating the size of the attentional focus in space (Hahn, Ross, & Stein, 2006). Briefly, participants were required to keep their eyes fixated on a central circle containing a fixation cross and to detect a target signal (500 ms) that could occur at any of four peripheral locations marked by empty circles (Figure 11) Cues were displayed in the central circle for a variable stimulus-onset-asynchrony (SOA) of 400, 700, 1000 or 1300 ms prior to target onset and remained on display until 500 ms after target termination. These cues consisted of quarters of the fixation circle turning black with their location, indicating the probable location of the peripheral target. One, two, three or four quarters could turn black simultaneously. Hence, predictability of the target location is varied across trials. Targets consisted of a peripheral circle filling with a checkerboard of grey and white squares of 3x3 pixels each. Upon occurrence of a target, subjects were instructed to press a button with their right index finger as

quickly as possible. The temporal unpredictability of targets required subjects to continuously allocate attention to cued locations in anticipation of a target.(Hahn et al., 2012, 2006).

## SARAT

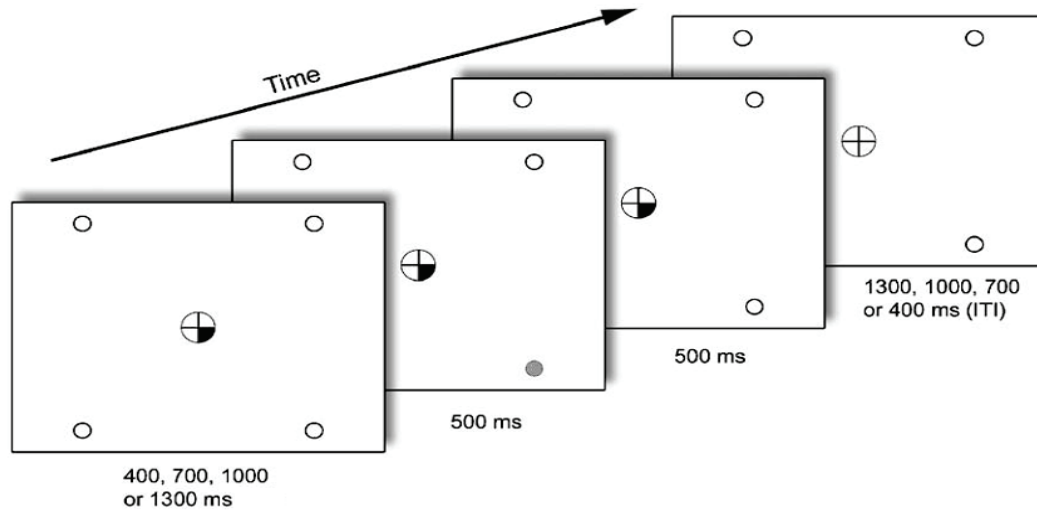
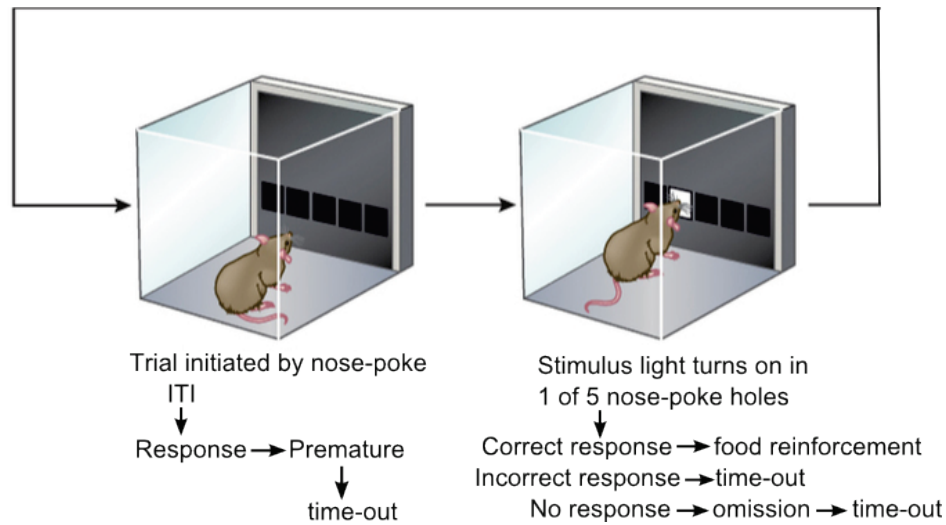


Figure 11 **Components of a single target trial in the SARAT.** Onset of a central cue preceded target onset by a variable SOA of 400, 700, 1000 or 1300 ms. The target was presented for 500 ms in the continuing presence of the cue, which remained on display until 500 ms after target offset. Only screen background was then presented for an inter-trial interval (iti) that varied in length such that total trial duration was always 2700 ms. No-target trials differed only in that no target occurred. One, two, three or all four target locations could be cued at the same time, thus varying the predictability of the target. [From (Hahn et al., 2006).]

The 5-CSRTT has been used for measuring effects of drugs and other manipulations on various aspects of attentional control over performance in rodents. The primary outputs measured in this test are: correct responses, accuracy, premature responses, perseverative responses and correct response latencies (Robbins, 2002). It has been widely used in studies of cognitive disruption with relevance to numerous clinical disorders such as attention deficit/hyperactivity disorder (ADHD) (Barbelivien, Ruotsalainen, & Sirviö, 2001; Davies et al., 2009; Navarra et al., 2008), Alzheimer's disease (Balducci, Nurra, Pietropoli, Samanin, & Carli, 2003; Lehmann, Grottick, Cassel, & Higgins, 2003), Parkinson's disease (Walitza et al., 2007), schizophrenia (Amitai & Markou, 2009; Amitai, Semenova, & Markou, 2007; Carli, Calcagno, Mainini, Arnt, & Invernizzi, 2011; Carli, Calcagno, Mainolfi, Mainini, & Invernizzi, 2011; Le Pen, Grottick, Higgins, & Moreau, 2003; Murphy, Dalley, & Robbins, 2005; Paine & Carlezon, 2009), among others. This task has been previously described by Robbins and coworkers (Robbins, 2002). Succinctly, the 5-CSRTT is implemented in a specially designed operant chamber with multiple target locations on a front wall allowing the measurement of possible nose-poke responses through

the use of infra-red beams placed inside the nose-poke targets. Food pellets were presented as food reinforcements delivered from a magazine at the rear of the chamber.



**Figure 12 The five-choice serial reaction time task (5-CSRTT).** Top: Representation of apparatus consisting of: a chamber with a rear magazine equipped with a pellet dispenser and a mechanism for detecting head entries (nose-pokes) into that magazine, an array of five equally spaced holes, each equipped with a light bulb and an infrared nose-poke detector on 1 wall in the front of the chamber. Bottom: possible trial sequences in the task. Subjects initiate trials by responding to the rear magazine; after an inter-trial delay (iti), a brief stimulus is presented in one of the five holes. Subjects must respond to that hole (correct response) within a certain time to be rewarded with a food reinforcement. If they respond to the wrong hole (incorrect response), respond before the stimulus is presented (premature response), or fail to respond (omission), they are subjected to a time-out (house-light turned off) before the next trial begins. [Modified from (Nithianantharajah et al., 2012).]

Some advantages of using the 5-CSRTT task include a low within- and between group subjects variance and although the performance generally reaches high levels (e.g. >80% accuracy; >80% correct responses), this is not at ceiling and it is still possible theoretically to detect improvements (Robbins, 2002). Moreover, the stable baselines of performance obtained with the 5-CSRTT have made it a useful test for the repeated assessment of effects of systemically administered drugs in groups of well-trained rodents, especially as performance often returns to baseline quite rapidly (Robbins, 2002). Another important advantage of the 5-CSRTT is that it is capable of measuring several different types of performance that include aspects of attention, impulse control, processing speed, and cognitive flexibility (Amitai & Markou, 2011; Robbins, 2002). This is done by devising different test procedures and configuring the test chamber in different ways which can increase the versatility of 5-CSRTT by selectively enhancing the cognitive load on different aspects of the task (Amitai & Markou, 2011; Robbins, 2002). In spite of the many advantages, there are a few limitations to this task. For example, this test usually requires long training periods and food restriction before starting the training. Imposed food-restriction has been found to introduce additional stress to animals (Ilott et al., 2014). Therefore, we set out to implement a modified training protocol that run three sessions during the night (when the mice are more active) on the 5-CSRTT apparatus that would

speed up the training process as compared to conventional 5-CSRTT protocols that run only 1 session a day with food restriction. Moreover, our new protocol does not require the mice to be food restricted before starting the training, as once they have learnt the task, they are able to eat enough during the training/test sessions to be maintained at equal to or more than their baseline weight. This has the advantage of decreasing the amount of stress on the mice through removal of the need to perform food restriction. Through communication with the TestDiet company (Tes), it has been assured that the food reward pellets (5TUL Purified rodent tablet, TestDiet) are a complete diet for rodents. For these reasons, we have chosen to use the 5-CSRTT as one of the cognitive tests in our lab to evaluate cognitive function in mice that are genetically modified for schizophrenia susceptibility genes.

#### **3.1.4 Dysbindin 1 and Dopamine D2 receptor interaction as a mouse model relevant to schizophrenia**

Dysbindin-1 (Dys) and the dopamine D2 receptor (D2) genes are two important candidate risk genes for schizophrenia. In particular, they are both involved in the regulation of dopamine signalling which is centrally implicated in the pathophysiology and treatment of schizophrenia. It has been established, in fact, that genetic mutations reducing Dys expression lead to an up-regulation of D2 receptors on the neuronal cell surface, thus, producing a D2-hyperdopaminergic functional state and a pattern of abnormal excitability in PFC circuits (Iizuka et al., 2007; Papaleo & Weinberger, 2011; Papaleo, Yang, et al., 2012). In schizophrenia, a decreased D1/D2 activation ratio due to excessive D2 signalling may induce alterations of cortical network excitability, leading to abnormal representation of external and internal stimuli and hence, to psychiatric symptoms and cognitive disabilities (Winterer & Weinberger, 2004). The D2 gene produces two different isoforms: the long (D2L) and the short form (D2S). These two isoforms differentially contribute to dopamine signalling in the PFC and striatum (Usiello et al., 2000). Moreover, in humans, polymorphisms in the D2 gene have been reported to affect its splicing, resulting in an altered expression of D2L versus D2S (Zhang et al., 2007). Notably, recent results indicate that these functional genetic variants in the D2 gene are differently expressed in patients with schizophrenia (Kaalund et al., 2013) and might modulate schizophrenia-related phenotypes by modifying D2S/D2L ratio (Bertolino et al., 2009).

In light of these results, our hypothesis is that synergistic effects of a reduced dys expression and an increased D2S/D2L ratio in the same subject might cause an excessive alteration in the dopamine/D2 signalling able to trigger cognitive deficits and psychotic symptoms. Using genetically-modified mice for D2L and dysbindin-1, a combined approach is employed to dissect the behavioral and functional alterations relevant to schizophrenia in these mouse models. Emphasis has been placed on cognitive deficits as these are core enduring symptoms in schizophrenia which dramatically contribute to poor functional outcomes in patients (Papaleo, Lipska, et al., 2012; Young, Powell, Risbrough, Marston, & Geyer, 2009).

Another main aim of this project was to assess the potential efficacy of CRF(6-33) in reverting cognitive deficits observed after DYS-D2L reduction in mice. We performed these tests with the CRF(6-33) in the 5-CSRTT manipulations SARAT and Impulsivity test.

## 3.2 Materials and Methods

### 3.2.1 Subjects

All procedures were approved by the Italian Ministry of Health (permit n. 230/2009-B) and strictly adhere to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in U.S.. C57BL/6J male mice between 12 to 20 weeks of age used in this study were obtained from Charles River Laboratories (France). Animals were housed two to four per cage, in a climate-controlled animal facility ( $22\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ) and maintained on a 12-hour light/dark cycle, with food and water available ad libitum throughout the experiments. All behavioral testing and procedures were conducted during the light phase of the cycle. The experimenter handled the mice on alternate days during the week preceding the first behavioral test. Experimenters were blind to the mouse genotypes and treatments during testing and behavioral scoring.

### 3.2.2 Intranasal CRF(6-33) administration

CRF(6-33) (Tocris Bioscience, Bristol, UK) was dissolved in distilled water and administered intranasally in a volume of  $5\text{ }\mu\text{l}$  to each mouse in dose of  $20\text{ }\mu\text{g}/5\text{ }\mu\text{l}$  ( $4\text{ mg/ml}$ ),  $10\text{ }\mu\text{g}/5\text{ }\mu\text{l}$  ( $2\text{ mg/ml}$ ) and  $2,5\text{ }\mu\text{g}/5\text{ }\mu\text{l}$  ( $0.5\text{ mg/ml}$ ) that corresponded respectively to  $\approx 6.21 \times 10^{-09}$ ,  $\approx 3.10 \times 10^{-09}$  and  $\approx 7.76 \times 10^{-10}$  mol CRF(6-33), for each administration of  $5\text{ }\mu\text{l}$  (CRF(6-33)  $20\text{ }\mu\text{g} \approx 526\text{ }\mu\text{g/kg}$ , CRF(6-33)  $10\text{ }\mu\text{g} \approx 263\text{ }\mu\text{g/kg}$ , CRF(6-33)  $2,5\text{ }\mu\text{g} \approx 65,8\text{ }\mu\text{g/kg}$ ). The dose of  $10\text{ }\mu\text{g}/5\text{ }\mu\text{l}$  was initially chosen in order to be one of the highest possible as there was no literature data on intranasal bioavailability of CRF(6-33). Tocris datasheet guaranteed  $1\text{ mg/ml}$  solubility that was also higher than the concentration used in literature by ICV administration (S C Heinrichs et al., 2001; Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003). We verified initially that the solubilization in water of CRF(6-33)  $2\text{ mg/ml}$  was possible. That concentration permitted, for  $5\text{ }\mu\text{l}$  administration (the optimal volume for adult mice (Huang et al., 2014)), an administration of a dose about 4 times higher than the one used in rats (normalized for body weight) and about 20 times lower than the common ICV dose used in the literature (S C Heinrichs et al., 2001; Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003).

A  $20\text{ }\mu\text{l}$  Eppendorf pipette with gel-loading tips (Costar no. 4853) were used for administration. Drops of the  $5\text{ }\mu\text{l}$  solution were gently placed equally on both nostrils of each mouse, and from there they were inhaled reflexively. Control mice received the same volume of distilled water (veh). Administration was rapid (less than 30 seconds) and handling was consistent across treatment groups. The detailed timelines of each procedural manipulation and CRF(6-33) treatments are reported in

Figure 13. Briefly, for the chronic intranasal treatments, mice were administered once a day at 6.15 PM, 45 minutes before first night session of test for 21 consecutive days and tested as described below, one hour after the last administration. For the acute intranasal treatments, mice were administered only once, 45 minutes before the first session of test. The acute test was performed in a latin square design with 6 days of washout between each treatment and the following one. The delay of 45 minutes was chosen based on evidence indicating that intranasal administration of peptides has usual peak of concentration between 10-80 minutes, with peaks of concentrations for bigger peptides as insulin between 20 and 40 minutes (Born et al., 2002). For the CRF(6-33) we expected a pharmacokinetic much slower than for oxytocin due to the bigger molecular size and due to the peculiar mod of action of CRF(6-33). The CRF(6-33), indeed, shows an indirect agonist effect due to displacement of the peptide from a binding protein.

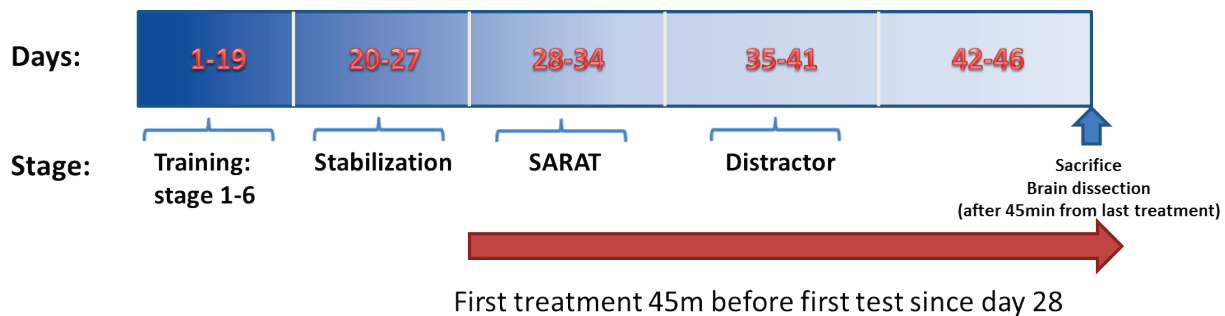


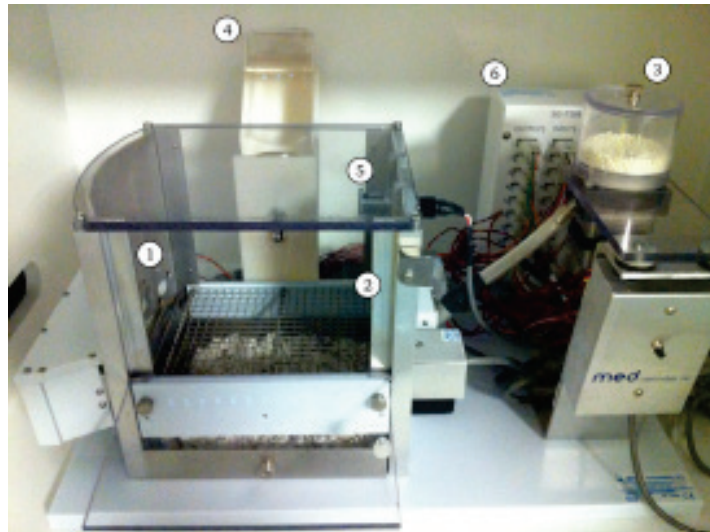
Figure 13 Timeline of treatment and manipulations for 5-CSRTT.

### 3.2.3 5-CSRTT

#### Apparatus

Training and testing was conducted in 12 operant chambers (ENV-307A; Med Associates, St. Albans, VT, USA), housed in sound-attenuating boxes each containing a fan, which provided ventilation and a constant low background noise. 2 strings of LED lights (1 providing warm light and the other cool light) were installed onto the ceiling of each of the sound-attenuating box controlled by a timer so that the 12-hour light/dark cycle can be regulated within each box. Each operant chamber contains, on 1 wall, 5 nose-poke holes (1 cm in diameter; ENV-115C-A) that are each outfitted with a recessed LED stimulus light (ENV-313M). 2 additional LED cue lights (red and green) are installed above each of the 5 nose-poke holes. Nose-pokes were detected by an infrared beam transecting the aperture of each hole. On the wall opposite the 5-hole array, a food magazine with an infrared beam (ENV-303M) and a head entry detector (ENV-303HDA) where food reinforcement in the form of a food pellet (5TUL Purified rodent tablet, TestDiet) was delivered by a pellet dispenser (ENV-203-14P). A water dispenser had been built into each operant chamber to ensure that the mice have access to water over the night during the training/test sessions. A house-light (ENV-315M) was located 7 cm above the food

magazine. The operant chambers are connected to a Smart Control Panel (SG-716B) and interfaced to a Windows computer equipped with a MED-PC IV software (Med Associates, St. Albans, VT, USA).



**Figure 14 5-CSRTT apparatus** 1. Wall with 5 nose-poke holes, each outfitted with a recessed LED stimulus light and 2 additional LED cue lights (red and green) above each of the 5 nose-poke holes. 2. Food magazine on wall opposite 5-hole array. 3. Food pellet dispenser. 4. Water dispenser. 5. House-light. 6. Smart Control Panel.

## Habituation

Mice were handled on alternate days during the week preceding the start of 5-CSRTT training (3 handling sessions; 1 min per mouse per session). During these handling sessions, the mice were weighed to obtain a baseline of their ad libitum body weight. No food restriction was imposed on the mice before training. After each of the handling sessions, each mouse was also presented with 10 pellets so that they were habituated to the food reinforcement. Throughout training and the test variations after, mice were placed into the operant chambers in the evening between 5 - 6 pm and taken out of the chambers the following morning between 10 - 10:30 am every day and placed into regular holding cages (in cages of 4 mice). When the animals were taken out of the operant chambers in the morning, they were weighed to check that their body weights were maintained above 90% of their ad libitum body weight. No food was given to the mice during the day when they were in the regular holding cages. The food reward pellets are appropriate as a complete diet for the animals. However, if any of the mice were not doing well enough in the training sessions and were losing weight (<85% of their ad libitum body weight), an appropriate amount of regular food pellets were given to them when they were placed in their regular holding cages in the morning.



## Training protocol

Each night, mice were presented with 3 sessions spread out over the dark phase (8 pm - 8 am).

The general protocol of each session is as follows:

A free reinforcement pellet is issued at the start of each session. When a head entry to retrieve the free reinforcement pellet has been detected, the first trial will begin with an inter-trial interval (ITI). Any nose-pokes during the ITI will be recorded as premature responses and will result in a time-out (default 5s) with the house-light turned on. At the end of the time-out, the house-light will be turned back off and the ITI will restart. Any nose-pokes during the time-out will reset the time-out.

At the end of the ITI, the program will randomly select a stimulus location (1 out of 5 stimulus lights) and turn on the corresponding stimulus light. The stimulus light will remain on for the stimulus duration (SD) value set. The animal has the limited hold (LH) time to nose-poke into the lit hole. If the animal nose-pokes into the correct lit hole before the LH time runs out, this is recorded as a correct response, the first response to stimulus will be recorded, the stimulus light will be turned off if not turned off earlier, the correct response latency will be recorded, and the pellet will be issued. The animal must now do a head entry to retrieve the pellet. Any nose-pokes before retrieving the reward will be recorded as perseverative responses.

If the animal responds to one of the nose-poke holes that is not lit, this is recorded as an incorrect response, the first response to stimulus will be recorded, the stimulus light will be turned off if not turned off earlier, the incorrect response latency will be recorded, and the house-light will be turned on for the time-out period. At the end of the time-out, the house-light will be turned back off and the ITI will start. Any nose-pokes during the time-out will reset the time-out.

If the animal does not nose-poke into any hole before the limited hold time runs out, this is recorded as an omission error, the first response to stimulus will be recorded as 0, the stimulus light will be turned off if not turned off earlier, the correct/incorrect response latency will be recorded as 0 (this value will not affect the average correct/incorrect response latencies), and the house-light will be turned on for the time-out period. At the end of the time-out, the house-light will be turned back off and the ITI will start. Any nose-pokes during the time-out will reset the time-out. If a pellet was issued, then when the head entry is detected, the program will record the latency to reward and then start timing the ITI. Any nose-pokes during the ITI will be recorded as premature responses and will result in a time-out with the house-light turned on. At the end of the time-out, the house-light will be turned back off and the ITI will restart. Any nose-pokes during the time-out will reset the time-out. After the ITI ends, the next trial will start immediately.

Training consists of 6 stages. To proceed to each subsequent stage, mice are required to reach the criterion for 2 consecutive sessions. Each stage is more challenging than the last, with the SD and LH

period decreasing while other criteria (such as the required number of correct trials) become more demanding. Each 30-minute session has a maximum limit of 100 trials.

1. Stage 1: SD=20 s; LH=30 s; ITI=2 s. Criteria:  $\geq 30$  correct trials;  $\geq 30\%$  correct.
2. Stage 2: SD=10 s; LH=30 s; ITI=2 s. Criteria:  $\geq 50$  correct trials;  $\geq 50\%$  correct.
3. Stage 3: SD=8 s; LH=20 s; ITI=5 s. Criteria:  $\geq 50$  correct trials;  $\geq 50\%$  correct
4. Stage 4: SD=4 s; LH=10 s; ITI=5 s. Criteria:  $\geq 50$  correct trials;  $\geq 80\%$  accuracy;  $\leq 50\%$  omission.
5. Stage 5: SD=2 s; LH=7 s; ITI=5 s. Criteria:  $\geq 50$  correct trials;  $\geq 80\%$  accuracy;  $\leq 40\%$  omission.
6. Stage 6: SD=1 s; LH=7 s; ITI=5 s. Criteria:  $\geq 50$  correct trials;  $\geq 80\%$  accuracy;  $\leq 40\%$  omission.

The following measures were recorded to assess task performance:

1. Percent choice accuracy: Number of correct responses divided by sum of number of correct and incorrect responses, multiplied by 100.
2. Percent correct responses: Number of correct responses divided by total number of trials, multiplied by 100.
3. Percent incorrect responses: Number of incorrect responses divided by total number of trials, multiplied by 100.
4. Percent omissions: Number of omissions divided by total number of trials, multiplied by 100.
5. Percent premature responses : Number of premature responses divided by sum of correct, incorrect, premature, perseverative and time-out responses (total number of responses), multiplied by 100.
6. Percent perseverative responses : Number of perseverative responses di-vided by total number of responses, multiplied by 100.
7. Number of time-out responses.
8. Average latency to a correct response: Total time from onset of light stimulus to the performance of a correct response divided by number of correct responses.
9. Average latency to collect a food reward: Total time from the performance of a correct response to the retrieval of the food reward from the food magazine divided by number of correct responses.

## **Stabilization**

Upon reaching stage 6, mice are subjected to 1 week of testing at Stage 6 with a maximum of 100 trials. After stable performance is achieved, mice are tested with a number of different test protocols.

SARAT

The mice are then submitted to two sessions of SARAT testing (with 3 different trial types randomly presented). Trial type 1 is the normal trial type as in Stage 6 where the stimulus light is turned on randomly in 1 of the 5 nose-poke holes. Trial type 2 is the same as trial type 1 with the addition of a red cue light over each stimulus light. In this trial type, the red cue light over the corresponding correct stimulus light is turned on from 1 s before to 1 s after the stimulus light duration. Trial type 3 is the same as trial type 1 with the addition of a red cue light over each stimulus light. In this trial type, the red cue lights over all five stimulus lights are turned on from 1 s before to 1 s after the stimulus cue duration. Any nose-pokes after the red LED cue light is lit, but before the stimulus light is presented, is considered a premature response and is not rewarded but results in a time-out (even if it was in the correct response hole). Each trial type is presented equally in a random fashion throughout each session

For chronic test experiments mice are treated since the first day of SARAT manipulation test, then chronically every day at 6.15 PM, 45 minutes before of the first session of testing. The mouse has to perform the SARAT protocol just the day 1 and the day 4 of test . Day 2,3, and 5,6 the mouse is tested in normal STAGE6 test.

For acute latin square test mice are treated at 6.15 PM (45 minutes before of first session of testing) just the day of the test . Mice are divided in 4 groups and they receive one of the 3 doses of CRF(6-33) or vehicle according to scheme of Table 1a. After 6 days of washout (when mouse is tested in normal STAGE6) the mouse is treated as before 45 minutes before the test and receives a different dose of CRF(6-33). This cyle is repeated 4 times, and each time with a different dose.

a

Days of test				
	DAY 1	DAY 2	DAY 3	DAY 4
Group1	VEH	Dose 0.5	Dose 2.0	Dose 4.0
Group2	Dose 0.5	Dose 2.0	Dose 4.0	VEH
Group3	Dose 2.0	Dose 4.0	VEH	Dose 0.5
Group4	Dose 4.0	VEH	Dose 0.5	Dose 2.0

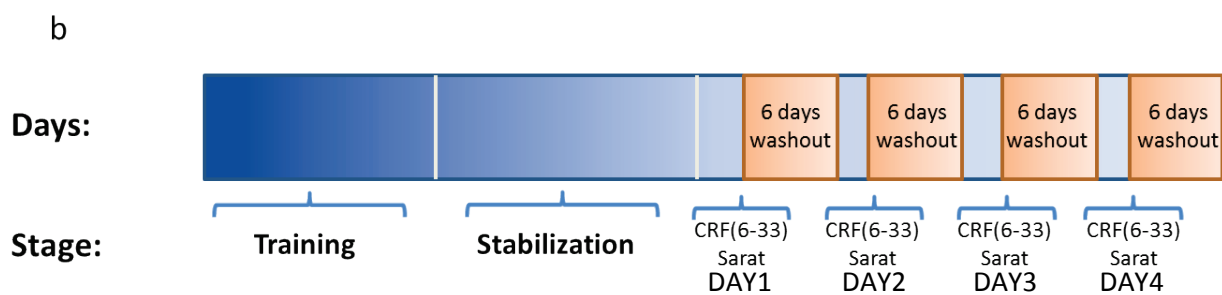
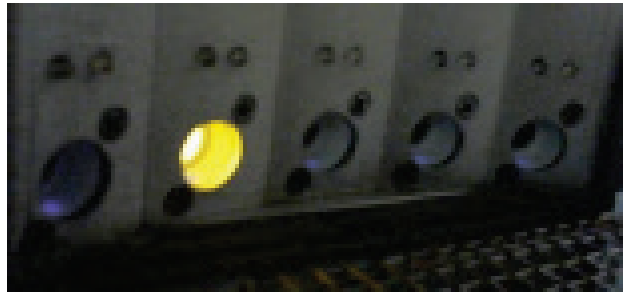


Table 1: Figure 6: Latin square scheme of treatment (a) and experimental timeline (b)

Trial Type 1



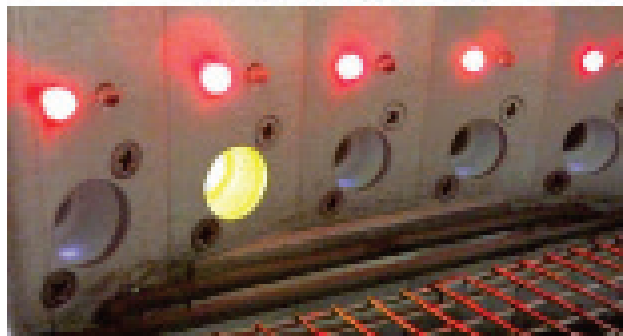
A stimulus light turns on randomly in 1 of 5 spatial locations (nose-poke holes).

Trial Type 2



A red LED cue light (randomly in 1 out of 5) will turn on 1 sec before the corresponding stimulus light turns on.

Trial Type 3



All 5 red LED cue lights will turn on 1 sec before a stimulus light turns on randomly in 1 of the 5 nose-poke holes.

Figure 15 SARAT protocol for mice

### Distractor test

This test was presented only in chronic treatment.

Subsequently to SARAT testing after 2 days of STAGE6 protocol mice are submitted to Distractor testing (with 2 different trial types randomly presented) (day 7, and day 10). In trial type 1 here the red cue light over the corresponding correct stimulus light is turned on from 1 s before to 1 s after the stimulus light duration. This trial type occurs 80% of the time. Trial type 2 occurs 20% of the time and is the same as trial type 1 with the addition of a green cue light over stimulus lights 1, 3, and 5. In this trial type, the green cue lights over the three stimulus lights are turned on flashing from 1 s before to 1 s after the stimulus cue duration. Any nose-poke occurring after the red/green LED cue lights are lit,

but before the stimulus light is presented, is considered a premature response and is not rewarded but results in a time-out (even if it was in the correct response hole). Mice are tested on Stage 6 protocol as normal on days 8, 9, and 11, 12 .

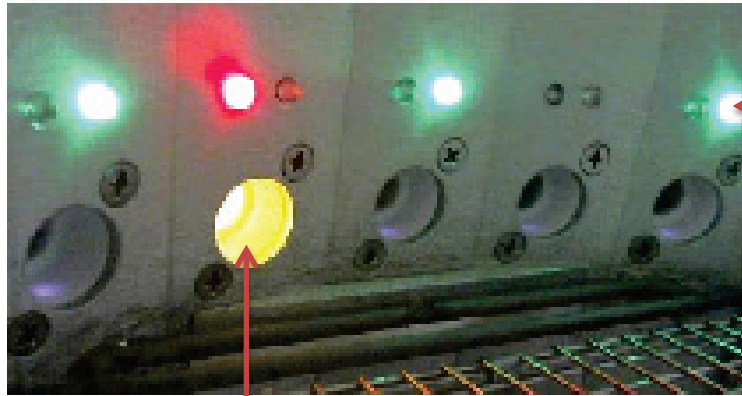
**Figure 16 Distractor protocol for mice**

**Trial Type1**



A red LED cue light (randomly in 1 out of 5) will turn on 1 sec before a corresponding stimulus light turns on

**Trial Type2**



3 green lights (DISTRACTORS) are flashing on and off 1 sec before to 1 sec after the stimulus light turns on

1 sec after the RED led cue light turns on (randomly in 1 out of 5), a corresponding stimulus light will turn on.

### Screening for impulsivity

The mice are then submitted to the impulsivity test (day 13 and day 16) .Mice are tested on Stage 6 as normal on days 14, 15 and 17.When the Impulsivity protocol is loaded the ITI is increased from 5 sec to 7 sec. This requires that mice now wait an additional 2 seconds between trials before the make a response. The SD and LH remain unchanged.

### 3.2.4 Molecular analyses

After 17 day of chronic treatment with 5µl of 2mg/ml CRF(6-33) mice have been administrated one last time 45 min before sacrifice and dissection of brain.

Olfactive bulbs, prefrontal cortex, striatum and hippocampus samples have been collected for each mouse and rapidly frozen in dry ice inside 1,5ml Eppendorf tubes, and kept in a freezer at -80°C until use.

#### **RNA extraction:**

RNA extraction was performed by GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) using the protocol provided by the producer. Briefly. Samples have been then measured at a Nanodrop 1000 (Thermo scientific) spectrometer for concentration and tested for eventual phenols contamination (260/230 absorbance). Samples with an absorbance of 260/230 lower than 1.7 were considered phenol contaminated and were precipitated again together with samples with a low concentration of RNA. RNA re-precipitation was performed adding 0.1 volume of 3M sodium acetate (ph5.2) and 2.2 volumes of ice cold ethanol 100%. After that, tubes have been kept for 20 minutes in freezer and centrifuged (14,000xg) for 20 minutes. Following this, the pellets of samples were washed with 500 µl of ice cold 70% ethanol, spinned for 5 minutes (12,000xg) and supernatant was discarded. The pellets have been then inverted on a towel 1 h to dry and were dissolved again in a appropriate volume of RNase free water. Concentration was then verified again at the Nanodrop.

#### **Retrotranscription:**

All samples have been retro transcribed by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) to a final concentration of about 45ng/2µl following the protocol of the kit producer.

#### **Real time PCR**

For real time PCR the following taqman probes (lifetechnologies) have been used :

Murine CRFr1 : Mm00432670\_m1

Murine CRFr2 : Mm00438303\_m1

Murine CRFbp : Mm01283832\_m1

HPRT (as internal reference) : Mm00446968\_m1

The probes have been used together with the TaqMan® Universal Master Mix II, with UNG (lifetechnologies, catalog number 4440042).

Each sample was tested in duplicate , according to producer protocol. Each assay used FAM functionalized taqman probes. Relative expression of samples treated with CRF(6-33) vs vehicle was tested using the  $2^{-\Delta\Delta Ct}$  method.

In this method it is defined a threshold of fluorescence, above the highest baseline value, in which amplification of samples is in exponential phase (linear doubling phase of amplification). According to this threshold definition, Ct (Threshold cycle) is the cycle number at which the fluorescence signal crosses threshold. That number inversely correlates with initial template concentrations.

With the method of  $2^{-\Delta\Delta Ct}$  it is possible to evaluate the relative expression of one gene in one group of samples (e.g. treated group) vs the same gene in another group (control group).

To calculate the  $2^{-\Delta\Delta Ct}$  first was calculated the  $\Delta C$  of each group, defined as difference between Ct value of target gene and Ct value of an internal control (that is supposed to be unchanged by the treatment)

$$\begin{aligned}\Delta Ct1 &= Ct (\text{Target A -treated}) - Ct (\text{Ref B-treated}) \\ \Delta Ct2 &= Ct (\text{Target A-control}) - Ct (\text{Ref B-control})\end{aligned}$$

Then it is performed difference between the two CT values  $\Delta\Delta Ct$

$$\Delta\Delta Ct = \Delta Ct1 (\text{treated}) - \Delta Ct2 (\text{control})$$

$2^{-\Delta\Delta Ct}$  gives an estimation of how the expression of a target gene varies in the treated group VS control (e.g.  $2^{-\Delta\Delta Ct} = 2$  means a doubling of expression in the treated group,  $2^{-\Delta\Delta Ct} = 0.5$  means half of expression).

Samples of PFC,hippocampus and olfactive bulbs have been tested for CRFr1, CRFr2 and CRFbp, while samples of striatum just for CRFr1. Results of CRFr2 from PFC and hippocampus have been discarded due to the low expression that led to a really high variability among both controls and treated samples.

### 3.2.5 Statistical analyses

Results are expressed as mean  $\pm$  standard error of the mean (sem) throughout all the experiments. One- or two-way analyses of variance (ANOVAs) were used for each single parameter measured. Newman-Keul's post-hoc test was used for making comparisons between groups when the ANOVA showed statistical significant differences for the main factors. The accepted value for significance was  $p \leq 0.05$ . All statistical analyses were performed using the software Statistica version 11 (Statistica, StatSoft, Inc.).

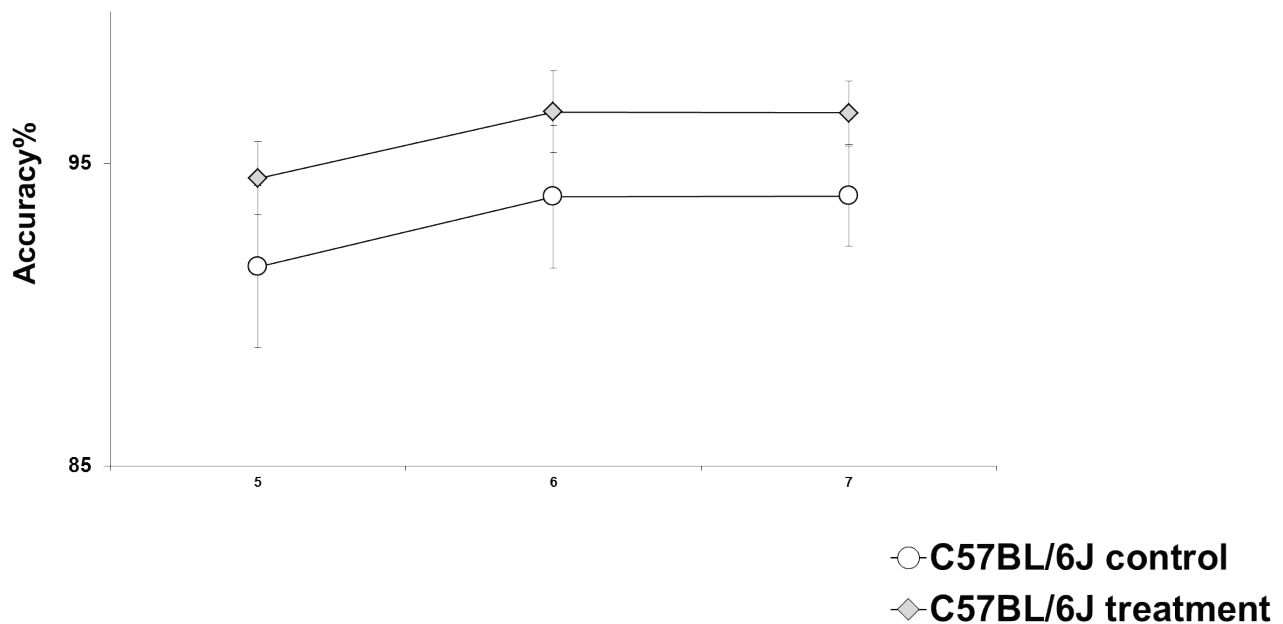


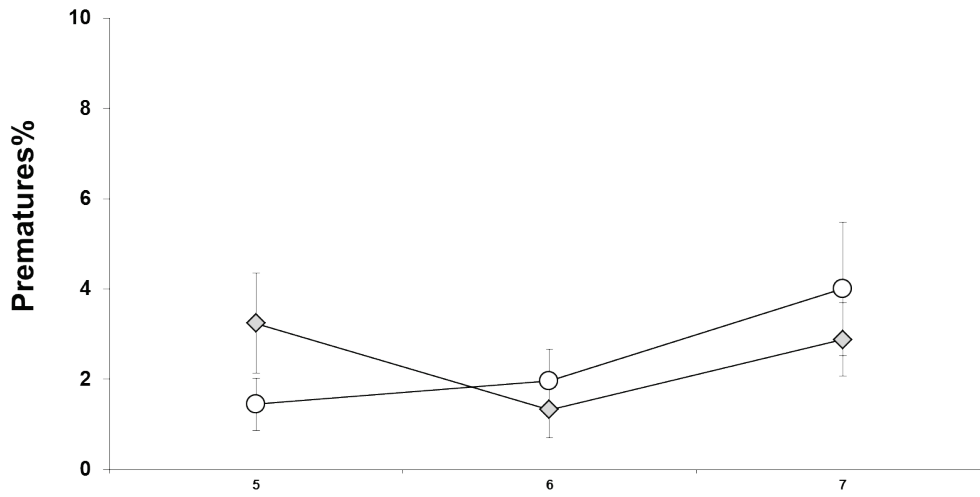
### 3.3 Results

#### 3.3.1 CRF (6-33) improves accuracy acutely and reduces impulsive response both acutely and chronically

To study cognitive performance and attention with the 5-CSRTT 12 C57BL/6j mice were initially recruited. After 21 days of training 3 mice have been excluded from the test due to their unreliable performance in poke pattern.

The remaining 9 mice received 7 additional days with normal training protocol (stage 6). In accordance to their performance of last 3 days of stabilization mice have been assigned to 2 balanced groups: 5 mice were assigned to treatment group, 4 to control group. As shown in **Figure 17** performance of two groups were comparable and there were no significant differences. In pictures is reported only accuracy and premature responses data in order to give a reference to following results, other parameters have been evaluated but none resulted significantly different in the two groups.

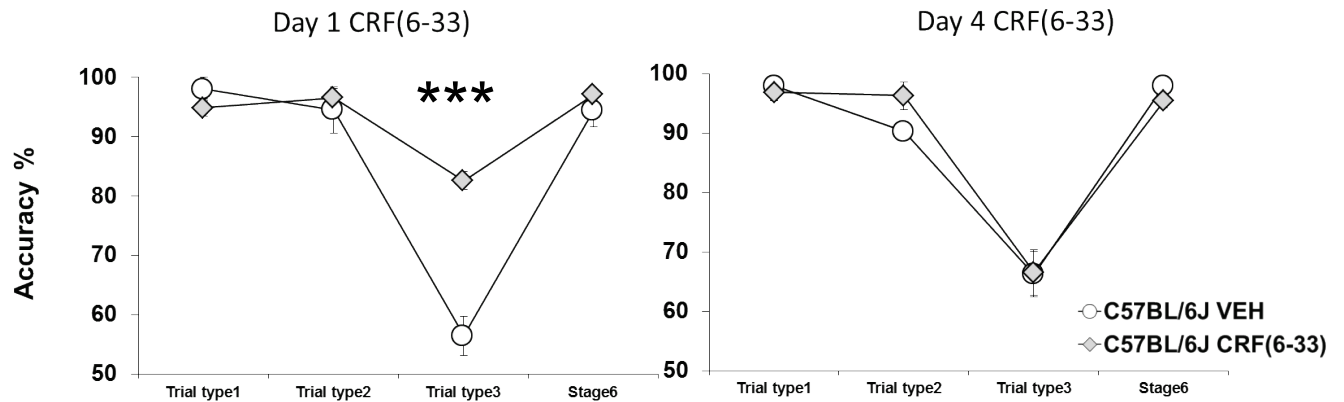




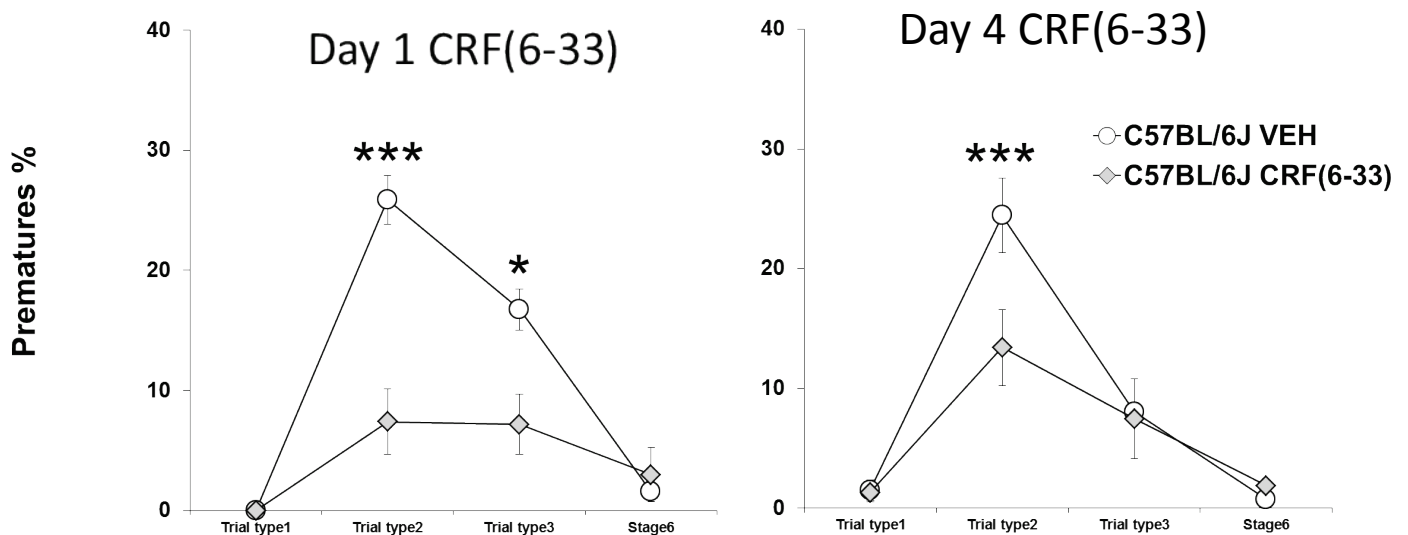
**Figure 17 Performance of treatment and control group last 3 days of stabilization.** None of the group has still received any treatment. Performance of the two groups are substantially similar. Treatment Group N=5, control group N=4

### 3.3.2 During SARAT manipulation CRF(6-33) improves accuracy and reduces impulsive responses

The first manipulation tested was the SARAT test. On the first day of intranasal administration mice showed a significant improvement of accuracy ( $p= 0.00014$ ), ascribable to an acute effect of administration. This effect in fact disappear after 4 days of chronic treatment.

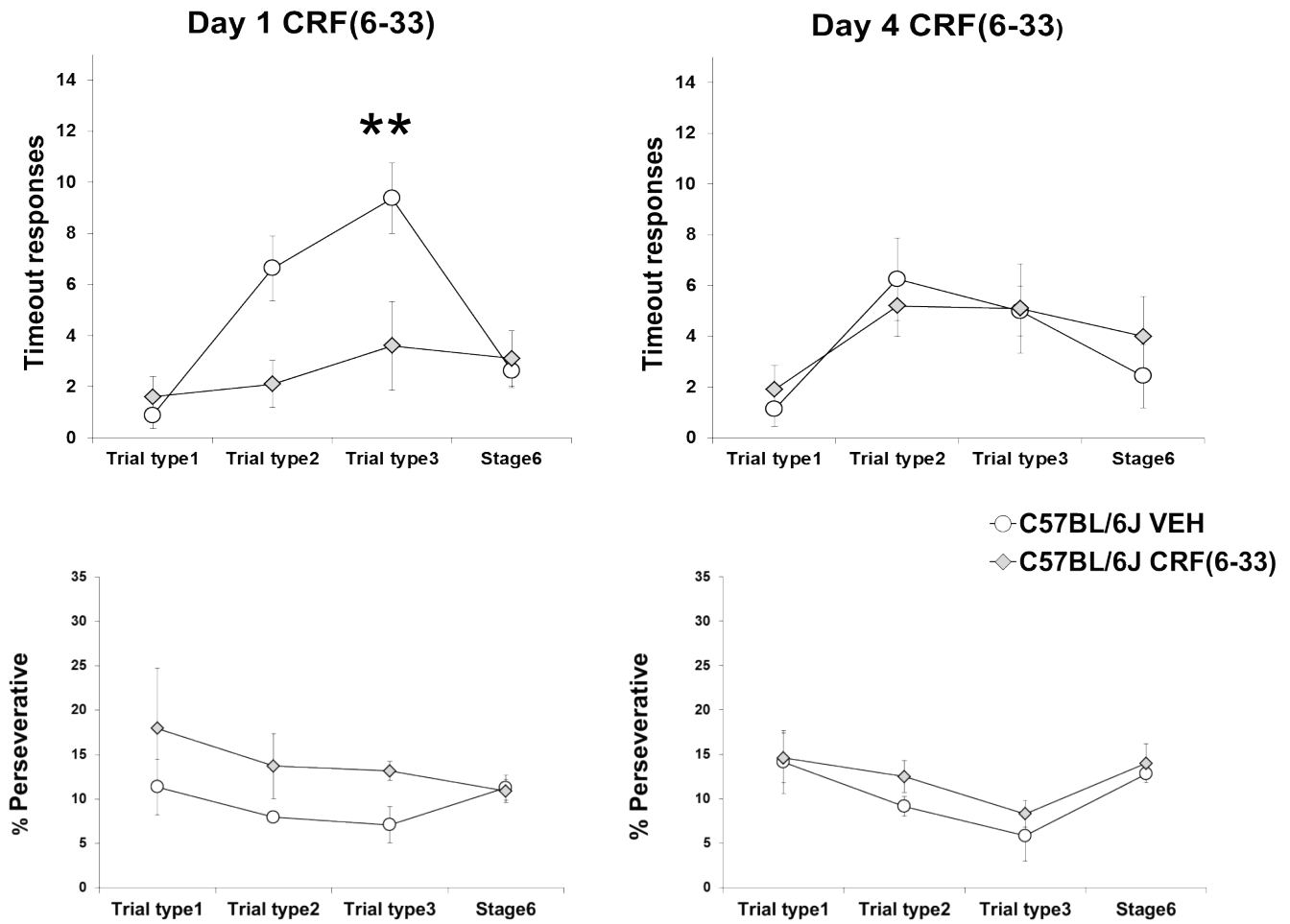


**Figure 18 Increased accuracy of response after acute administration of CRF(6-33) during SARAT manipulation.** The CRF(6-33) treated group (N=5) had a significant (\*\*\*)  $P<0.0005$  improvement of performance the first day of treatment VS the control group (N=4)



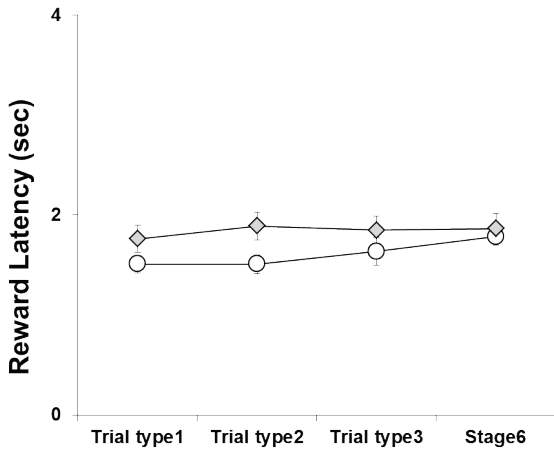
**Figure 19 Reduction of premature responses after acute administration of CRF(6-33) during SARAT manipulation.** The CRF(6-33) treated group (n=5) had a significant reduction of premature responses vs the control group (n=4) that is maintained after 4 days of treatment  
 \*\*\*  $P<0.0005$  , \*  $P<0.05$

Interestingly in this test was observed a significant reduction in premature responses both the first day of treatment ( $p= 0.00018$ ) and after 4 days of chronic treatment ( $p=0.0014$ ). Time out responses are significantly reduced in CRF(6-33) group only for acute administration. All the other parameters evaluated during this manipulation are unchanged in the 2 groups, confirming that the alterations that we see are specific for some cognitive features.



**Figure 20 Timeout responses and % of perseverative responses:**reduction of timeout responses after acute administration of CRF(6-33) during SARAT manipulation (\*\* P<0.005). Reduction of timeout responses was observed only acutely and disappeared the 4<sup>th</sup> day of administration. Ns: VEH=4, CRF(6-33) =5

Day 1 CRF(6-33)



Day 4 CRF(6-33)

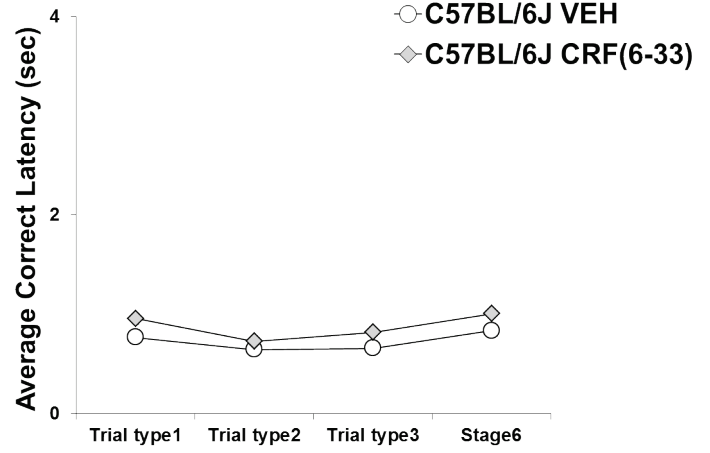
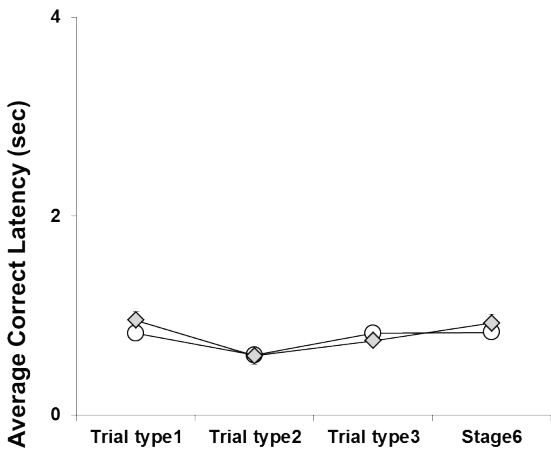
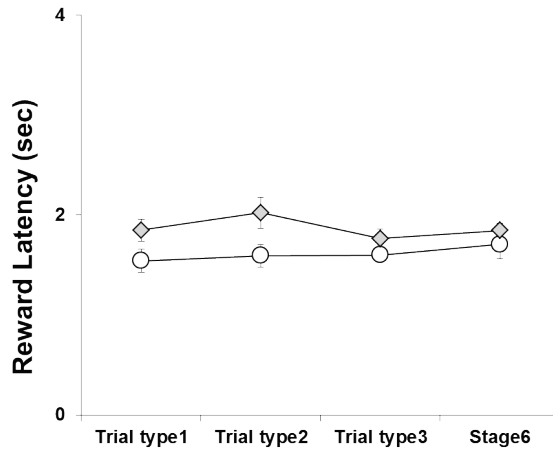


Figure 21 latency to reward collection and latency to correct response are unaffected during the first 4 days of treatment CRF(6-33): n=5; Control group: n=4

### 3.3.3 During DISTRACTOR manipulation CRF(6-33) still reduces impulsive responses

On the 7<sup>th</sup> day of chronic administration mice received their first session of DISTRACTOR manipulation test.

In this test is commonly evaluated the effect on accuracy during trial type 2 (DISTRACTOR test).

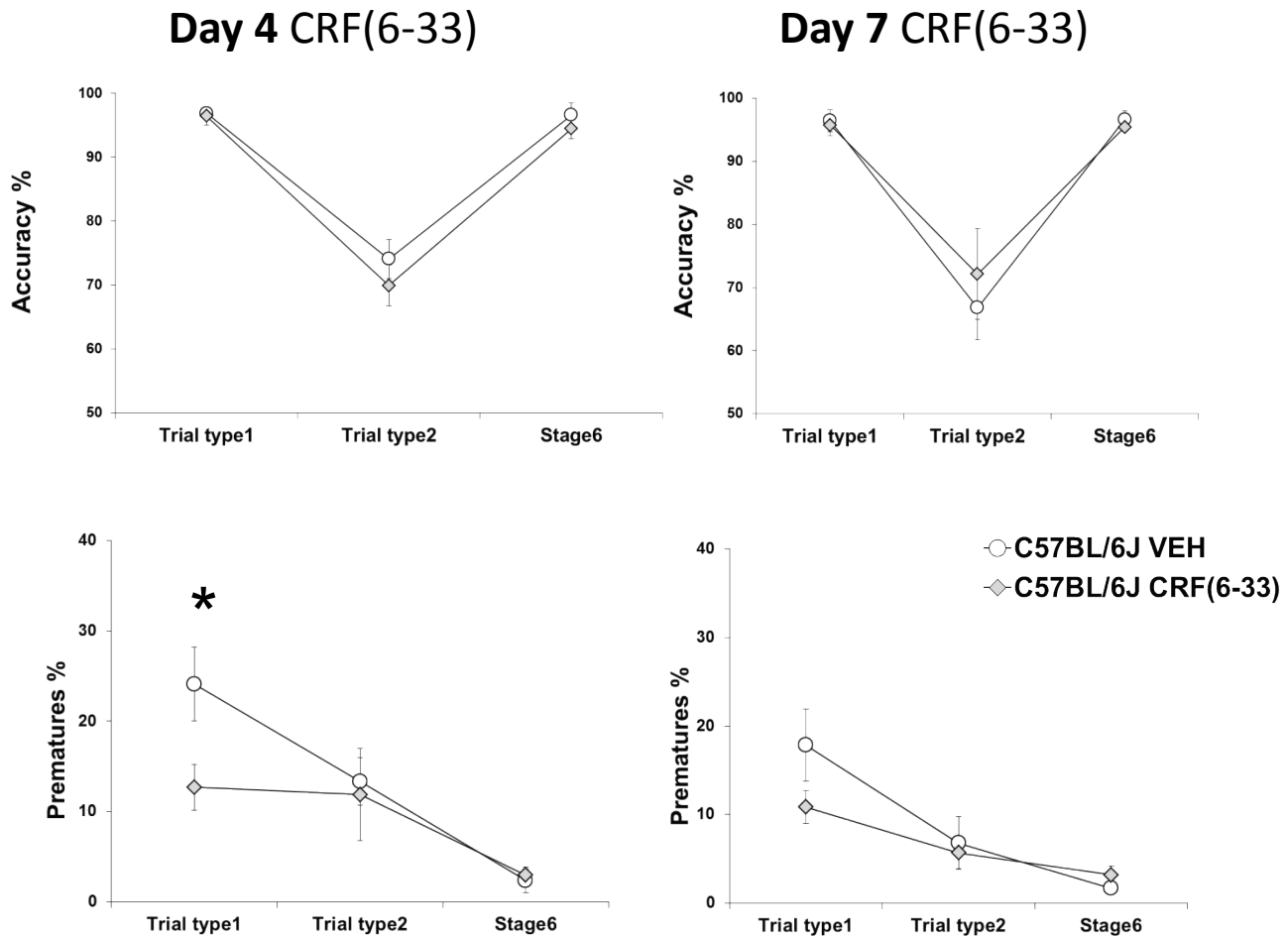
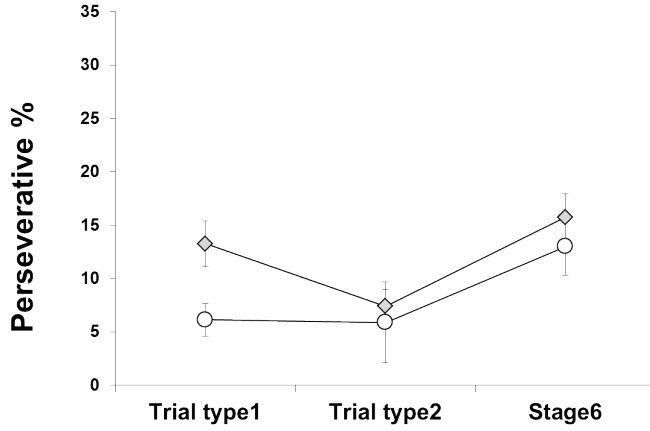


Figure 22 **Accuracy and Premature responses performance during distractor manipulation.** Premature responses after 4 days of intranasal treatment with CRF(6-33) are still significantly reduced (\* $p < 0.05$ ). No more effects on accuracy are visible.

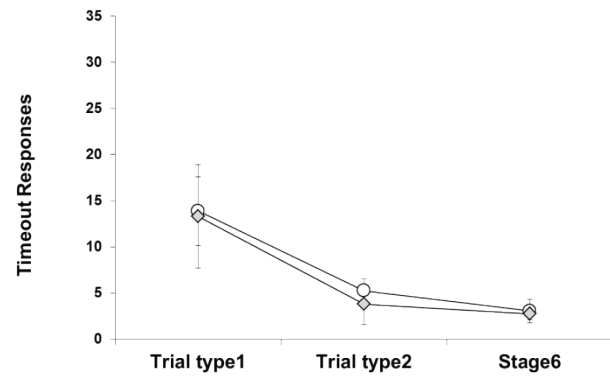
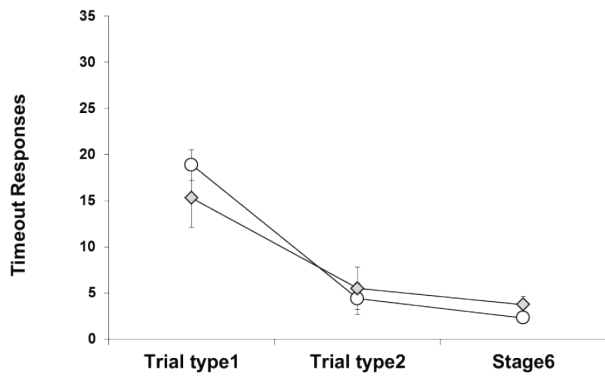
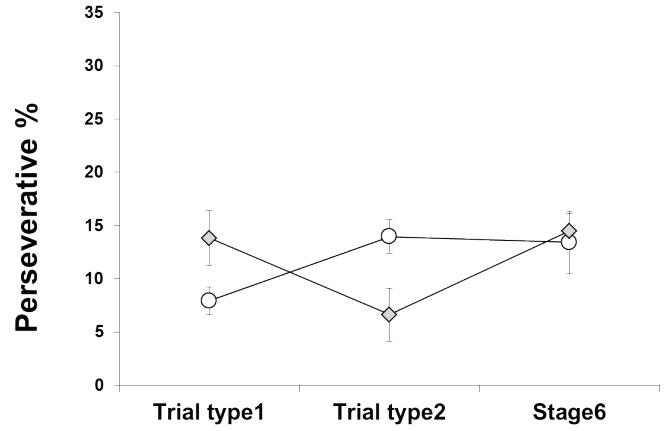
Values represent mean  $\pm$  SEM throughout all Figures.

In this context accuracy was not modified by the treatment. Interestingly instead premature responses are still significantly reduced ( $p \leq 0.05$ ) during trial type 1 (red led cued trial in the context of this manipulation). This trial is analogous to the trial type 2 of SARAT manipulation where this kind of effect was observed, suggesting an effect on impulsivity maintained after 7 days of chronic administration. This effect disappeared the day 10 in concomitance with the 2<sup>nd</sup> presentation of DISTRACTOR protocol test, suggesting an adaptive mechanism to chronic administration of CRF(6-33).

### Day 4 CRF(6-33)



### Day 7 CRF(6-33)



**Figure 23 Perseverative % and timeout responses during distractor manipulation.**

CRF(6-33): N=5; Control group: N=4 Values represent mean  $\pm$  SEM throughout all Figures.

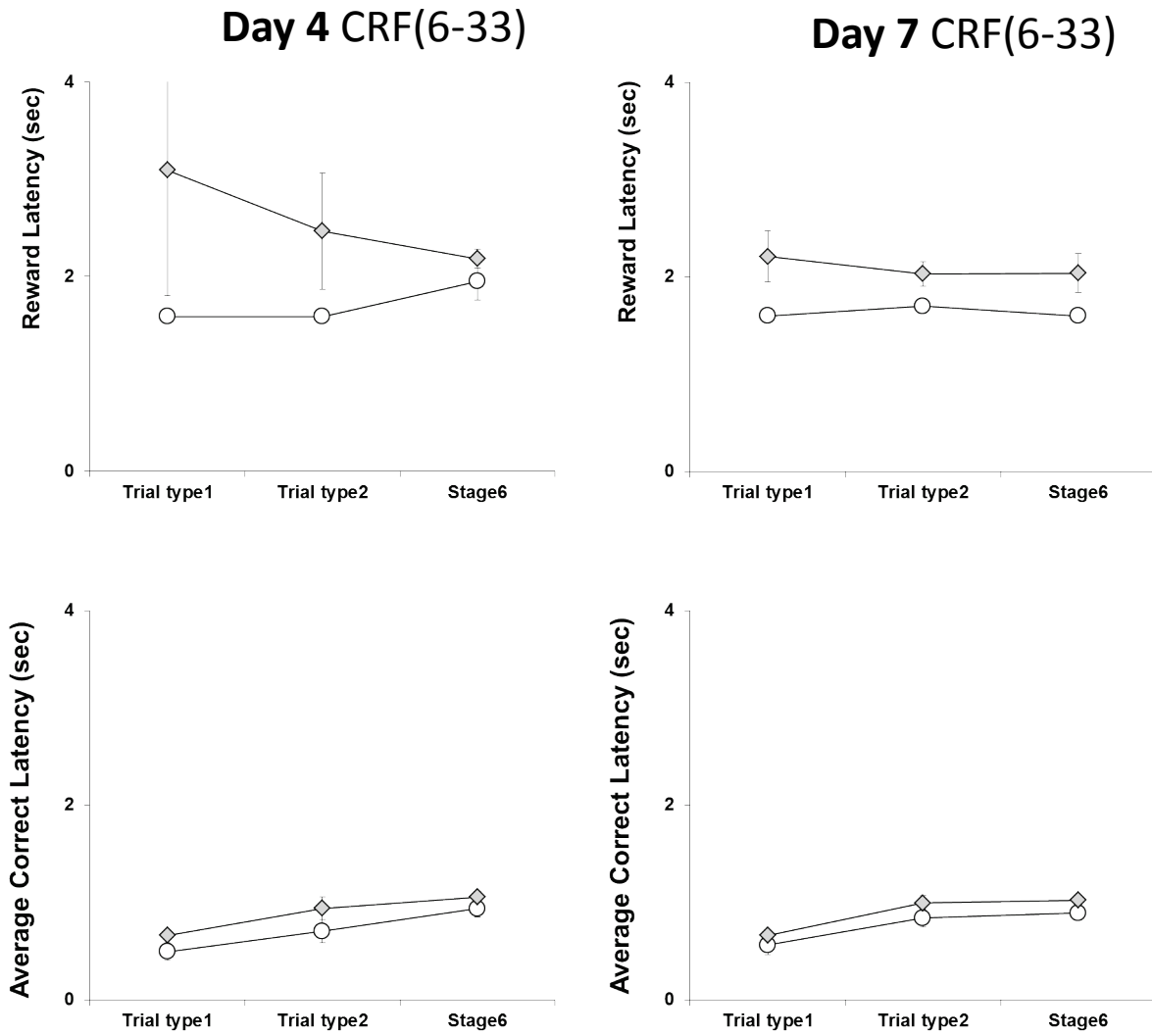


Figure 24 Latency to reward collection and latency to correct response are unaffected during DISTRACTOR manipulation after 7 days of treatment: CRF(6-33): n=5; Control group: n=4



### 3.3.4 IMPULSIVITY manipulation results:

During the impulsivity manipulation the relevant parameter evaluated (premature responses) was unchanged in the two groups.

Interestingly on latencies times was observed an unexpected effect: both latency to correct response ( $p < 0.05$ ) and latency to reward retrieval (trend) were increased in treated groups.

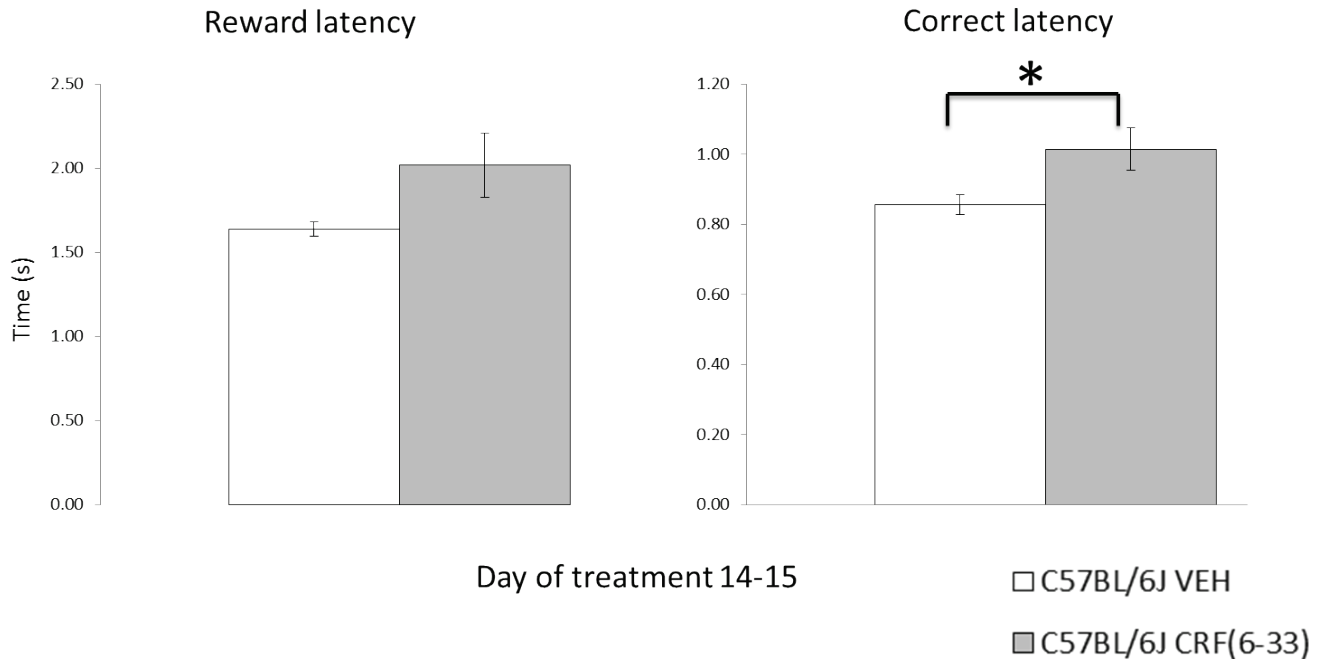


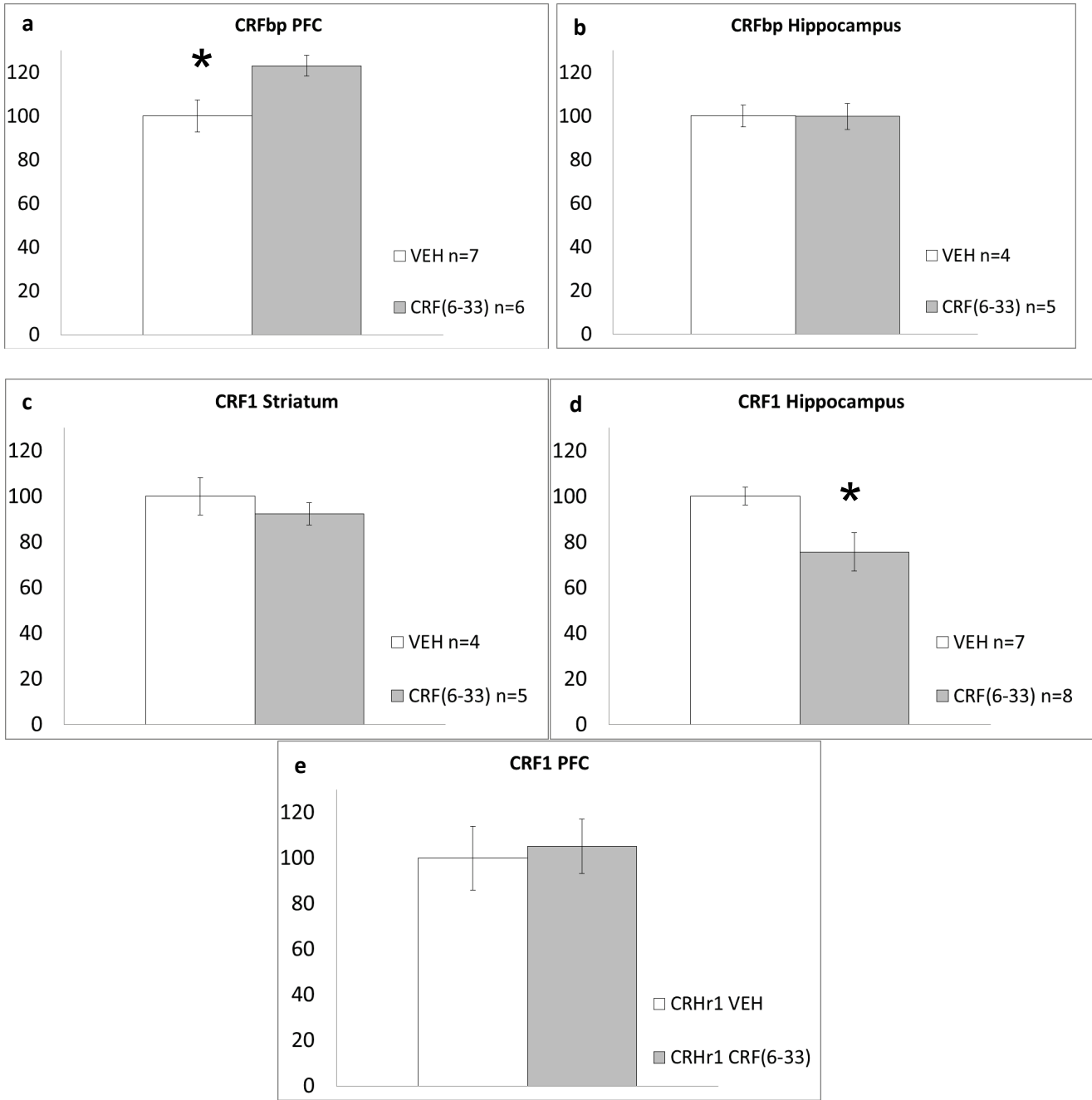
Figure 25: **Latencies are increased by CRF(6-33) after 2 weeks of chronic administration .**

Latency to correct response was significantly increased ( $*p < 0.05$ ) for for CRF(6-33) groups control. Latency to reward collection shows a trend of increase .

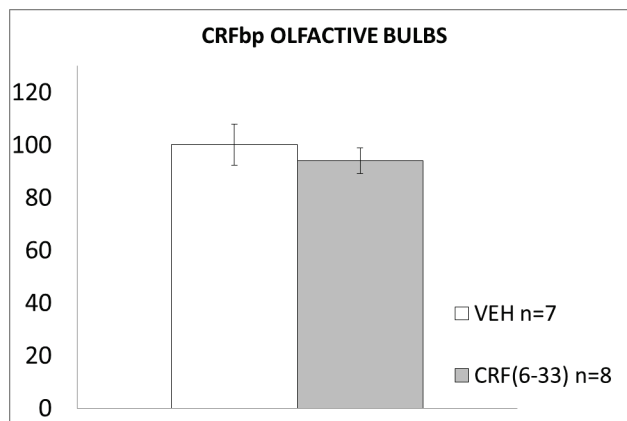
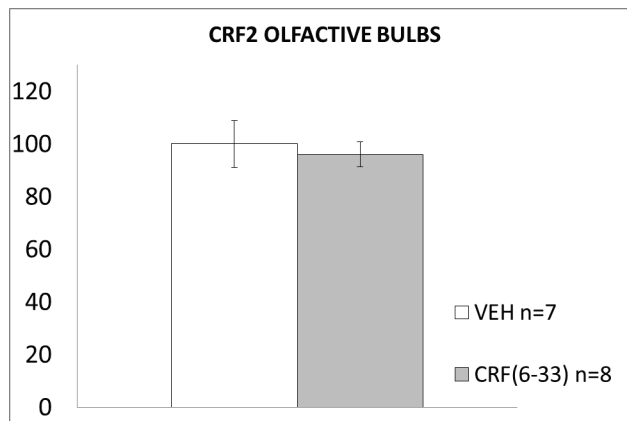
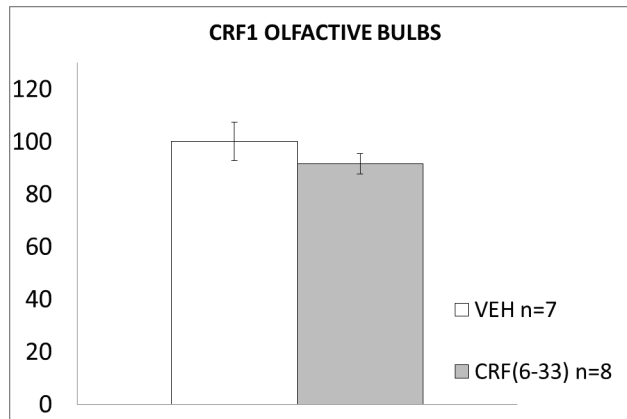
CRF(6-33): n=5; Control group: n=4

### 3.3.5 Real time PCR analysis confirms the ability of intranasal CRF(6-33) to modify expression of key molecules for the CRF system.

From real time PCR analysis we observed a significant increase of CRFbp expression of 23% ( $p < 0.05$ ) selectively in PFC (**Figure 26 a**). No effect of CRF(6-33) was observed in Hippocampus. Regarding to CRFr1 we observed a 25% significant ( $p < 0.05$ ) decrease of receptor selectively in Hippocampus (**Figure 26 d**). We tested also olfactory bulbs for CRFr1, CRFr2 and CRFbp, but none of them was significantly modified in this samples (**Figure 27**).



**Figure 26 CRF(6-33) induces a significant relative mRNA increase of expression for CRFbp in PFC (\*p<0.05) and a significant decrease of expression of CRF1 in Hippocampus>(\*p<0.05) in Real Time PCR analysis.**

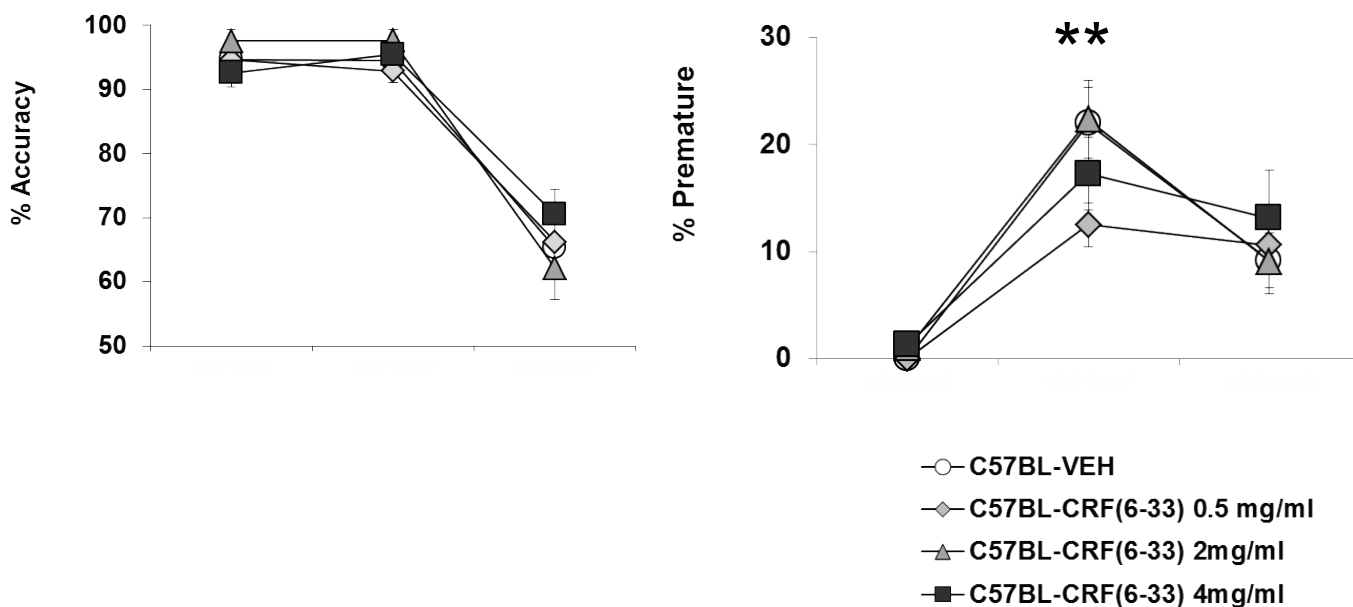


**Figure 27 CRF(6-33) doesn't affect a significantly relative mRNA expression for CRF1, CRF2 and CRFbp of olfactive bulbs in Real Time analysis.**

### 3.3.6 Acute lower dose of CRF(6-33) is still able to reduce impulsive behaviour

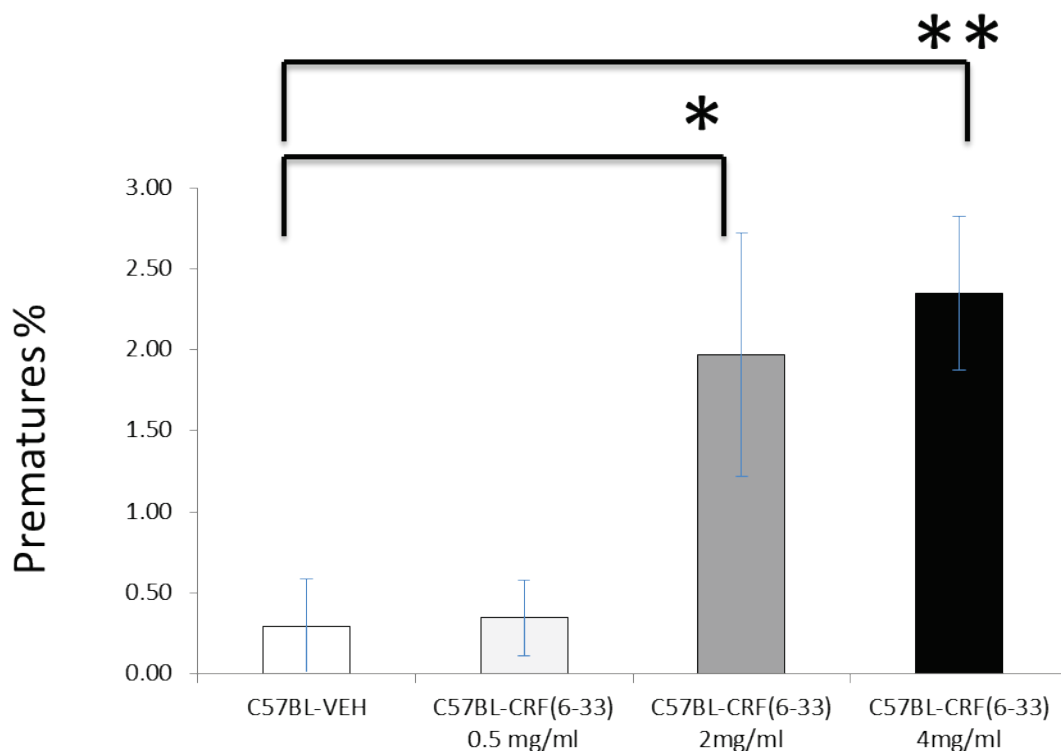
We tested acutely CRF(6-33) in SARAT manipulation using a Latin square paradigm in the 5-CSRTT. Treatment have been performed just the day of SARAT manipulation test, while the following days mice had to perform just the normal stage6 training test without additional administrations for 6 days of washout.

As in previous paradigm we observed a significant reduction (\*\*P<0.01 ) of premature responses for the lowest dose



**Figure 28 0.5mg/ml CRF(6-33) administration rduces significantly % of premature responses in a latin square protocol 5-CSRTT**  
 (\*\*p<0.01) (N=12)

Surprisingly that effect was observed only for the lowest dose and not for the higher. Following that observation we hypothesized a probable residual effect of previous treatments. Probably the mice were not completely recovered from the washout period (6 days). To eventual releable effect we checked performance of mice during stage6. We checked mice performance during the day following the administration and we observed a dose dependent increase of premature responses for the 2 highest dose groups evidencing a relevant residual effect the day after administration ( 2mg/ml p<0.05, 4mg/ml p<0.01 ) suggesting a rebound effect .



**Figure 29** Premature responses the day following administration in the latin square protocol; CRF(6-33) groups show a significant increase of premature responses (\* $P < 0.05$ , \*\* $p < 0.01$ )

### 3.3.7 Administration of acute intranasal CRF(6-33) may interact in opposite way with schizophrenia relevant mutations Dys+/- and D2L+/-

We tested acutely CRF(6-33) in the IMPULSIVITY manipulation of 5-CSRTT to test specifically effects on impulsivity responses that was the most promising effect observed so far for this drug. We chose to use the double mutant mouse model for genes Dys and D2L. They were chosen for a specific impulsivity deficit associated to double mutant mouse. Moreover we tested the single mutants to observe effects of interaction of CRF(6-33) with each single mutation.

Mice have been trained to the criteria as described in 3.2.3 for 5-choice protocol. Then they have been tested once for IMPULSIVITY without any treatment.

After 7 days they received intranasal administration of CRF(6-33) 45 minutes before the test.

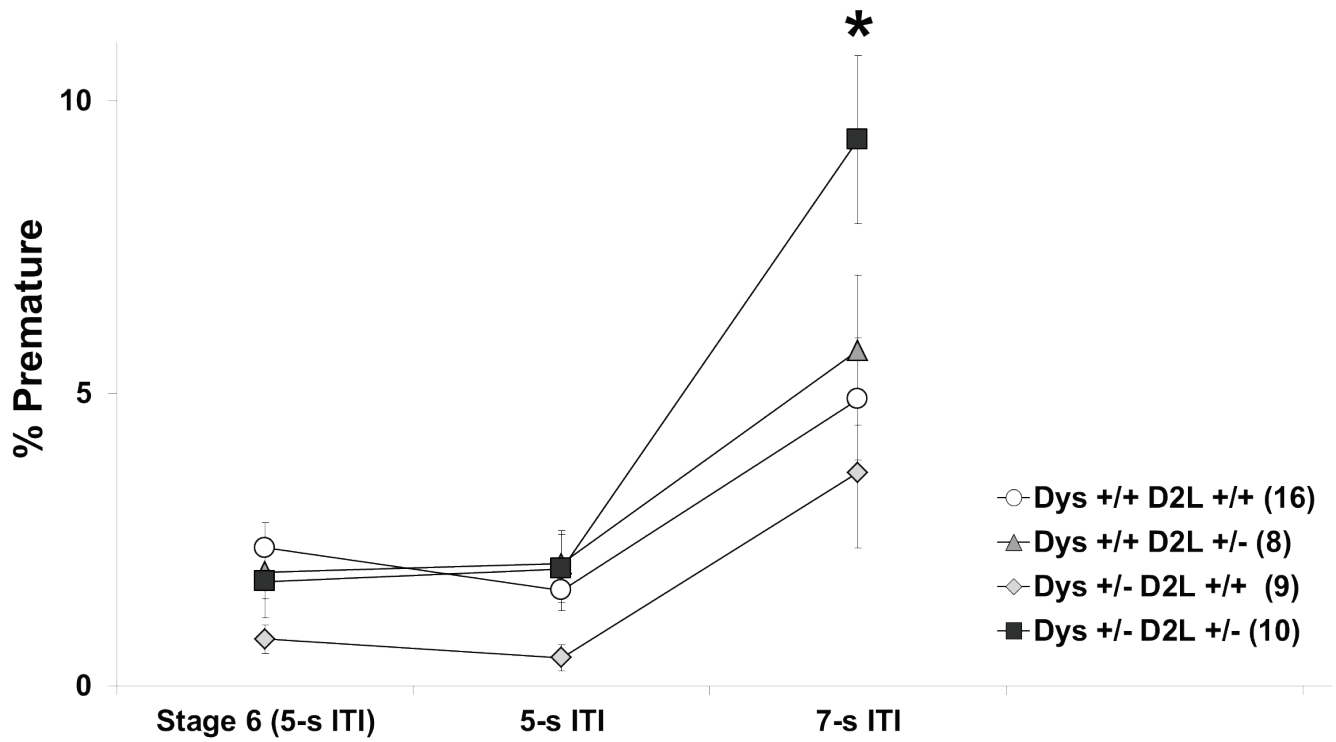
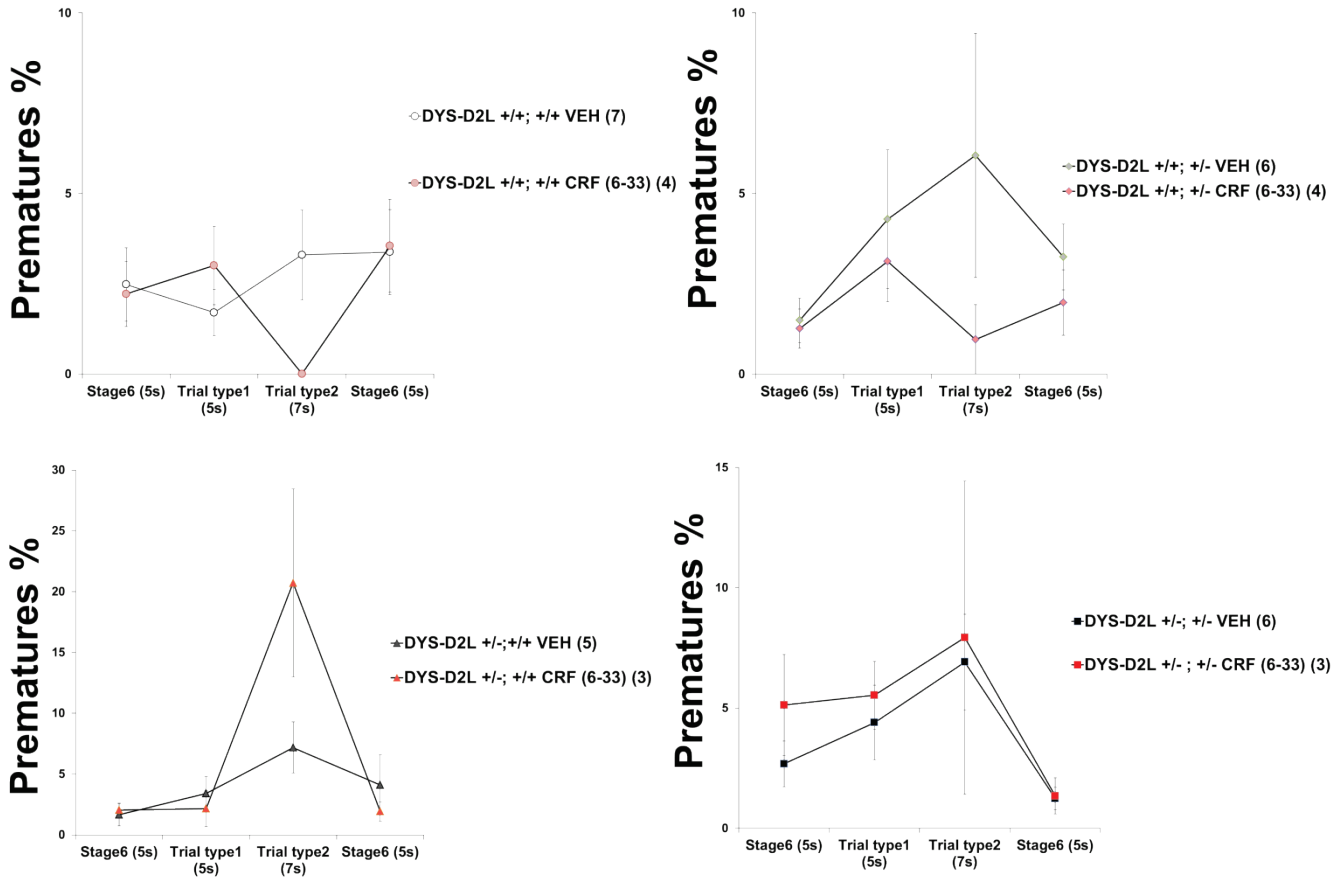


Figure 30 Dys +/- , D2L +/- show a significant impulsive behaviour (p<0.05)



**Figure 31** Effect of CRF(6-33) on schizophrenia relevant mice during IMPULSIVITY manipulation in *Dys* +/- *D2L* +/-, *Dys* +/- . *D2L* +/-, *Dys* +/- *D2L* +/-, *Dys* +/- *D2L* +/- . None of the groups has shown significant differences VS control. Wild type show a trend of improvement ( $p=0.08$  in T-Test) and *Dys* +/- show a trend of worsening of the phenotype(  $p=0.07$  In T-Test)

None of the group has shown a significant change of performance after CRF(6-33) administration. However, these results have to be considered preliminary data due to the low number of subjects in CRF(6-33) treated groups. It is discernible a trend of improvement in WT group, that is in accordance with previously observed data on WT and a trend of worsening of phenotype in *Dys* +/- *D2L* +/- group that, if confirmed might indicate a negative interaction with *Dys* deficiency.

### 3.4 Discussion

In men, CRF was proven to be an important molecule for cognition. This has been confirmed in studies of Alzheimer's patients (De Souza et al., 1986; Pomara et al., 1989) and in rodent studies in which CRF was administered via ICV (Behan et al., 1995; Eckart et al., 1999; S C Heinrichs et al., 1997; Stephen C Heinrichs & Koob, 2004; Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003; Koob & Bloom, 1985; Lee et al., 1993; Lee & Sung, 1989; Thompson et al., 2004). In an experimental, pre-clinical context in which there was acute ICV administration of CRF(6-33), CRF was proven to be effective in eliciting pro-cognitive effects on spatial memory (Behan et al., 1995). This was achieved without causing the undesired anxiogenic effects of the CRF itself that had been observed in rodents (Behan et al., 1995). In humans in pathophysiological conditions leading to a contextual increase in CRF was observed an increase of the state of anxiety (Mitchell, 1998; Takahashi, 2001) and in stressful conditions, such as the withdrawal from abused drugs and personal stress, which can cause hyperstimulation of the CRF system.

Since ICV administration of CRF is not suitable for human psychiatric therapy, our first aim was to determine whether it was possible to use this molecule by intranasal administration. Our second aim was to determine the proper therapeutic dose and assess any chronic effects of its use.

To assess cognitive performance, we chose to use a modified version of 5-CSRTT that was previously validated in our lab (data not yet published) with the advantage of reducing the training period from 39-day-to-several month sessions to 10-to-30-day sessions, which eventually resulted in a real operational session of 14 to 21 days (de Bruin, Franssen, Duytschaever, Grantham, & Megens, 2006; Papaleo, Erickson, Liu, Chen, & Weinberger, 2012; Patel, Stolerman, Asherson, & Sluyter, 2006). Additionally, our modification of the 5-CSRTT paradigm included the introduction of two new kinds of manipulations, i.e., 1) SARAT, primarily designed to assess broad monitoring abilities and 2) distractor manipulation, which permits us to assess the ability of patients to focus their attention in the presence of distractive events. This modification was introduced because both of these abilities are impaired in schizophrenic patients. The third kind of manipulation, the impulsivity test, was used specifically to assess impulsivity, which also is altered in schizophrenic patients.

Initially, we chose to test the drug in a dose of 5µl with a concentration of 2mg/ml. This was about 20 times higher than the minimal effective dose tested in rats via ICV (normalized by weight). This dosage was chosen to provide the highest chance of achieving an effective therapeutic concentration in the brain. The first battery of tests was performed in C57BL/6j mice, because this strain of mice is the strain that is most extensively used for genetic and behavioural studies. First, acute administration did, in fact, increase the accuracy in SARAT manipulation significantly, and it decreased impulsivity. After acute intranasal administration of CRF(6-33), the results were comparable to the results that were observed using ICV administration in rats (Behan et al., 1995). However, after sub-chronic



administration, this initial effect became unstable, but the effect on impulsivity was maintained much longer, i.e., for as long as seven days of sub-chronic administration. Moreover, the progression of the chronic administration of the drug did not lead to any additional cognitive improvement, but it increased the latency times required for the treated mice to respond and collect the reward.

These data indicated that the most promising and constant effect of the intranasal administration of CRF(6-33) was on impulsive behavior. The data indicated that prolonged treatment, i.e., more than a week, may lead to the worsening of general performance.

Our initial observations allowed us to answer the first question, i.e., CRF(6-33) can be delivered in active doses by intranasal administration. To confirm this observation that was made in the behavioural test, we performed molecular tests of real time PCR on dissected areas of the mice brain. In this experiment, we observed that animals treated with CRF(6-33) had a significant decrease in the expression of CRF1 receptors in the hippocampus and increased expression of CRFbp in PFC. These effects, together with the behavioural effects, clearly confirmed the ability of CRF(6-33) to reach the brain intranasally and partially explained the decrease in the response to the local increase of CRF. Interestingly, in different parts of the brain, the compensatory responses that were observed were quite different, i.e., PFC increased the binding protein, which likely would result in the reduction of free CRF, but in the hippocampus, they led to a reduction of CRF1 receptor expression, which likely reduced the responsivity of the neurons to the CRF. These two compensatory effects probably have a different timing profile, which likely resulted in the different time of compensation observed in the behavioural performance. This should be confirmed by additional temporal-specific molecular studies. These results, also suggest the possibility of interfering with one (or both) of these molecular adaptation mechanisms to obtain either a more specific or a more prolonged effect.

Secondly, based on our initial results, we decided to assess CRF(6-33) as an acute treatment and to define a proper therapeutic dose for the following studies. Thus, we performed a 5-CSRTT in the SARAT manipulation, which already has been shown to be sensitive to pharmacological manipulation in the first pilot test, in an acute latin square design. In this design, we tested all the groups for all available doses. We tested doses of 5  $\mu$ l of the vehicle, CRF(6-33) solutions with concentrations of 0.5, 2, and 4 mg/ml. In this latin square paradigm, we were able to replicate the reduction of impulsive responses for the lowest dose that was administered. Curiously, a lack of response was observed for other doses, raising the prospect that, even after acute administration, there would likely be some residual compensatory effects that were not completely reinstated after one week of washout. That did not affect the lowest dose group since, according to the treatment schedule, every time a subgroup received the lowest dose the week after it had received the vehicle. This led to an effective 13-day washout period instead of six days, as was the case in the other groups. So, we observed premature response performances among the different treatment groups the day after treatment. For the highest dose groups, we observed a rebound effect of the premature responses the day after the administration, which was indicative of an increase in impulsivity. In addition, we

observed that the lowest dose still was able to evocate the reduction of premature responses, but it did not seem to evocate the rebound response that was observed for the other two groups. For the effectiveness and apparent safety of this dose, we used the dose of 0.5 mg/ml for the subsequent studies in mouse models for schizophrenia.

The third step was to test the effect of CRF(6-33) administration in schizophrenia-relevant mice models. The ideal candidate for the test had to be a schizophrenia-relevant model characterized by an increased level of impulsivity as the main observed effect for CRF(6-33) in C57BL/6j was a reduction of impulsive behaviour. We chose to test Dys/D2l double heterozygous mice that, in previous tests in our lab, had shown increased impulsive behaviour. We also tested single Dys heterozygous and D2L hetero mice that seemed to have both a trend of increased impulsivity and also to check the effects of the administration of CRF(6-33) on the single mutations. It was possible to replicate the genotype deficit on the double heterozygous Dys/D2L mice. For this purpose, we performed pharmacological manipulation in the paradigm of the impulsivity test because it was designed specifically to assess impulsive behaviour.

After performing the administrations of CRF(6-33), the preliminary data seemed to suggest a decreasing trend in impulsive behaviour in the WT and in the D2L group. However, there was no effect in the Dys/D2L double heterozygote model, and there was an increase in impulsive behaviour in the Dys het group. If this is confirmed by an increase of the number of samples of the groups, these results suggest a detrimental interaction between the pharmacological manipulation of CRF(6-33) and the Dysbindin mutation. To confirm these results, an increase in the populations is required, but this result would give additional incentive for the genetic characterization of the different forms of schizophrenia and of the molecular mechanism that underlies the observed phenotypes. Concerning similar treatments, we can expect diametrically opposed results due to the interaction with the genotype, and it would be desirable, advantageous, and beneficial to know how the interactions with genotypes after pharmacological manipulation will modify the outcome of treatments

# Chapter 4

## Conclusion and Future Directions

### 4.1 Conclusion

Since the intranasal route of administration was demonstrated to be an option (Born et al., 2002) to deliver drugs to the brain, exciting new frontiers to pharmacological research have been opened. Indeed, intranasal administrations can be used to bypass different restrictions of other route, mostly linked to the Blood Brain Barrier: elevated lipophilicity, small molecular weight (below 400- to 600-Da), low number of Hydrogen bonding donors or acceptors, low polar surface area (60–70 Å generally, maximum limit is 90 Å), reduced number of rotatable bonds and low flexibility, pKa between 4 and 10 (Pajouhesh & Lenz, 2005).

In particular, these requisites previously precluded the use of peptide molecules as they are of high molecular weight, high Hydrogen bonding, high polar surface, flexible molecules. Importantly, these very versatile and largely unexplored molecules embed a numerous potentiality to influence brain functions (Tsomaia, 2015).

Being able to deliver these molecules to CNS, bypassing BBB, in a non-invasive might open new perspectives for neurological and psychiatric disease therapy. There are plenty of natural peptides such as, OXT, VPS, insulin (Salameh et al., 2015) that have central effects and can be potentially used for therapy. Moreover, some of them (e.g. OXT and insulin) have important and undesired peripheral effects if injected systemically. Intranasal route of administration will avoid also this issue. Furthermore, instead of natural peptides, we can engineer peptides (Sato, Viswanathan, Kent, & Wood, 2006) to obtain different effects from natural molecules, e.g. ligand specificity (Sutton et al., 1995), when originator natural peptide has multiple ligands or different intrinsic activity (i.e. antagonist activity from an agonist peptide) Or some properties of natural peptides can be obtained in a more straight-forward way (Tsomaia, 2015).

In this thesis work we explored 2 representative and different examples taking advantage of the intranasal ways. On one side OXT, a natural peptide with central social-related effects On the other hand CRF(6-33) an engineered peptide, table-bench designed for a specific action on a target in the brain that was previously studied only by ICV administrations (Behan et al., 1995; Eckart et al., 1999; S C Heinrichs et al., 1997; Stephen C Heinrichs & Koob, 2004; Stephen C. Heinrichs & Joppa, 2001;

Stephen C. Heinrichs, 2003; Koob & Bloom, 1985; Lee, Lee, Wang, & Lin, 1993; Lee & Sung, 1989; Thompson, Erickson, Schulkin, & Rosen, 2004).

The overall goal was to study, through the intranasal administration of neuropeptides, therapeutical strategies targeting two of the main and still untreatable alterations relevant to schizophrenia, that is, social and cognitive deficits (Arguello & Gogos, 2006; Kvajo, McKellar, & Gogos, 2012).

We demonstrated that OXT intranasal administrations can reliably modulate the oxytocinergic system to selectively modulate social behaviour. Specifically, chronic intranasal OXT was able to ameliorate the social deficits found in dys +/- mice, as demonstrated by both males social interaction vs opposite sex and female-female interactions. Thus, Dysbindin-1 genetics could be used in the future to predict the behavioral responses to intranasal Oxytocin.

OXT seems to be effective only for treatments of social deficits, but not for cognitive impairment (even if more studies should selectively check this). Thus, for cognitive functions we focused more on the CRF(6-33), as previous literature had shown that it might ameliorate cognitive deficits by ICV administrations in mice.

CRF(6-33) our first concern was if it was possible to reach active concentration into the brain. Indeed, due to its mechanism of action, it needs to reach relatively high concentration to exert an effect. In fact, it implies to dissociate CRF from its binding protein while  $K_i$  between CRFbp-CRF is comparable to  $K_i$  of CRFbp-CRF(6-33) (Sutton et al., 1995)

Stemming from literature data on rats (S C Heinrichs et al., 1997; Stephen C. Heinrichs & Joppa, 2001; Stephen C. Heinrichs, 2003), we expected that active ICV dose for a mouse should range from 0.5 to 2.5  $\mu\text{g}$ . And in a 5  $\mu\text{l}$  volume we were limited initially to maximum 10  $\mu\text{g}$  for administration. Nevertheless, we obtained an improvement of accuracy since the first experiment on C57BL/6j, and a reduction of impulsivity suggesting we succeeded in obtaining a penetration of an active dose of CRF(6-33). A further support came from the molecular observations from real time PCR on CRF1 receptor and CRFbp. Importantly, the strongest and most stable effect was found on impulsivity

Thus, we demonstrated that intranasal CRF(6-33) can reach the brain and can modulate some cognitive parameters relevant for schizophrenia, as accuracy and premature responses. However, our findings warn that these improvements might rapidly disappear or even have some rebound effects. A plausible interpretation of these results is that these are probably negative feedback mechanisms to reduce CRF activity in response to local increase of CRF. Particularly, even if the triggering condition for that response is the displacement of CRF from CRFbp, the response observed is really different in different brain areas: in PFC there is augmented sequestration of free CRF by an increase of CRFbp expression while in hippocampus responsiveness to CRF it is decreased itself by the reduction of available receptors.

These mechanisms should be confirmed by other molecular data and will require further investigations. A possible hypothesis is that downregulation of hippocampal receptors is the fast event and is responsible for the rapid loss of improvement on accuracy during SARAT manipulation (that was observed after just 4 days of administration) while increase in CRFbp expression is probably the slower event as putative responsible for the loss of improvement on premature responses.

Based on these evidence in wild type mice, because in previous studies (data unpublished) we found that our schizophrenia relevant mouse model double heterozygous *Dys +/- D2L +/-* (receptor for dopamine Long form) was impulsive, we tested their impulsive behaviour after acute administration of CRF(6-33).

In this context, preliminary results seem to suggest no effects on impulsivity on the genotype *D2L +/- Dys +/-* mice while both WT controls and *D2L +/-* showed a tendency to reduction of premature responses and *Dys +/-* were found to have a higher percentage of premature responses, compared with littermates vehicle treated controls. Preliminary results of acute intranasal CRF(6-33) treatment in these mice show that it decreased the impulsivity trait both in *D2L +/-* and in WT, while interactions of pharmacological manipulation with *Dys +/-* genotype lead to a worsening of the impulsive trait. In the double heterozygote mouse model, the effect of pharmacological manipulation on the genotype seems to be a sum of positive effects on *D2L +/-* and negative effects on *Dys +/-* with a resultant sum of opposite effects almost inexistent.

In the light of this, the best way of using CRF(6-33) and the proper target should still be better explored. Our preliminary studies on schizophrenia relevant mouse models highlight that this molecule can interact in different way with different genetic backgrounds this might be important in the context of developing personalized healthcare based on the genetic background of each subject.



## 4.2 Future Directions

While current results on the effects of intranasal OXT have been encouraging, there is still considerable uncertainty as to the route of action and distribution of OXT following intranasal delivery. It is not clear whether behavioural effects that follow peripheral (olfactory route or trigeminal system) administration of OXT are driven by increased central concentrations of OXT due to the OXT entering the CNS, or to an indirect peripheral effect driving central production and release of OXT, or that peripheral effects of OXT may drive indirect behavioural effects through unknown mechanisms (Evans, Dal Monte, Noble, & Averbeck, 2014). Further studies will be needed to determine the route and distribution of OXT following intranasal delivery. A possible experiment to clarify this, could be one where, in conjunction with intranasal OXT administration, to apply a peripheral OXT antagonist that does not cross the blood brain barrier which would then eliminate any peripheral actions (by blocking peripheral OXT receptors) that might confound interpretation of central effects of OXT (Evans et al., 2014).

On top of that, as the pharmacokinetics of OXT are still not fully understood, therefore, an accurate, specific, and readily available method for measuring OXT that can be adopted as the standard in the field is urgently required for progress in our understanding of OXT's roles in behaviour and cognition (McCullough et al., 2013).

For CRF(6-33) instead ability of the peptide to reach its sites of action in the brain seems much less prone to misinterpretations. CRF(6-33) is itself a synthetic peptide designed to have no other interaction than with CRFbp (Sutton et al., 1995), it has no significant bind affinity for any of CRF receptors (Sutton et al., 1995) and molecular result cannot be explained without brain penetration. While we can know almost exactly where the peptide can act and that his effect is mediated by a local CRF agonism still it is not completely understood the underlying mechanism that induces the pro cognitive phenotypes observed in C57BL/6j mice and interaction with different genotypes. Further studies will be needed to determine the kinetic of distribution in the brain tissue and of brain adaptation to CRF system stimulation.

The understanding of the interactions between multiple schizophrenia risk genes will aid in the development of new and more efficient ad personam therapeutic strategies to ameliorate negative symptoms and cognitive dysfunction in schizophrenia.





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