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Hippocampus Dysfunction in a Quinpirole Sensitization Model of Obsessive-Compulsive Disorder

Narušená Funkce Hipokampu u Modelu Obsedantně-Kompulsivní Poruchy Vyvolané Quinpirolem

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Podpis:

LIST OF ABBREVIATIONS

ACC - Anterior Cingulate Cortex
ACQ - acquisition
ANOVA – Analysis of Variance
Arc - gene coding for Activity Regulated Cytoskeleton Associated Protein
CA1 - Cornu Ammonis area 1
CC - Cage Control
CMI - clomipramine
COMT - Catechol-O-methyltransferase
CSTC - Cortico-Striato-Thalamo-Cortical
CREB – cAMP-response element binding
D_1R - Dopamine Receptor D1
D_2R - Dopamine Receptor D2
dACC - dorsal Anterior Cingulate Cortex
DBS - Deep Brain Stimulation
DLPFC - Dorsolateral Prefronal Cortex
FEF - Frontal Eye Field
FISH - Fluorescence in Situ Hybridization
GABHS - Group-A Beta-Hemolytic Streptococcal infections
GPe - external segment of Globus Pallidus
GPi - internal segment of Globus Pallidus
HTR2A - Serotonin Receptor 2A
LA, LG, S - long A, long G and short alleles
MSN - Medium Spiny Neuron
NAcc - Nucleus Accumbens
OCD - Obsessive Compulsive Disorder
OF - Open Field
OFC - Orbitofrontal Cortex
PANDAS - Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections
PAS - Prepare And Select (model of action selection)
QNP - Quinpirole
QSM - Quinpirole Sensitization Model
REV - reversal
RIS - risperidone
SAL - saline
SMA - Supplementary Motor Area
SNr - Substantia Nigra pars reticulata
SRI - Serotonin Reuptake Inhibitors
SSC - citrate buffer
SSRI - Selective Serotonin Reuptake Inhibitor
STN - Subthalamic Nucleus
TPH2 - Tryptophan Hydroxylase 2
VTA - Ventral Tegmental Area

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PREFACE

Obsessive Compulsive Disorder (OCD) is a well-known and serious mental disorder. Despite general public awareness or the disorder, it is often taken lightly and with a humor it does not deserve. Most probably, such out-of-place humor can be tracked to pop-culture representations of this disorder (Sheldon Cooper from Big Bang Theory or Hercule Poirot from a well-known British series). OCD is one of the leading causes of disability, and has an immense impact on the wellbeing of both OCD patients and their families and friends. There are several successful treatments that reduce OCD symptoms, but not many cure OCD completely. Discovery of such treatments has been delayed by a lack of understanding of the disorder, and prevention is hindered by obliviousness to direct causes. As with many mental diseases, there are only rare times when a cause of a mental disease can be confidently identified, and causes often go unnoticed or are not even noticeable. Animal models are thus indispensable in allowing us to controllably reproduce a suspected insult and observe behavioral and neurophysiological changes that follow.

By serendipitous coincidence, two seemingly unrelated lines of research are underway in our laboratory that converge in this thesis – basic research on memory function and translational research on animal models of psychiatric disorders. Without this concurrence, it is unlikely my work on OCD would lead to my findings regarding hippocampal deficiency in an animal model of OCD.

Methodologies and results described here are not in chronological order. Experiments presented as first were actually conducted last based on results from the latter two studies. Yet, I thought that this order would be more digestible for the reader. Hopefully, this minor alteration for the sake of the story is not punishable.

What follows is general information about Obsessive Compulsive Disorder – its variability, proposed causes and description of the animal model that this work was conducted on. Knowledge gaps will also be described. It is possible that a more knowledgeable reader than I will find a simpler and more fitting explanation of our findings. But, it is possible that there may be some substance to hypotheses proposed here – because when observing neuropsychiatric diseases (or any disease for that matter), it often may be the consequences and not the causes that grab our attention.

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1 ABSTRACT

Obsessive-compulsive disorder (OCD) is a serious psychiatric condition manifested by repeated thoughts followed by stereotypic compulsive behavior. Alterations to cortico-thalamo-striato-cortical circuits are most often implicated in the pathophysiology of OCD. However, many studies have also found a changed volume, shape and activity of the hippocampus in OCD patients. This work focused on the activity of hippocampal CA1 cells during stereotypical checking behavior and on cognitive flexibility in a quinpirole (QNP) sensitization model of OCD.

The activity of CA1 hippocampal cells during stereotypical checking was assessed in an enriched open-field test in QNP sensitized rats. *Arc*+ (activity - regulated cytoskeletal associated protein, or Arg 3.1) mRNA expression profiles were determined in CA1 coronal hippocampal sections following stereotypical checking. After the establishment of stereotypical checking (10 sessions), rats were exposed to the arena and sacrificed after 5 minutes. QNP sensitized animals visited the same objects with the same frequency as during previous sessions, while control rats did not. Locomotor activity was comparable between QNP treated rats and controls. Following sacrifice, rat brains were flash frozen and sliced to 20 μm thick sections. Sections, mounted on slides, were hybridized with anti-*Arc* riboprobes, and visualized using tyramid amplification. Both control rats and rats treated with QNP displayed low baseline *Arc*+ positive cells in CA1. Importantly, there was a significant interaction between QNP and the environment – QNP treated rats displayed a lower number of *Arc*+ nuclei in CA1 during exploring/checking the open-field compared to control rats; while in the baseline condition there was no significant difference in *Arc*+ cells in CA1 between QNP treated rats and control rats.

To assess cognitive flexibility, a hippocampus-dependent Carousel arena task with reversal was employed. Animals were to avoid a sector on rotating arena that was not directly perceptible and could only be localized by spatial relationships to distal landmarks. The number of entrances into the sector was used as a measure of learning. Rats treated with QNP displayed a severe, but transient, increase in the number of errors in reversal. Treatment with clomipramine, a drug commonly used to treat OCD, further impaired reversal and impaired acquisition of the Carousel arena task. On the other hand, a combination of clomipramine and risperidone improved the rats' performance. Furthermore, two-way active avoidance task confirmed hippocampal impairment in QNP treated rats.

Although direct causality cannot be inferred from present results, this work highlights the possibility of hippocampal involvement in generation of stereotypical behavior similar to behavior observed in OCD patients.

2 ABSTRAKT

Obsedantně kompulzivní porucha (OCD) je závažné psychiatrické onemocnění, které se projevuje opakovanými nutkavými myšlenkami a následně stereotypním kompulzivním chováním. Do patofyziologie OCD jsou zapojeny změny kortiko-thalamo-striato-kortikálních obvodů. Mnoho studií však také zjistilo změněný objem, tvar a aktivitu hipokampu u pacientů s OCD. Tato práce byla zaměřena na aktivitu hipokampálních CA1 buněk během stereotypního kontrolního chování podobného kompulzivní kontrole a na kognitivní flexibilitu v potkaním modelu sensitizace dopaminovim D2 agonistem quinpirolem (QNP).

U sensitizovaných potkanů a kontrol byla hodnocena aktivita hipokampálních buněk v oblasti CA1 během stereotypní kontroly v otevřeném poli s vloženými objekty. Stanovili jsme profily exprese ranneho genu *Arc*+ (aktivitou regulovaného cytoskeletálního proteinu neboli Arg 3.1) v koronálních hipokampálních řezech v oblasti CA1. Po ustavení stereotypního kontrolovani (10 sezení) byli potkani vystaveni aréně a přesně po 5 minutách humánně usmrceni. Sensitizovaná zvířata navštěvovala konkrétní objekty v jednotlivých sezeních s konsistentní preferencí, zatímco kontrolní tuto preferenci měnila od jednoho sezení k druhému. Lokomoční aktivita byla v testovacím sezení mezi oběma skupinami srovnatelná. Po usmrcení byly mozky potkanů bleskově zmrazeny a nakrájeny na řezy o tloušťce 20µm. Řezy na sklíčkách byly inkubovány s anti-*Arc* probami a exprese byla vizualizována pomocí tyramidové amplifikace. Jak kontrolní skupina, tak potkani sensitizovaní QNP, kteří nebyli vystaveni prostředí, vykazovali bazální nízké počty *Arc*+ buněk v CA1. Důležité je, že došlo k významné interakci mezi QNP a prostředím – potkani sensitizovaní pomocí QNP vykazovaly nižší počet *Arc*+ jader v CA1 během explorace/kontroly otevřeného pole ve srovnání s kontrolními potkany při této exploraci.

Pro otestování kognitivní flexibility byla použita úloha aktivního vyhýbání se místu s přeučením na rotující aréně (Kolotoči) závislá na hipokampu. Zvířata se měla za úkol vyhýbat sektoru na rotující aréně, který nebyl přímo viditelný, a jehož polohu šlo určit pouze prostorovými vztahy vůči vzdáleným orientačním bodům. Počet vstupů do tohoto sektoru byl použit jako měřítko učení a flexibility. Potkani vystavení QNP vykazovali významné, ale přechodné zvýšení počtu chyb při přeučení. Léčba klomipraminem, léčivem běžně používaným k terapii OCD, zhoršila počáteční učení i přeučení na rotující aréně. Na druhou stranu kombinace klomipraminu a risperidonu zachovala výkonnost potkanů na úrovni kontrol. Kromě toho výsledky z neprostorové úlohy aktivního obousměrného vyhýbání se (two-way active avoidance) také podpořily koncept narušení hipokampu u potkanů sensitizovaných QNP.

Ačkoli z dosavadních výsledků nelze odvodit přímou příčinnou souvislost, tato práce zdůrazňuje možnost klíčové role hipokampu při vytváření stereotypního chování podobného kompulzivním projevům u pacientů s OCD.

3 INTRODUCTION

3.1 About obsessive-compulsive disorder

Every so often, people are uncertain if they turned off appliances or locked doors. And every so often they go and check if they really did. However, when these and similar thoughts and checking behaviors cross a certain threshold, a person is suspected of suffering from Obsessive-Compulsive Disorder (OCD). In fact, OCD is relatively common – it is the 4th most frequent psychiatric disorder, with a lifetime prevalence of about 1-3% (Ruscio et al., 2010; Wahl et al., 2010). OCD is characterized by two groups of often-complementary symptoms – obsessions and compulsions. Obsessions are characterized by uncontrollable intrusive thoughts. The contents of obsessive thoughts are mostly idiosyncratic, although there are recurrent themes of 'security' (Woody and Szechtman, 2011). Specifically, obsessions often include fears of contamination, robbery, household fires or other harm coming to ones self or others (Williams et al., 2013). Obsessions are often followed by compulsive behaviors that offer temporary relief (Van Schalkwyk et al., 2016), and these compulsions are often the only visible symptoms of OCD. Similarly to obsessions, compulsions are very individual and range from invisible mental acts to complex physical rituals (Sibrava et al., 2011). Common compulsions include washing, checking, ordering and counting.

3.2 Symptom dimensions

Although the manifestations of OCD are easily graspable, more systematic analyses of symptoms are necessary for scientific research. Cluster analyses have indicated that patterns in the above-described OCD symptoms can be broken down into classes, or symptom dimensions of obsessions and compulsions, which often occur together (Williams et al., 2013). Four main OCD dimensions are recognized – contamination/cleaning; checking/aggressive thoughts; symmetry/ordering; and sexual/religious thoughts (Leckman et al., 2010). Analyses that have been used to cluster these symptom dimensions have yielded very consistent results across studies (Bloch et al., 2008). Throughout a lifetime, symptom dimensions usually stay constant, although changes of symptoms within one dimension can occur (Skoog and Skoog, 1999; Mataix-Cols et al., 2002).

3.3 Age of onset

Heterogeneity in OCD is also apparent in the age of onset. Based on the time of first symptom manifestation, patients can be divided into two subgroups: an adult-onset subgroup, where symptoms appear approximately at the age of twenty, and a pediatric subgroup, where first symptoms occur when a patient is around 8-12 years old (Heyman et al., 2001). Pediatric patients account for 30-50% of OCD cases (Zohar, 1999), and they display similar types and intensities of symptoms as adult onset patients (Delorme et al., 2005), with the exception of a slight increase in compulsions unaccompanied by obsessions in the pediatric patient group (Swedo et al., 2004). Males and females are equally affected in adult onset patients, but boys are more commonly affected in pediatric cases (Geller, 2006). Moreover, OCD has a more abrupt onset in pediatric patients (Swedo et al., 2004). In conclusion, despite symptom similarities, there are several important differences between adult and pediatric onset OCD.

3.4 Comorbidities

Various comorbid diseases often burden OCD patients. Of these, the most common are anxiety, depression, tics, social phobia and eating disorders (Torresan et al., 2013). Conversely, obsessive and compulsive symptoms are often a comorbidity of other psychiatric disorders, such as Tourette syndrome, Sydenham's chorea (Hounie et al., 2004), trichotillomania (Keuthen et al., 2016) and schizophrenia (Veras et al., 2017). In fact, Tourette syndrome is present in 30% of OCD cases (Sinopoli et al., 2017). Importantly, it appears that many comorbid disorders do not arise as a consequence of each other but are due to common genetic etiology. For example, Tourette syndrome is genetically very closely related to OCD (Ferrão et al., 2009). As shown by twin studies, shared genetic factors between OCD and depression account for the close relationship between the two diseases (Bolhuis et al., 2014). This notion is also supported by the efficiency of selective serotonin reuptake inhibitors (SSRIs) in both OCD and depression (Murphy et al., 2008).

3.5 Causes of OCD

Several different causes of OCD have been proposed. In many cases it is presumed that environmental factors are responsible for the development of OCD. The most commonly discussed of these are life traumas and streptococcal infections. However, genetic predispositions make people more vulnerable to developing certain conditions following environmental triggers. It is estimated that genetic factors account for at least 48% of the variance of OCD (Browne et al., 2014; Monzani et al., 2014).

3.5.1 Environmental factors

Two known environmental factors associated with OCD are life traumas and streptococcal infections. Traumas are related to OCD in both pediatric and adult onset OCD, while streptococcal infection is considered to specifically trigger pediatric patients.

It is well established that stressful life events are associated with the manifestation of OCD (Rosso et al., 2012). Although intuitively sound, a systematic assessment of traumatic events was only developed relatively lately. Specifically, new tools such as Paykel's Recent Life Events Interview, have enabled studying the link between trauma and OCD in a more systematic manner (McKeon et al., 1984). Using this assessment tool, it was shown that 6 months prior to onset, patients often experienced a higher frequency of important life events, with a peak at 1 month before OCD onset (McKeon et al., 1984). Moreover, many patients are diagnosed with OCD along with post-traumatic stress disorder (Dinn et al., 1999; Fontenelle et al., 2011). The causality of this link was indicated by the rise in new OCD cases after road-traffic accidents, sexual assaults, combat exposures and personal violence (de Silva and Marks, 1999), but see (Grabe et al., 2008). However, no mechanism of how life trauma might trigger OCD has currently been proposed.

The second environmental factor proposed to trigger OCD is an immune response after streptococcal infection. In the 1990's, Dr. Swedo from the National Institutes of Mental Health observed 50 pediatric patients who displayed a rapid onset of OCD after a Group-A Beta-Hemolytic Streptococcal infections (GABHS) induced by *Streptococcus pyogenes* (Swedo et al., 1998). The authors coined the term Pediatric Autoimmune Neuropsychiatric Disorders Associated

with Streptococcal infections (PANDAS) for this subgroup of patients. Although still not an official diagnosis in many countries, OCD triggered by streptococcal infection is finally being recognized. In fact, PANDAS are etiologically and symptomatically related to well-known Sydenham chorea. Apart from the iconic motor manifestations of St. Vitus dance, neurological symptoms of Sydenham chorea include many OCD-like behaviors (Pérez-Vigil et al., 2016). Moreover, the presumed trigger of both disorders is an abnormal immune reaction following these infections. Anti-neuronal autoantibodies in both Sydenham chorea and PANDAS react with antigens present on dopamine receptors (Brimberg et al., 2012; Cox et al., 2013). Importantly, Streptococcus pyogenes have been specifically shown to produce antibodies that react with dopamine D_2 receptors in laboratory rats (Brimberg et al., 2012; Cox et al., 2013). Moreover, there is a direct relationship between the level of anti-streptococcal antibodies and symptom intensity in OCD (Murphy et al., 2015).

To sum up, evidence supports the idea that environmental factors (life traumas and infection) have a significant influence on the development of OCD. However, susceptibility to environmental triggers, especially traumas, is potentiated by the genetic makeup of an individual.

3.5.2 Genetic factors

There is consensus that OCD is, at least partially, genetically determined. The higher prevalence of OCD among relatives of already diagnosed OCD patients (do Rosario-Campos et al., 2005; Grabe et al., 2006) and twin studies (van Grootheest et al., 2005) suggest that there is a strong genetic component to OCD. Moreover, research indicates that the heritable component appears to be larger in pediatric patients (45-65%) compared to patients with adult onset (25-47%; in van Grootheest et al., 2005; Nestadt et al., 2010). Numerous attempts have been made to decipher the genetic basis of this disorder. The most frequent targets of these studies include genes for receptors, transporters and enzymes involved in the serotonin, dopamine and glutamate systems. Dopamine related genes are described next, as stereotypes triggered by alterations in the dopamine system are the focus of this thesis.

3.5.2.1 Dopamine-related genes

Dopamine-related genes have been one focus of searches for the genetic underpinnings of OCD. Dopamine dysfunction has been implicated in OCD pathogenesis for a long time, triggered by observations such as the induction of stereotypical behaviors by dopaminergic agonists (Ridley, 1994), the effectiveness of antipsychotic augmentation to SSRI treatment (Hollander et al., 2003), and the altered binding of D2 receptors in the striatum of OCD patients (Denys et al., 2004b). As mentioned above, antibodies against D2 receptors trigger OCD behavior in PANDAS patients. Moreover, dopamine constitutes the main neuromodulator regulating the cortico-striato-thalamo-cortical circuits – a set of interconnected brain regions that are consistently implicated in the OCD pathology (Koo et al., 2010). From the standpoint of genetics, OCD has repeatedly been found to be related to a polymorphism of dopamine receptor 4 (Millet et al., 2003; Reshma et al., 2013). Moreover, genes involved in the degradation of dopamine have also been associated with OCD. Specifically, a monoamine oxidase A polymorphism was associated with female OCD patients with depression (Camarena et al., 2001; Hemmings et al., 2003) and low catechol-o-methyltransferase (COMT) activity was associated with OCD in males (Karayiorgou et al., 1997;

Gothelf et al., 2004). Associations between a COMT polymorphism in males and MAO-A with OCD was confirmed by meta-analyses (Taylor, 2013; Melo-Felippe et al., 2016). However, studies that did not separate male and female participants failed to find an association between COMT and OCD, illustrating the difficulties involved in drawing general conclusions about the hypothetical, all-encompassing population of 'OCD patients' (Erdal et al., 2003; Sampaio et al., 2015).

3.6 Neuroanatomy of OCD

Although the underlying 'causes' of OCD are unknown, the OCD etiology converges to a common defect in the functioning of cortico-striato-thalamo-cortical (CSTC) circuits (Milad and Rauch, 2012). All CSTC circuits have a common scheme of projection – they link cortical regions with striatum, thalamus and back with cortex (Figure 1). CSTC circuits are crucial for action selection, where actions are initiated in the cortex and are selected by the striatum (Redgrave et al., 1999; Mink, 2018). Perhaps more importantly, CSTC circuits are essential in action suppression. After selecting an action, a multitude of competing actions must be suppressed to prevent interference with the desired action (Mink, 1996).

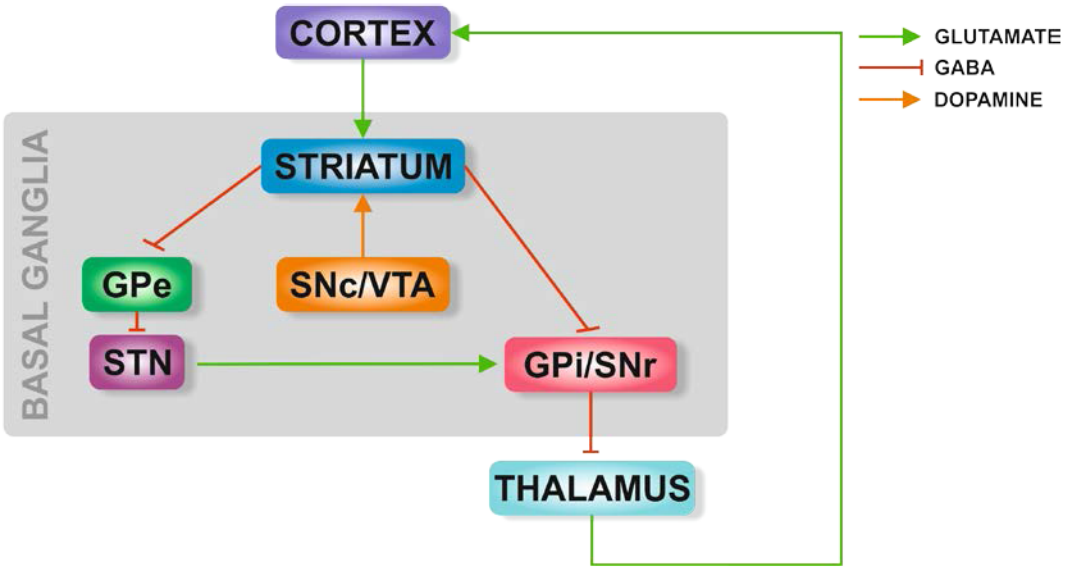

Figure 1 | A simplified CSTC circuit. The cortex sends excitatory signals to the striatum, which inhibits external and internal segments of the globulus pallidus and the substantia nigra reticulata (GPe, GPi, SNr, respectively). The substantia nigra compacta (SNc) and ventral tegmental area (VTA) modulate striatal activity by tonic and phasic dopamine release. GPe activates the subthalamic nucleus (STN), which in turn inhibits the thalamus, while GPi/SNr activates the thalamus. The active thalamus completes the CSTC circuit by inhibiting the cortex.

3.6.1 Dopamine as an orchestrator of CSTC circuits

One putative modulator of CSTC circuits is dopamine, which is thought to play an essential role in reward processing and action selection – hallmark functions attributed to CSTC circuits (Graybiel, 2008). The ventral tegmental area (VTA) and substantia nigra pars reticulata (SNr) supply dopamine to the CSTC circuitry (Albanese et al., 1986). Dopamine release is increased following the appearance of unexpected rewards or following cues predicting those rewards (Schultz, 2016). Otherwise, dopamine is released tonically and regulates overall responsiveness to reward-related dopamine release (Grace, 1991). In general, feedback regulates dopamine release by activating dopamine (D_2) autoreceptors present on the VTA and SNc neurons. Activation of D_2 autoreceptors decreases membrane potential by activating K_i inward rectifying channels (Beaulieu and Gainetdinov, 2011). Although dopamine modulates the activity of all structures within CTCS circuits, its role in the modulation of activity in the striatum is the most prominent and also the most studied.

3.6.2 Five parallel CSTC circuits

Dopamine input is integrated with information originating in cortical regions and in the striatum (Goto and Grace, 2008). The striatum consists of the nucleus accumbens, caudate and putamen (Parent and Hazrati, 1995a). Different cortical areas project to the nucleus accumbens, caudate and putamen, and are essential for different forms of action selection. Namely, the putamen receives inputs from the premotor, motor and supplementary motor area; the ventromedial caudate nucleus receives inputs from the orbitofrontal cortex; the dorsolateral caudate receives input from the dorsolateral prefrontal cortex; while the nucleus accumbens receives input from the dorsal anterior cingulate cortex (ACC) (Alexander et al., 1986) as shown in Figure 2; adapted from (Provost et al., 2015).

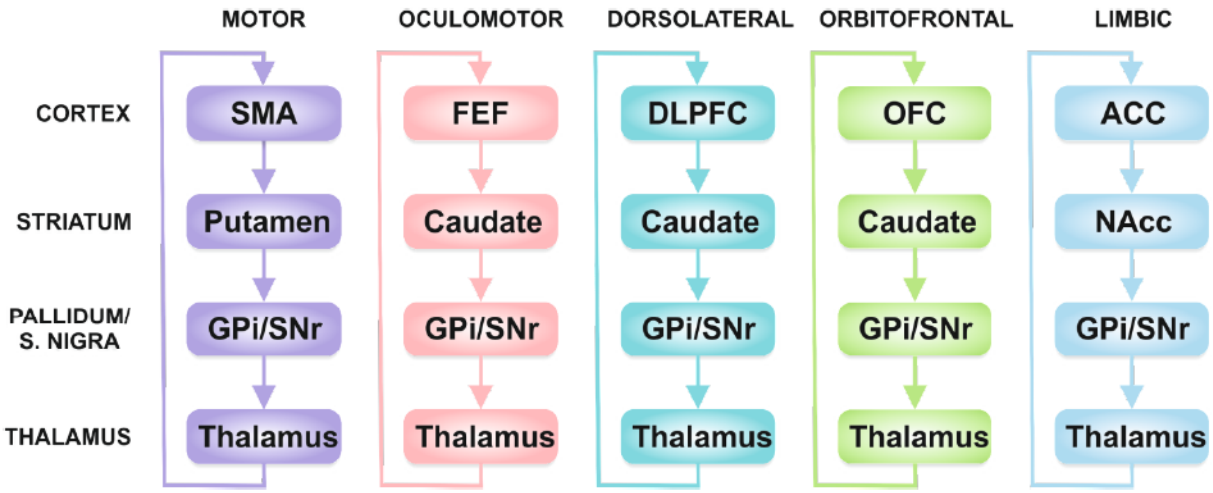

Figure 2 | Illustrates specific CSTC circuits and subregions of the cortex and striatum involved. The motor CSTC circuit is utilized by the supplementary motor area (SMA) and sends afferents to the putamen. The oculomotor CSTC circuit is utilized by the frontal eye field (FEF) and sends afferents to the caudate nucleus. The dorsolateral CSTC is utilized by the dorsolateral prefrontal cortex (DLPFC) and sends afferents to the caudate nucleus. The orbitofrontal CSTC circuit is utilized by the orbitofrontal cortex (OFC) and sends afferents to the caudate nucleus. Lastly, the limbic CSTC circuit is utilized by the anterior cingulate cortex (ACC) and sends afferents to the nucleus accumbens (NAcc). All CSTC circuits project to topographical segregated regions of the internal segment of the globulus pallidus (GPi) or substantia nigra reticulata (SNr) and then to topographically segregated regions of the thalamus.

3.6.3 Medium Spiny Neurons (MSNs) of the striatum

MSNs are the most common type of neurons in the striatum (Matamales et al., 2009). There are of two kinds, based on the expression of D_1 or D_2 receptors (D_1R and D_2R, respectively). Striatal D_1 receptors increase the activity of adenylylcyclase, which usually depolarizes neurons. Striatal D_2 receptors, on the other hand, decrease adenylylcyclase activity in MSNs and lead to the hyperopolarization of neurons (Stoof and Kebabian, 1981). D_1 and D_2 receptors are expressed on non-overlapping, pseudo-randomly distributed populations of striatal MSNs (Keeler et al., 2014). Within each CSTC circuit, striatal MSNs constitute the beginnings of two parallel striato-thalamic pathways (Figure 3). D_1R expressing MSNs are the beginning of the 'direct' striato-thalamic pathway, and D_2R expressing MSNs are the beginning of the 'indirect' striato-thalamic pathway (Surmeier et al., 2007).

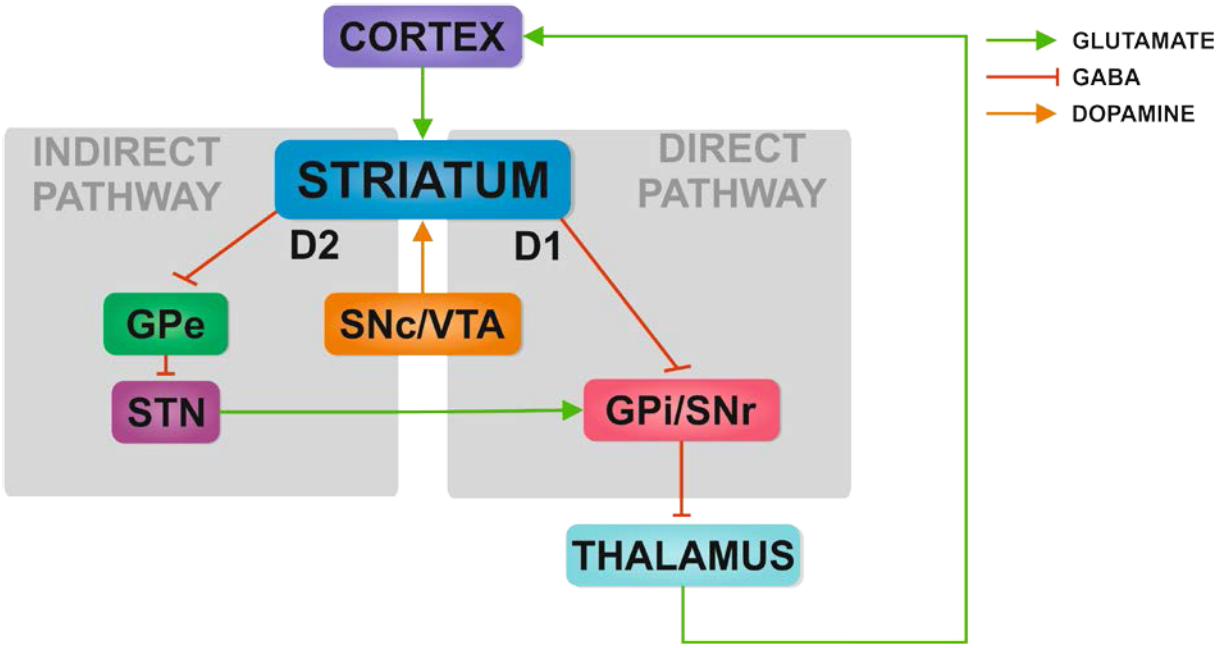

Figure 3 | Illustrates differneces between the 'direct' and 'indirect' striato-thalamic pathways. These pathways are segregated at the level of the striatum, where the 'direct' pathway commences by activation of the dopamine 1 (D_1) receptor expressing medium spiny neurons (MSNs), and the 'indirect' by activation of the dopamine 2 (D_2) receptor expressing MSNs. The indirect pathway proceeds by increasing activation of the external segment of the globulus pallidus (GPe), which in turn increases activation of the subthalamic nucleus (STN). The STN increases inhibition of the thalamus by increasing the inhibitory action of the globulus pallidus (GPi) or the substantia nigra reticulata (SNr). The direct pathway produces the opposite effect by activating the thalamus via increased activation of the GPi/SNr. Balance between the direct and indirect pathways results in the initiation of desired actions (direct) and suppression of unwanted actions (indirect).

3.6.3.1 Direct pathway

D_1R-expressing MSNs project GABAergic inhibitory afferents to the internal segment of the globus pallidus and substantia nigra pars reticulata (GPi/SNr), constituting the direct pathway. Next, the GPi/SNr exerts tonic inhibitory activity on the thalamus (Alexander et al., 1986). When this direct pathway is activated, via D_1R-expressing MSNs, inhibition of the thalamus is lifted. Increased activity of the thalamus in turn activates the cerebral cortex through direct glutamatergic projections (Goldberg et al., 2013). D_1 receptors have relatively low affinity to dopamine, therefore the direct pathway is activated in situations when dopamine levels are high (phasic dopamine release) and promotes the execution of selected motor/cognitive programs (Dreyer et al., 2010).

3.6.3.2 Indirect pathway

On the other hand, the indirect pathway was long considered to be constitutively inhibited by baseline dopamine levels maintained by the tonic release of dopamine, due to the higher affinity of inhibitory D_2 receptors to dopamine (Dreyer et al., 2010; Surmeier et al., 2011). However, this notion was recently challenged, as it was shown that D_2 receptors, which are most often in low affinity state, also respond to phasic dopamine release despite being activated by tonic dopamine release (Marcott et al., 2014). This information has not yet been incorporated into the theoretical framework of basal ganglia function. In current models, the indirect pathway is essential in the inhibition of unwanted motor/cognitive programs via inhibiting the thalamus.

D_2R-expressing MSNs project to the external segment of the globus pallidus (GPe) (Calabresi et al., 2014). When dopamine levels increase, D_2R-expressing MSNs are inhibited and disinhibit target neurons in the GPe. Disinhibited GPe neurons send inhibitory signals to corresponding STN neurons and also to corresponding neurons in the GPi/SNr (Calabresi et al., 2014). The STN sends glutamatergic afferents to all basal ganglia structures, including the GPe and GPi/SNr (Koshimizu et al., 2013). In fact, STN connections to the GPe are more numerous than connections to the GPi/SNr (Sato et al., 2000; Koshimizu et al., 2013). Increased excitability of GPi/SNr neurons by the STN potentiates its inhibitory effect on the thalamus. However, the STN also sends glutamatergic back-projection to the GPe, which increases the GABAergic inhibitory tone of the GPe on the GPi/SNr (Chu et al., 2015).

3.6.3.3 Models of action selection by CSTC circuits

At present, there are two models that describe action selection by CSTCs – the 'Go/No-Go' model and the 'prepare and select' model.

The traditional model of CSTC proposes that when the direct pathway overrides the activity of the indirect pathway, tonic inhibition of the GPi/SNr (by the indirect pathway) on the thalamus is decreased and motor/cognitive programs are facilitated (Frank, 2005). This theory is exemplified by the Go/No-Go model (Frank, 2005). Only in situations when dopamine levels are low, such as in cases of absent expected rewards, D_2 expressing neurons of the indirect pathway become active and inhibit the GPe, with a subsequent increase of inhibition of the thalamus and suppression of motor/cognitive programs (Parent and Hazrati, 1995b). Activation of the indirect pathway was speculated to facilitate a broader behavioral repertoire that aids in finding novel rewarded behaviors (Humphries et al., 2012).

The newer 'prepare and select' (PAS) model of basal ganglia proposes that the indirect pathway does not inhibit action, but in fact promotes it, because previously activated D_2R-expressing MSNs are D_2R free due to D_2R internalization. The function of the direct pathway in this model is to offer a range of possible behaviors, and the function of the indirect pathway is to select the most rewarded action based on prior experience (Keeler et al., 2014).

3.6.4 CSTC circuits in OCD

It has been suggested that in OCD the direct pathway is hyperactive compared to indirect pathway, resulting in the absence of a 'stop signal' following successful execution of a cognitive/motor program (Pauls et al., 2014; Karas et al., 2019). Figure 4 illustrates the disbalance between the direct and indirect pathways in OCD.

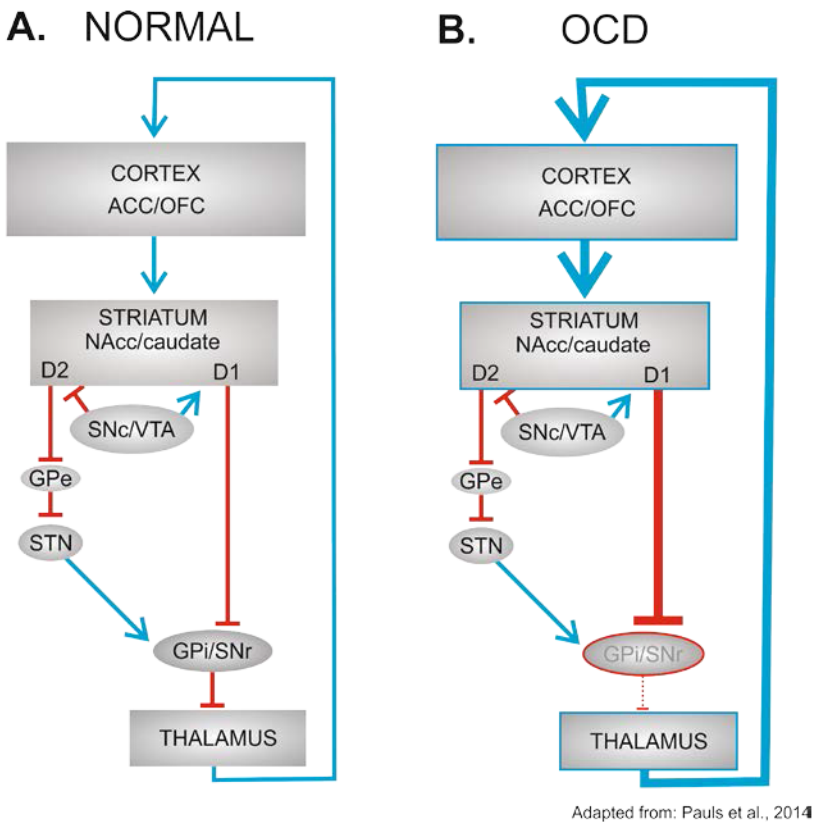

Figure 4 | In a normally functioning CSTC circuit (panel A), direct (D_1R expressing) and indirect (D_2R expressing) pathways increase or decrease inhibition of the thalamus (GPi/SNr), respectively, in a balanced way. In OCD (panel B), hyperactivation of the direct pathway non-proportionally to the indirect pathway induces a pathologic hyperactivity of cortical regions resulting in an excitatory loop. Blue arrows signify glutamatergic transmission. Red arrows signify GABAergic transmission. Abbreviations: GPe – external segment of the globus pallidus; GPi – internal segment of the globus pallidus; SNr – substantia nigra pars reticulata; STN – subthalamic nucleus; NAcc – nucleus accumbens; ACC – anterior cingulate cortex; OFC – orbitofrontal cortex.

Although CSTC circuits are traditionally described as separate, there is a functional and anatomical overlap between them (Haber, 2016). Nonetheless, in the context of OCD it is advantageous to describe the circuits separately, with each circuit named after the cortical area that it 'supplies' (orbitofrontal, anterior cingulate, dorsolateral prefrontal, motor, and oculomotor).

Of these circuits, the orbitofrontal (OFC) and anterior cingulate (ACC) cortices are involved in the pathology of OCD (Maia et al., 2008; Piras et al., 2013). These cortices have been shown to contribute to ritual formation and error detection, respectively (Hollerman et al., 2000; Van Veen and Carter, 2002). As mentioned above, 'corresponding' dorsal and ventral striatal regions are caudate for the OFC and the nucleus accumbens for ACC (Provost et al., 2015).

Findings in OCD patients suggest that the cortical structures implied to be abnormal in OCD are the orbitofrontal and anterior cingulate cortices (Maia et al., 2008; Bokor and Anderson, 2014). Moreover, subcortical abnormalities have also been consistently found in the caudate nucleus of the striatum (Calabrese et al., 1993; Hansen et al., 2002; Menzies et al., 2008). Importantly, alterations within the temporal lobe structures, such as the hippocampus and amygdala, are also present in OCD patients (Atmaca, 2011; Ullman and Pullman, 2015).
Observed abnormalities in the above-mentioned structures include structural changes, changes in volume, changes in white matter density and altered metabolism at rest, during symptom provocation, and also in cognitive tasks. Since the present thesis focuses on the activity of the hippocampus in an animal model of OCD and on cognitive flexibility, only alterations in the hippocampal region in OCD will be described.

3.6.5 The hippocampus in OCD

There are several brain regions that are recurrently implicated in OCD. These include regions that are part of the aforementioned CSTC loops, namely the lateral orbitofrontal cortex, anterior cingulate cortex, and caudate nucleus. However other structures that are not part of CSTC loops are also involved in OCD. These include the hippocampus and amygdala. The hippocampus is a well-studied brain region implicated in memory formation, consolidation, retrieval and in spatial orientation (Moscovitch et al., 2016). Importantly, animals with lesions of the hippocampus display stereotypical behavior such as a decrease of spontaneous alternation behavior (Lalonde, 2002).

Supporting its role in stereotypical behaviors, hippocampal aberrations have repeatedly been observed in OCD. Reduced hippocampal volume has been observed in patients with OCD (Kwon, 2003; Atmaca et al., 2008). Additionally, OCD patients have exhibited increased activation in the left posterior hippocampus compared to control subjects during reward based learning (Marsh et al., 2015) and greater activation of the right hippocampus during implicit sequence learning (Rauch et al., 2001, 2007). Moreover, activity of the right hippocampus is increased after symptom provocation (Adler et al., 2000) and hippocampal hyperactivity decreased after successful SSRI treatment (Kang et al., 2003).
However, medial temporal lobe structures do not fit within the established theoretical framework of OCD etiology. One theory that considers the hippocampus in OCD pathology gives it a role in compensating for deficiencies in procedural memory in OCD (Ullman and Pullman, 2015).

Many brain regions implicated in OCD are important in cognitive flexibility, reward-based decision-making, response inhibition and task switching (Pauls et al., 2014). Therefore, it is worth asking if OCD patients are also impaired in tasks that test these abilities. Since OCD behavior is often described as very inflexible, it is not surprising that flexibility has become a cognitive domain that has received much attention. The next chapter will describe how different forms of cognitive flexibility are compromised in OCD patients.

3.7 Cognitive flexibility in OCD

Cognitive flexibility is the ability to change a behavior in response to a changing environment (Dajani and Uddin, 2015). Adaptation to new situations is quintessential to the survival of almost every species on Earth. However, inflexible or rigid behavior can also be adaptive. When conditions are stable, the formation of automatic behavioral patterns (rituals) decreases the cognitive load for many otherwise mundane tasks. OCD itself is marked by very inflexible, rigid behavior (both obsessions and compulsions rarely change). In fact, OCD has been considered to be an exaggeration of the natural tendency to form adaptive habits (Gillan et al., 2011).

As hinted above, many of the brain regions implicated in pathology of OCD are also implicated in cognitive flexibility. These brain regions include the orbitofrontal cortex (Schoenbaum et al., 2007), anterior cingulate cortex (Ragozzino and Rozman, 2007), amygdala (Schoenbaum et al., 2003) and hippocampus (Rubin et al., 2014). Not surprisingly then, cognitive flexibility has become an intensively studied characteristic of OCD (Gillan et al., 2011). Although an impairment of cognitive flexibility is currently considered to be integral part of OCD, it is not true that all forms of cognitive flexibility are compromised in OCD. The next section discusses the three most common types of cognitive flexibility – as tested in experimental environments – and how they relate to OCD.

3.7.1 Attentional Set Shifting

Attentional set shifting tests the capability of switching attention from one aspect of a stimulus to another without receiving a cue regarding contingency changes. However, feedback is available if the selection was 'correct' or 'wrong'. Only changes in feedback can be used to determine changes in contingencies. Set shifting is an essential aspect of cognitive flexibility (Dajani and Uddin, 2015) and it is commonly tested by Intra-dimensional/Extra-dimensional shift tasks. In these tasks, patients are usually presented with cards representing objects with two different dimensions – attributes such as *color* and *shape*. The patient determines a rule (which color/shape is rewarded) and applies the rule until the feedback changes. After a feedback change patients are challenged to determine a new rule and follow it. Intra-dimensional set shifting occurs within a dimension – when a *red color*, for example, was a rewarded attribute and it changes to a *blue color*. Extra-dimensional set shifting, on the other hand, is tested when the change of the rule happen outside of the dimension – for example when the *red color*, which was previously the rewarded attribute, stops being rewarded in favor of, for example, *square shaped objects*.

In OCD patients, some studies have reported reduced performance in extra-dimensional set shifts (Veale et al., 1996; Watkins et al., 2005; Chamberlain, 2007), while others have reported deficits in intra-dimensional set shifts (Veale et al., 1996; Fenger et al., 2005). Overall, deficits in set shifting in OCD patients present as an increase in perseverative errors – patients more often follow an old rule despite a change in feedback (Shin et al., 2014; Snyder et al., 2015). Recruitment of brain regions in OCD patients during set shifting was not reported in these studies, however.

3.7.2 Reversal learning

Reversal learning is considered to be the simplest test of cognitive flexibility. Generally, it is similar to intra-dimensional shifting, but there are only two options to choose from. Subjects are reinforced to respond to one choice/spatial location until a criterion is reached. Next, reinforcement changes to another choice/spatial location (Izquierdo et al., 2017). Damage to the OFC specifically is associated with impaired reversal learning (Dias et al., 1996; Hornak et al., 2004; Boulougouris et al., 2007), therefore, the performance of OCD patients in reversal learning has been of great interest.

Researchers have often observed no behavioral deficiencies in reversal learning in OCD patients; however, they have often observed changes in the recruitment of brain regions within CTSC loops during such tasks (Chamberlain et al., 2008). Remijnse and colleagues (Remijnse et al., 2006a) showed a reduced responsiveness of the OFC and the caudate during rewarded switching reversal tasks in OCD patients. Another study reported a lower lateral activity in the OFC and lateral prefrontal cortex in patients with OCD during a reversal task (Menzies et al., 2008). Behaviorally, however, apart from a slower response, no performance deficiencies during the task were detected (Valerius et al., 2008; Ersche et al., 2011). The observed slowness may suggest that OCD patients require more processing time when challenged with altered response-reward contingencies and have an altered processing of cognitive information during reversal learning.

3.7.3 Task switching

Task switching is represented by cued switching tasks (Kortte et al., 2002). During the task, the subject has to work on two parallel tasks that alternate. Rules of the task are explicitly stated and errors are marked. Immediate feedback is also provided when errors are made.

OCD patients showed increased error rates and lower activation of the OFC, ACC, and caudate nucleus during cued task switching compared to control subjects (Gu et al., 2007). Reminjse and colleagues also found hypoactivation of the OFC in OCD patients during a similar switching task (Remijnse et al., 2013). However, in contrast to the previous study, these authors found hyperactivation of the ACC. Although Remijnse and colleagues observed slower reaction times, the error rate was lower in OCD patients compared to controls. Similarly, Vriend and colleagues found a more accurate, but slower, performance of OCD patients on a similar switching task (Vriend et al., 2013). These results were explained as a strategic tradeoff for the sake of not making mistakes in OCD patients.

Cognitive flexibility, when measured in experimental settings, is generally not impaired in OCD patients; however, general slowness and abnormalities in brain activations has often been observed. Slowness of responses in OCD possibly indicates a higher cognitive load. It is therefore possible that a more difficult task would reveal a behavioral deficit. Moreover, when tested, a similar alteration in brain circuitry was also observed in unaffected relatives of OCD patients (Chamberlain, 2007; Menzies et al., 2008; Rajender et al., 2011). Therefore, abnormal neurophysiological processing during tasks requiring cognitive flexibility could be an endophenotype of OCD (Gruner and Pittenger, 2017). Importantly, cognitive flexibility is much more easily tested compared to obsessive thoughts and compulsive behaviors in animal models.

3.8 Studying neuronal substrates of OCD using animals models

Many brain regions are implicated in OCD, yet where and how stereotypical behavior is generated is still unknown. Causal relationships can mostly only be deciphered through intentional manipulation of an attribute that is being tested as causative. Of course, such manipulations are unethical to perform in humans, so animal models must be used. Apart from the possibility to test causal relationships, animal models allow the use of much more invasive methods to probe brain function.

Exploring brain activity using high-resolution molecular imaging using in situ fluorescence hybridization (FISH) of immediate early gene (IEG) mRNA is of great help in this regard. It allows the exploration of brain activity at single cell resolution during any selected behavior in many brain regions at once (Kubik et al., 2007). This method is similar to magnetic resonance imaging in the sense that it allows the visualization of brain activity in many brain areas.

Changes in the expression of genes related to neuronal activity – IEGs – can be used to map preceding neuronal activity (Okuno, 2011). Upregulation of the expression of these genes is triggered by the activation of cAMP-response element binding proteins (CREB) preceded by a Ca_2 influx into the neuron (Mermelstein et al., 2000; Vazdarjanova et al., 2006). Because of this expression, IEGs, such as *Arc* and *cFos* are used as markers of neuronal plasticity (Minatohara et al., 2015). *Arc* (activity - regulated cytoskeletal associated protein, or Arg 3.1) mRNA can be detected in the nucleus in a very short time window – 2-5 minutes following neuronal activation (Kubik et al., 2007). For this reason, high-resolution molecular imaging of *Arc* mRNA is advantageous for detecting neuronal correlates of discrete bouts of behavioral activity. Methods such as this offer unique information about neuronal activity accompanying behavior in animal models.

3.8.1 The quinpirole sensitization model of OCD

Although there are numerous models of obsessive-compulsive disorder, few of them possess such striking similarity to OCD behavior as the quinpirole-sensitized model (QSM). Rats sensitized with the dopamine D_2/D_3 receptor agonist quinpirole (QNP) produce checking behavior very similar to OCD patients. Because of this, and because this model is the one within which experiments in this work were conducted, it is the only OCD animal model described here.

The best way to describe an animal model is by its validity – how well it models a certain disease. Validity can be subdivided into three components – face validity, construct validity and predictive validity (Willner, 1986). Face validity corresponds to the similarity in disease manifestation. In models of psychiatric disorders, face validity is mainly concerned with similarities in behavior. Construct validity is present when the same physiological substrates are affected in the disease and the animal model. These include similar alterations in neurotransmitter systems, brain structures and morphology (Albelda and Joel, 2012). Last, predictive validity is most important if an animal model is to be useful in aiding the finding of novel treatments. It corresponds to the effectiveness of the same treatments in both the disease and the animal model. No animal model is valid in all aspects. However, depending on the aim of the study, a certain type of validity may be preferred over the others. In this work, where we aim to decipher neurophysiological correlates of stereotypical checking, face validity is the most important aspect of the model.

3.8.1.1 QSM face validity

Repeated administration of quinpirole induces several stereotypical behaviors, suggesting a high face validity of this model (Stuchlik et al., 2016). When repeatedly introduced into an object-enriched open-field arena, quinpirole-sensitized animals display substantial stereotypy of behavior compared to controls. Namely, these animals make many more returns to one or two selected objects in the open-field (Szechtman et al., 2001).

Similarly, OCD patients are frequently involved in a selected few repetitive behaviors – compulsions – aimed to relieve obsessive recurrent thoughts (Stein et al., 2016). To appreciate the similarity between quinpirole sensitized animals and OCD, Szechtman and colleagues asked readers to view human behavior in ethological terms (Szechtman et al., 1998). They proposed five criteria to assess checking behavior in both rodents and humans:

1. There are one or two preferred location/objects of visiting.
2. The animal/patient returns to this location/object more often.
3. The animal/patient visits fewer other location/objects prior to the return to the favorite location/object.
4. The animal/patient preforms a characteristic set of acts when visiting these locations/objects.
5. The animal/patient behavior changes when the environment is changed.

All these criteria are met by both OCD patients and QNP sensitized rats (Szechtman et al., 2001). Apart from the striking similarity to patients in checking behaviors, the QNP sensitization model of OCD also displays a deficit in cognitive/behavioral flexibility, a hallmark of OCD – as was discussed in the previous chapter.

3.8.1.2 Cognitive inflexibility in QSM

Specifically, QNP sensitized animals show reduced spontaneous alteration (Einat and Szechtman, 1995). As already mentioned, spontaneous alteration utilizes the natural tendency of animals to explore new environments (Deacon and Rawlins, 2006). In an experimental setup in a T-maze, flexibility is manifested as choosing the opposite arm to an arm visited on a previous trial when given a free choice. Cognitive inflexibility is also manifested by a deficit in a paradigm known as contrafreeloading. QSM animals choose to work for a resource, instead of obtaining it for free (Amato et al., 2008; De Carolis et al., 2011). In short, water deprived animals are first trained to obtain a drop of water by pressing a lever. Next, animals are offered a choice between pressing a lever and drinking water that is freely available (Jensen, 1963). Despite the benefits of free water access, QSM animals continue to press the lever to receive water. Continuous pressing in QSM animals is unrelated to their thirst, as these animals do not drink all the water they were offered and even drink less than control animals (De Carolis et al., 2011; Schepisi et al., 2014). Moreover, cognitive flexibility insufficiency in QSM was observed as a deficit in a two-lever spatial discrimination reversal learning task (Boulougouris et al., 2009). Clearly, mounting evidence indicates that sensitization by QNP produces two hallmark characteristics of OCD – stereotypical attendance to locations and objects, and cognitive inflexibility.

3.8.1.3 QSM construct validity

Compared to face validity, the construct validity of QSM is somewhat less convincing. In this model we consider the involvement of same neurotransmitter systems (dopamine) and same brain regions (CSTC structures) as support for construct validity.

The decrease of D2/D3 receptor binding using [11C]raclopride in QSM supports the construct validity of this model. Raclopride is a derivate of a selective antagonist of D_2 receptors. By labeling raclopride with the radioactive isotope 11C, it is possible to visualize the density of D_2 receptors (Ikoma et al., 2010). A decrease of D_2/D_3 occupancy in striatum has been shown in both OCD patients (Denys et al., 2004b) and in QNP sensitized animals (Servaes et al., 2017); but see (Culver et al., 2008).

As aforementioned, the brain regions that are repeatedly found to display alterations in OCD are the orbitofrontal cortex, anterior cingulate cortex, and caudate nucleus. In QSM, it appears that similar structures are involved, although in some cases in an opposite manner as is implicated in OCD.

Several studies have explored the activity of numerous brain regions after repeated QNP treatment. Most of these studies used local cerebral glucose utilization (LCGU) by quantitative radiography to visualize the metabolism of cortical structures after sensitization to QNP. These studies confirmed the involvement of CSTC structures in QSM; however, instead of elevated glucose utilization, as would be expected in a constructively valid OCD model, decreased utilization of glucose was observed. Carpenter and colleagues (Carpenter et al., 2003) observed a 14% decrease of LCGU in the caudate/putamen of QNP sensitized animals. This study also confirmed a decrease of activity in the nucleus accumbens. Another study that focused on anterior cortical areas showed an overall decrease of cortical activity (Richards et al., 2005). Glucose utilization was reduced in the anterior cingulate cortex (by 19%), lateral orbitofrontal cortex (by 18%) and in the medial and ventral orbitofrontal cortices (by 17%). Another study using multichannel electrophysiological recordings showed that QNP dose-dependently decreased the activity of the anterior cingulate cortex and striatum in the first 15 minutes after quinpirole application (Huang et al., 2013). Another study utilizing [18F]-FDG PET neuroimaging did not replicate the decreased cortical activity in quinpirole sensitized animals, but found a significant reduction of cerebral glucose utilization in the hippocampus (Servaes et al., 2016).

Possibly, as some authors suggest, it is possible to consider the QSM model as valid merely on the grounds of involvement of the ACC, OFC and striatal regions. Although a single study found over-activation of the ACC and striatum in later stages of QNP sensitization, most studies present a different picture – that the opposite directions of metabolic changes in OCD patients and the QNP model are very consistent, and that reconciliation is hardly possible.

3.8.1.4 QSM predictive validity

The predictive validity of QSM has not yet been explored in any great detail. However, the effectiveness of clomipramine, DBS of the nucleus accumbens and high frequency stimulation (HFS) of subthalamic nucleus supports QSM as a good predictive model of OCD. Namely, clomipramine was shown to postpone the development of checking behavior (Szechtman et al., 1998) and also to attenuate contrafreeloading (De Carolis et al., 2011). Importantly, antipsychotics alone were not capable of attenuating contrafreeloading (De Carolis et al., 2011).

On the other hand, fluoxetine, an SSRI used to alleviate OCD symptoms and to validate other animal models of OCD, did not ameliorate stereotypic behavior (Collu et al., 1997). However, DBS of the nucleus accumbens shell and core reduced checking symptoms (Mundt et al., 2009). DBS of the nucleus accumbens is a successful treatment method for OCD (Islam et al., 2014). Moreover, a successful reduction of quinpirole-induced checking was also observed after HFS of another proven target of DBS in human OCD patients – the subthalamic nucleus (Klavir et al., 2009).

3.9 Knowledge Gaps

As is clear from the text above, OCD is a relatively poorly understood multifaceted psychiatric disorder. Moreover, treatment of OCD is complicated. To treat OCD more effectively, preclinical research using valid animal models is necessary. We described a popular animal model of OCD – stereotypical checking following sensitization with D_2R agonist quinpirole. Though to the best of our knowledge dopamine levels or its metabolites have not yet been assessed in OCD patients, indirect evidence suggests the involvement of the dopamine system in OCD. Childhood OCD triggered by streptococcal infection is characterized by the production of antibodies against D_2 striatal receptors (Brimberg et al., 2012; Cox et al., 2013). Moreover, reduced D_2R binding was found in the left caudate nucleus of OCD patients using [11C]raclopride binding (Denys et al., 2004a). Also, both enhanced and reduced dopamine transporter density has been observed in OCD patients (van der Wee et al., 2004; Kim et al., 2007). Involvement of dopamine in OCD is also indicated by the efficiency of antipsychotic augmentation of SSRI treatment (Koo et al., 2010). The involvement of dopamine in OCD is therefore very likely in light of current knowledge. In this context, the QNP induced model of OCD is very promising. Since the hippocampus is often implicated in OCD but does not receive much attention by the scientific community, the focus of this work is on the activity of the hippocampus during QNP-induced stereotypical checking. Similarly, although a cognitive flexibility deficit in OCD is implicated, not many studies have addressed cognitive flexibility in QNP sensitized animals. This work assessed cognitive flexibility in QNP treated animals using a hippocampus-dependent Carousel maze task and hippocampus independent two-way active avoidance in shuttle boxes.

4 AIMS of the THESIS

AIM 1: To characterize checking behavior in quinpirole sensitized rats in an enriched open-field arena, as described by Szechtman (1998).

AIM 2: To determine if checking in quinpirole sensitized rats is associated with changes in the expression of the immediate-early gene *Arc* in the hippocampal CA1 area.

AIM 3: To determine if quinpirole sensitized rats display inflexible behavior in a hippocampus dependent Carousel maze task.

AIM 4: To determine if clomipramine, risperidone or a combination thereof ameliorates cognitive flexibility deficits in quinpirole sensitized rats.

AIM 5: To determine how quinpirole and clomipramine treatments affect performance in a hippocampus independent two-way active avoidance in shuttle boxes.

5 METHODS

5.1 Animals

Adult male Long-Evans rats from the breeding colony of the Institute of Physiology CAS were used. All rats weighed 300-400g at the start of experiment and were 12-15 weeks of age. Rats were housed 2-3 rats per cage in an air-conditioned rat room with a stable temperature of 22°C, constant humidity and 12/12-light/dark cycles. All experiments were conducted in the light phase of the day. Food and water were freely available. Prior to the experiments rats were handled for 2-min daily for 3 days. All rat manipulations were conducted in accordance with the Animal Protection Code of the Czech Republic and the corresponding directive of the European Community Council on the use of laboratory animals (2010/63/EC).

5.2 Stereotypical checking in QSM

5.2.1 Apparatus – enriched open-field arena

The open-field arena was made of white waterproof plastic surrounded by black plastic unreflective walls (arena: 95x95cm; walls: 95x50cm). Two objects were placed inside at random locations between the walls and the center (Figure 5). The whole open-field arena was elevated 1m above the floor with a video camera placed above it. During the animal testing, a trained experimenter observed the live video in an adjacent room, which was also simultaneously recorded.

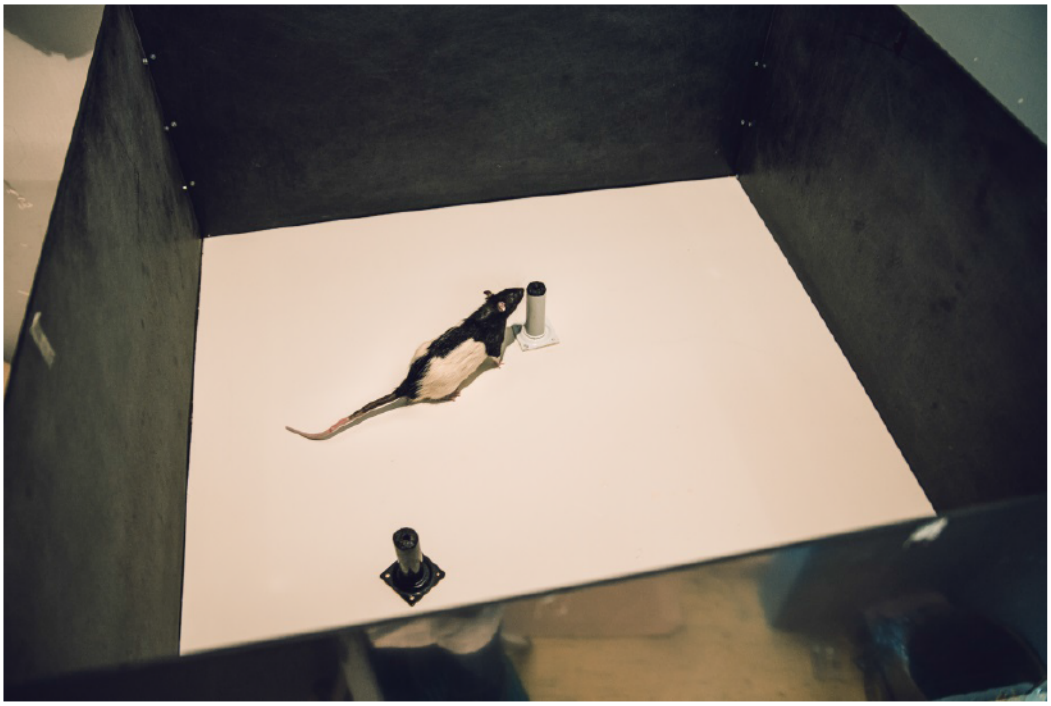

Figure 5 | Photograph of the enriched open-field arena. Photo: Kristina Maleninska, M.Sc.

5.2.2 Behavioral procedure

The spontaneous behavior of animals in the enriched open-field was observed in ten 50min daily sessions following quinpirole (QNP; n = 13) or saline (SAL; n = 13) administration. QNP was dissolved in saline and administered subcutaneously 50min prior to behavioral testing at a dose of 0.5mg/mL/kg. In the control group saline was administered at a volume of 1mL/kg 50min prior to behavioral testing. Following injection, animals were left undisturbed for 50min in their home cage. Next, each animal was placed into the most proximal corner of the enriched open-field arena facing a wall. After the experimenter left the room, a 50min recording of animals' spontaneous activity commenced.

5.2.3 Measured parameters and statistical analysis

Obtained recordings were analyzed using a video-tracking system monitoring the position of the mouse head, body and tail (Viewer2, Biobserve BmbH). The focus of analysis was on the most important objects of checking – two objects and all four corners of the arena – and the number of visits to each object/location ('zone') was the main output parameter of interest.

After obtaining the frequency of visits, zones were re-labeled based on frequencies of visits. The most popular zone was labeled 'A'; the second favorite 'B' and so on. The sum of visits in all 10 sessions was used to determine the preference of a zone for each animal separately (see Figure 6). Thus, data from each group (either QNP or SAL) could be pooled and analyzed together. Such an analysis is an extended version of previous analyses where visits to key-locale were analyzed, with the key-locale being the most frequently visited zone for each animal (De Haas et al., 2011).

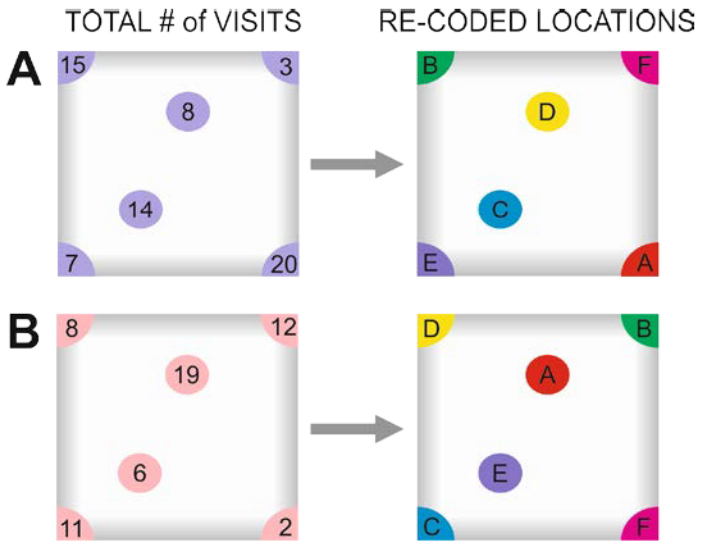

Figure 6 | Illustration of zone labeling. Panels **A** and **B** represent visits to zones by two different animals. For each animal all visits for each zone were summed from all sessions ('total # of visits'). For each rat zones were re-labeled based on the total number of visits to a given zone ('re-coded zones'). Label 'A' was given to most frequented zone, label 'B' to the second most frequented zone, etc.

5.3 *Arc* expression during checking behavior in the CA1 area of the hippocampus

This part of experiment was conducted after animals were repeatedly exposed to the arena and a stable checking pattern was established in QNP treated animals. 12 hours prior to the experiment all rats were single-housed in opaque white cages and handled very carefully. Animals received either QNP or saline based on their group designation as during sensitization sessions. Next, rats were placed into the enriched open-field in the same manner as during previous 50min sensitization sessions. This time, the session was only 5min long to equalize the locomotor activity between QNP treated and control rats. Five QNP sensitized rats and three control rats were designated as cage controls (CC groups). These animals were left in the opaque cages throughout the whole experiment. Following 5min of exploration (OF groups) or 55min following injection in opaque cage (CC groups), the rats were deeply anesthetized with isoflurane and decapitated. Their brains were quickly removed, flash-frozen in a dry-ice-cooled isopentane bath and stored in a -80°C freezer.

5.3.1 Tissue preparation

Upon tempering to -20°C, four-millimeter segments from 6-8 animals containing the hippocampi of right hemispheres were arranged in blocks, maximizing the number of within-block and between-group comparisons. Blocks were embedded in optimal cutting temperature medium (OCT; Sakura), sectioned at 20µm in a cryostat (Leica CM 1850, Germany) and mounted on gelatin-coated superfrost slides (Fisher). Later, sections were processed for fluorescence in situ hybridization as previously described (Vazdarjanova and Guzowski, 2004; Kubik et al., 2012). The protocol used is briefly described below.

Hybridization buffer, which was also used as a prehybridization buffer, was made a day in advance. Hybridization buffer was prepared from formamide (50%), saline-sodium citrate (SSC) buffer, RNAse-free water, dextran sulfate (0.05g/ml), 50X Denhardt solution (resulting concentration 1X), yeast RNA (250µl/ml) and sonicated salmon sperm DNA (500µl /ml). Glass slides with brain sections were submerged in racks into the following solutions (in 250ml containers) in the order listed:

- 4% chilled paraformaldehyde solution (5min)
- SSC buffer (4min)
- Acetic anhydride solution (10min) – this solution was prepared by dissolving 2.3g of NaCl, 3.7ml of triethanolamine in 250ml of RNAse free water, which was immediately before use enriched with 1.25ml of acetic anhydride.
- RNAse free water (dip)
- Chilled acetone/methanol mixture (1:1, 5 min)
- SSC buffer (4min)

Tissue preparation was followed by pre-hybridization. 120 µl of hybridization buffer was applied to each glass slide and cover-slipped (to ensure that liquid stayed on the glass, it was lined with two hydrophobic paraffin lines). Glass slides were covered by a cover glass and incubated at room temperature for 30min.

Meanwhile, digoxigenin-labeled *Arc* antisense riboprobes were prepared for hybridization. The probe was placed into the prepared hybridization buffer to achieve a concentration of 1ng/µm. Hybridization buffer with probe was heated to 90°C for 6 minutes to denature the riboprobes. Again, 120µl of hybridization buffer with riboprobes was applied to sections and cover-slipped. Glass slides were then placed on a rack and left to incubate at 56°C for 16 hours. During this time, *Arc* antisense riboprobes hybridized with RNA fragments of *Arc* RNA.

The next day, cover glasses were removed by dipping the slides in SSC buffer, and the brain sections were washed in 2X SSC buffer (0.3M NaCl and 0.03M sodium citrate) for 5 and 10min, consecutively. Sections were then incubated with 25 µl RNAse1 (ThermoFisher scientific EN0602) in 250ml SSC buffer, 15min in 37°C) to rinse off all un-hybridized riboprobes. Subsequently, glass slides were rinsed in 2X SSC buffer for 3 and 5min consecutively and then for 30min in warm 0.5X SSC buffer (75mM NacL and 7.5mM sodium citrate; 56°C). Next, endogenous peroxidase activity was inhibited by saturating brain sections with a 3% hydrogen peroxide solution (in 250ml of 2X SSC buffer for 15min). Then sections were rinsed twice in 2X SSC buffer for 5min. Next, glass slides were incubated for 10min in blocking buffer (TSA Perkin Elmer kit NEL404A) produced by dissolving 0.1g of blocking buffer powder in 250ml of Tris buffer saline (TBS)). Anti-digoxigenin antibody (Anti-Dig-POD, Fab fragments from sheep, Roche) conjugated with horseradish peroxidase (HPR) was then diluted 1:300 in blocking buffer. 150 µl of diluted antibody was applied to each slide, cover-slipped and left to incubate for 2h at room temperature. Following incubation cover slips were washed away in TBS. Glass slides were then washed in TBS-T (TBS enriched with 0.5mL/L Tween20) three times for 5min.

Next, the antibody signal was potentiated using a Tyramide Signal Amplification (TSA) system. Tyramide-Flourescein concentrated stock solution (TSA Perkin Elmer kit NEL404) was dissolved 1:50 in amplification diluent (part of the kit from Perkin Elmer). 90 µl of this solution was applied to each slide and left to incubate for 30min. Following incubation, the cover glass was removed in TBS and glass slides were washed twice for 5min in TBS-T. Lastly, nuclei were labeled using DAPI counterstain. Glass slides were washed with TBS for 5min and then in 1:10000 diluted DAPI in TBS for 15min. Following staining, sections were consecutively rinsed in TBS for 5 and 10min. Sections were cover-slipped using a glycerol based mounting medium (Vectashield, Vector labs, H1000) and clear nail polish to prevent drying.

5.3.2 Image acquisition and analysis

Confocal stacks were acquired from the CA1 region of the hippocampus on a Leica TCS SP8 laser-scanning microscope with an apochromatic HCX PL APO 20× immersion objective. Each stack was composed of 21 horizontal sections. The blue signal (DAPI) was imaged using 405nm excitation and a 415 – 490nm bandpass filter, and the orange/red signal (TSA-Cy3) with 555nm excitation and a 565 – 665nm bandpass filter. The laser power, gain, and offset were always set individually for the whole slide. The settings were optimized to obtain bright intra-nuclear foci of *Arc* positive cells. Three images from the CA1 were analyzed from each animal. The identity of images was blinded during analysis by a custom macro for ImageJ kindly provided by RNDr.

Stepan Kubik PhD. The proportions of *Arc*+ to *Arc*- neurons were used to map neurons active during the test session. The difference in activities of CA1 neurons between QNP treated and control groups was analyzed separately using two-way ANOVA.

5.4 Reversal learning on a Carousel arena task

5.4.1 Drug administrations

For administration of all drugs animals were removed from their home cage, injected, and returned back to the home cage. Specifically, quinpirole (QNP, SigmaAldrich, Czechia, Cat. No. Q102) was always administered subcutaneously, 30min prior to the experiment at a dose of 0.5mg/kg (dissolved in saline). Clomipramine (CMI) was administered intraperitoneally; always 1.5h prior to the experiment at a dose of 10mg/kg. Risperidone (RIS) was also administered subcutaneously 1.5h prior to the experiment at a dose of 0.25mg/kg dissolved in a drop of acetic acid and diluted with saline; final pH 3.0. Groups that did not have a scheduled administration of a drug at a certain time received saline solution in a volume appropriate for their body weight (Figure 8).

5.4.2 Experimental design

5.4.2.1 The Carousel arena

The Carousel arena was a circular metallic disk (82-cm diameter) elevated one meter above the floor with a low rim (Figure 7A). The arena was surrounded by 60-cm-high transparent Plexiglas wall and rotated at one revolution/min in a clockwise direction. An unmarked 60º wide to-be-avoided sector was defined in stable room-frame coordinates on the rotating arena (Figure 7B). Whenever a rat entered the sector for more than 300ms, constant-current regulated electric foot-shocks (AC, 50Hz, 200 – 600 μA) were delivered at 1200ms intervals until the rat left the sector. Shocks were administrated through a subcutaneous needle connector implanted on the back of the rat. The highest voltage drop of the current passing through the rat was at the high-impedance contact between the paws and grounded metal floor. The appropriate current was individualized for each rat in order to elicit a rapid escape reaction but prevent freezing. This aversive procedure has been shown to be effective and safe in previous studies (for review see (Stuchlík et al., 2013)). Each rat was allowed to move freely within the arena boundaries. To locate the sector, rats had to navigate purely using distant extra-arena landmarks, because proximal intra-arena landmarks (such as scents, marks on arena walls, urine marks or feces) were made irrelevant by arena rotation. During acquisition (ACQ) sessions the to-be-avoided sector was arbitrarily defined. During reversal (REV) sessions the sector was relocated to the opposite side of the arena in relation to acquisition sector (rotated 180°), while the direction of arena rotation remained unaltered.

The constant-current-regulated source that carries current for the shock (through the intradermal needle on the rat's back) also contained a unit for powering a light-emitting diode (LED), attached by a latex harness to the rat's back, signaling the position of the rat to an overhead camera and a computer. A second LED diode was placed on the arena periphery signaling arena rotation. The analogue signal from an overhead infrared camera was digitized by a

DT-3155 card (Data Translation, USA) and processed by Tracker software (Biosignal Group, USA), which sampled the rat´s position at the rate of 25Hz.

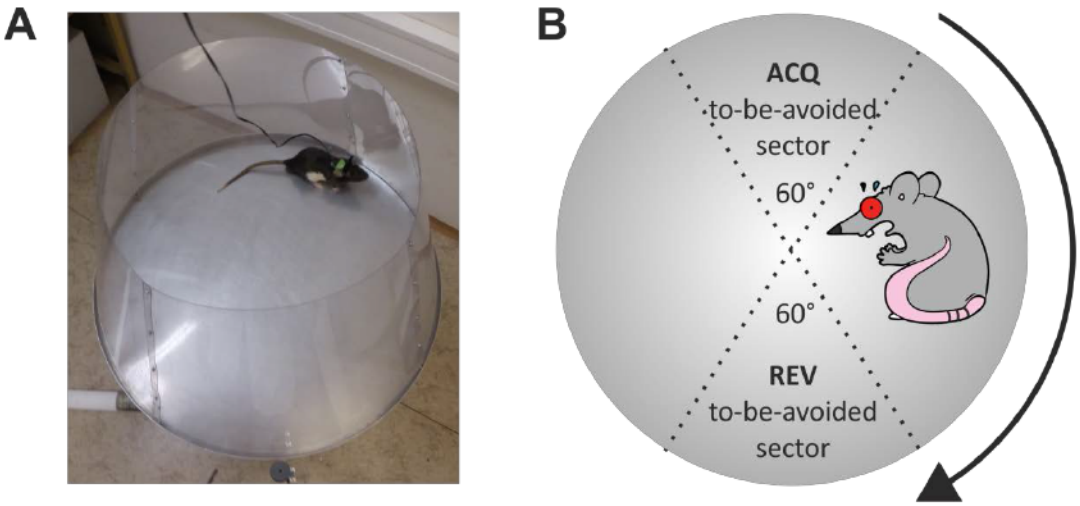

Figure 7 | Experimental setup. A. The Carousel arena is a rotating metallic disk with a 60° sector defined where rats received a mild electric foot-shock. The sector was not directly perceptible, but had to be located only using extra-arena cues, which were abundant in the experimental room. So as not to disturb the rats, the experimenter observed their movement on a TV screen from a different room. **B.** Schematic illustration of the acquisition (ACQ) and reversal (REV) to-be-avoided sectors on the Carousel arena. Arena rotated in a clockwise manner.

5.4.2.2 Procedure – acquisition and reversal testing

Behavioral testing included three phases – habituation (HAB), acquisition (ACQ) and reversal (REV) (Figure 8). All phases were composed of 30min sessions conducted every other day.

Prior to avoidance testing, rats were habituated to the apparatus and sensitized to QNP (HAB sessions). If animals were to also receive other pharmaceuticals they were administered prior to each HAB session as described in chapter **3.4.1 Drug administrations**. After the injection of drugs and elapsed appropriate post-injection interval, each rat was placed into the arena opposite to the location of the shock sector, facing the experimenter. Carousel rotation and tracking was turned on immediately after the experimenter left the room.

The schedule of drug administration remained the same for the ACQ and REV phases, when the to-be-avoided sector was already present. Since the arena rotated independently of the to-be-avoided sector, the best strategy for a rat to avoid a shock was to walk constantly or intermittently in a counter-clockwise direction to avoid being transported into the shock sector by arena rotation.

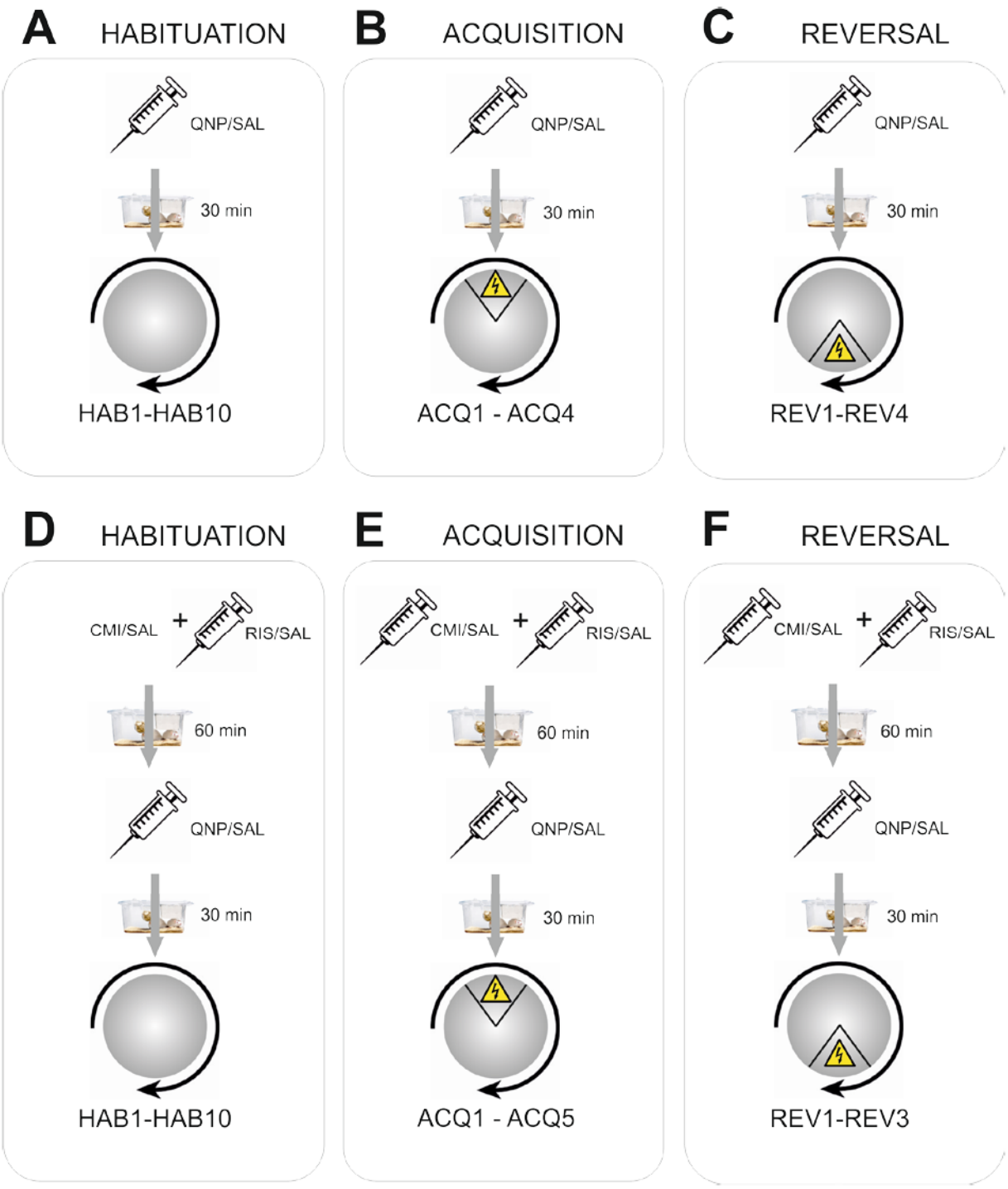

Figure 8 | Experimental scheme. Panels **A**, **B** and **C** illustrate the first experiment - testing the effect of QNP on acquisition of the Carousel maze task and reversal. Panels **D**, **E** and **F** illustrate the second experiment, testing the effects of drugs commonly used in the treatment of OCD on cognitive flexibility. Times of drug administration and numbers of acquisition and reversal sessions slightly differed between these two experiments.

5.4.2.3 Measured parameters and statistical analysis

Behavioral parameters were extracted from Tracker software (Biosignal Group, USA) and analyzed using the open-source Carousel Maze Manager (Bahník, 2013). Output parameters used to interpret the Carousel arena task performance were: locomotor activity, measured as the distance walked throughout the session in meters (movement of arena detected by peripheral LED diode was subtracted from total locomotion); number of errors (entrances into the to-be-avoided sector); time to the first error; and the percentage of time spent in the former to-be-avoided sector during reversal (in the second Carousel arena task experiment only the numbers of errors were analyzed, as the number of errors was a parameter shown to be affected by QNP treatment in the first experiment).

To assure that the variation in the number of errors between groups was not associated with higher locomotion in the QNP group, the presence of correlations between these two parameters was also assessed. For correlation analyses Pearson's product moment coefficient was used.

To discern types of errors animals made when the to-be-avoided sector was reversed, the ratio of the time spent in the to-be-avoided sector during acquisition was assessed for the first day of reversal (REV1). The calculation was similar to one described in detail by Petrasek and colleagues (Petrasek et al., 2013). In short, the arena was divided into six 60°-sectors. Apart from sectors that served as a to-be-avoided sector in acquisition and reversal there were four other sectors, n1 – n4, located in pairs between the acquisition and reversal to-be-avoided sectors, where shocks were never given. The perseverative behavior was indicated by the ratio of time spent in the acquisition to-be-avoided sector divided by the average time spent in always-safe sectors (A / (n1– n4 + A)). The reversed to-be-avoided sector was excluded from the calculation because the electric shock affected the time spent in this sector. Since perseveration can be quickly overridden by re-learning the new sector position, only the initial 10min of the first reversal session were analyzed.

Every batch of rats used in this study included rats that did not learn how to prevent entering a to-be-avoided sector. Rats that did not acquire an effective learning strategy during acquisition training were excluded from the REV phase (it is not possible to reverse learning that did not take place). The criterion for the exclusion of rats in reversal was more than 10 errors during the last 30min session (ACQ4 or ACQ5 depending on the experiment).

For the assessment of learning in ACQ and REV sessions, a two-way repeated measure ANOVA was conducted using the 'sessions' as a repeated within-subject measure and 'group' as a between-subject factor (QNP vs. SAL). When a session x group interaction was significant it was followed by simple-effects analysis. If necessary, acquisition learning was analyzed twice, once with included and once with excluded 'non-learners'. This was to uncover any bias that could be present due to the exclusion of non-learning rats (i.e. in situations where 'non-learners' had a greater impact in one group than in the other). When data were not normally distributed or did not meet the assumption of homogeneity of variance, the appropriate transformation was conducted. If no transformation was able to transform the data into a parametric data set, differences between the groups were assessed by a non-parametric Mann-Whitney sum ranks test with Bonferroni correction applied to the level of test significance. All statistical tests were considered significant at the threshold of $p<.05$ (two tailed). All statistical analyses were conducted using SPSS software (SPSS Inc., version 23, USA).

5.5 Hippocampus independent two-way active avoidance in shuttle boxes in quinpirole treated rats and augmentation with clomipramine

5.5.1 Two-way active avoidance

The apparatus (Multi Conditioning system, TSE, Germany) was a sound proof, well-lit (10Lx) and ventilated 90 x 90 x 90cm box with plain black non-transparent walls. The floor consisted of a metallic grid made of 0.5cm diameter stainless steel rods, with centers spaced 1.5cm apart. This grid was used to deliver electric shocks to animals. The box was divided into two compartments by a black insert with a 7 x 7cm cutout opening in the middle. In this task, a rat had to make an association between an acoustic conditioned stimulus (CS; 5s tone; 70db) and an unconditioned stimulus (US; an electric foot shock; 0.5mA; 500Hz, AC) separated by a 10 sec time gap. Each session began with a 60s habituation period. Immediately after habituation, CS was administered. US followed after a 10s delay. CS and US were always administered in the compartment where the animal was present at that moment. The electric foot shock (US) was terminated when the animal left the compartment. In case the animal did not leave compartment during the foot shock it was automatically terminated after 5s. The inter-trial interval (ITI) was variable, with an average of 30s (± 60%).

5.5.2 Behavioral procedure in the two-way active avoidance task

30 daily CS-US trials were performed for 5 consecutive days. Prior to each experiment, animals were sensitized to QNP and CMI for 10 consecutive days in their home cage. During testing animals also received drugs in their home cage and were placed into the apparatus 1.5h after CMI/SAL administration and 30min after QNP/SAL administration. All drugs were administered at doses described above in chapter **3.4.1 Drug administrations**. There were four treatment groups in the experiment: QNP alone, (QNP, n = 6); QNP in combination with CMI, (QNP+CMI, n = 6); CMI alone, CMI (n = 6); and control, (SAL, n = 7).

5.5.3 Measured parameters and statistical analysis

Key parameters measured were the number of escapes after the acoustic conditioned stimulus (conditioned stimulus escape; CSE) and escapes after electric foot shocks (unconditioned stimulus escape; USE). The ratio of USE to CSE was analyzed using the formula USE / (CSE + 1). Also, the number of escape failures was recorded. Three-way ANOVA was used to analyze the results, with 'group' (CMI/SAL or QNP/SAL) a between-subject variable and 'time' a within-subject repeated measure variable. All statistical tests were considered significant at the threshold of $p < .05$ (two-tailed). All statistical analyses were conducted using SPSS software (SPSS Inc., version 23, USA).

6 RESULTS

6.1 Enriched open-field checking in QSM

To determine the stability of checking behavior we analyzed the checking behavior of SAL (n = 13) and QNP (n = 13) treated rats. We expected QNP animals to show a more uniform session-to-session preference of zones compared to SAL animals. We utilized a general mixed linear model to determine if there were overall effects of 'treatment' (QNP or SAL) 'session' (sessions 1 – 10) and 're-coded zone' ('A'-'F'). Session and zone were entered as repeated measures into the model. The analysis revealed significant effects of treatment, session, zone, session x zone, session x treatment, zone x treatment and session x zone x treatment interactions (Table1, panel A). We the performed a highest order, three-way interaction (session x zone x treatment) by simple analysis of variance (ANOVA) to analyze the checking frequency of zones for each treatment within each session separately. In SAL treated animals there was no significant difference between zone visits in any of the sessions (Table 1B, Figure 9A). On the other hand, in the QNP group visits to zones were significantly different in sessions 1, 4, 5, 6, 7, 8, 9 and 10 (Table 1C). The Bonferroni post-hoc test revealed that significant tests in the QNP treated group were due to significantly more visits to zone 'A' compared to zone 'B' in sessions 1, 5, 7, 9 and 10; zone 'A' compared to zone 'C' in sessions 1, 5, 7, 8, 9 and 10; zone 'A' compared to zone 'D' in sessions 4 to 10; zone 'A' compared to zone 'E' in sessions 1 and 4 to 10; and zone 'A' compared to zone 'F' in sessions 1 and 4 to 10 (Table 1C, Figure 9B). Clearly, the post-hoc test revealed very similar sets of differences in most of the sessions. Moreover, during the last session QNP animals showed significantly more visits to zone 'B' compared to zones 'D', 'E' and 'F' (Table 1C, Figure 9B).

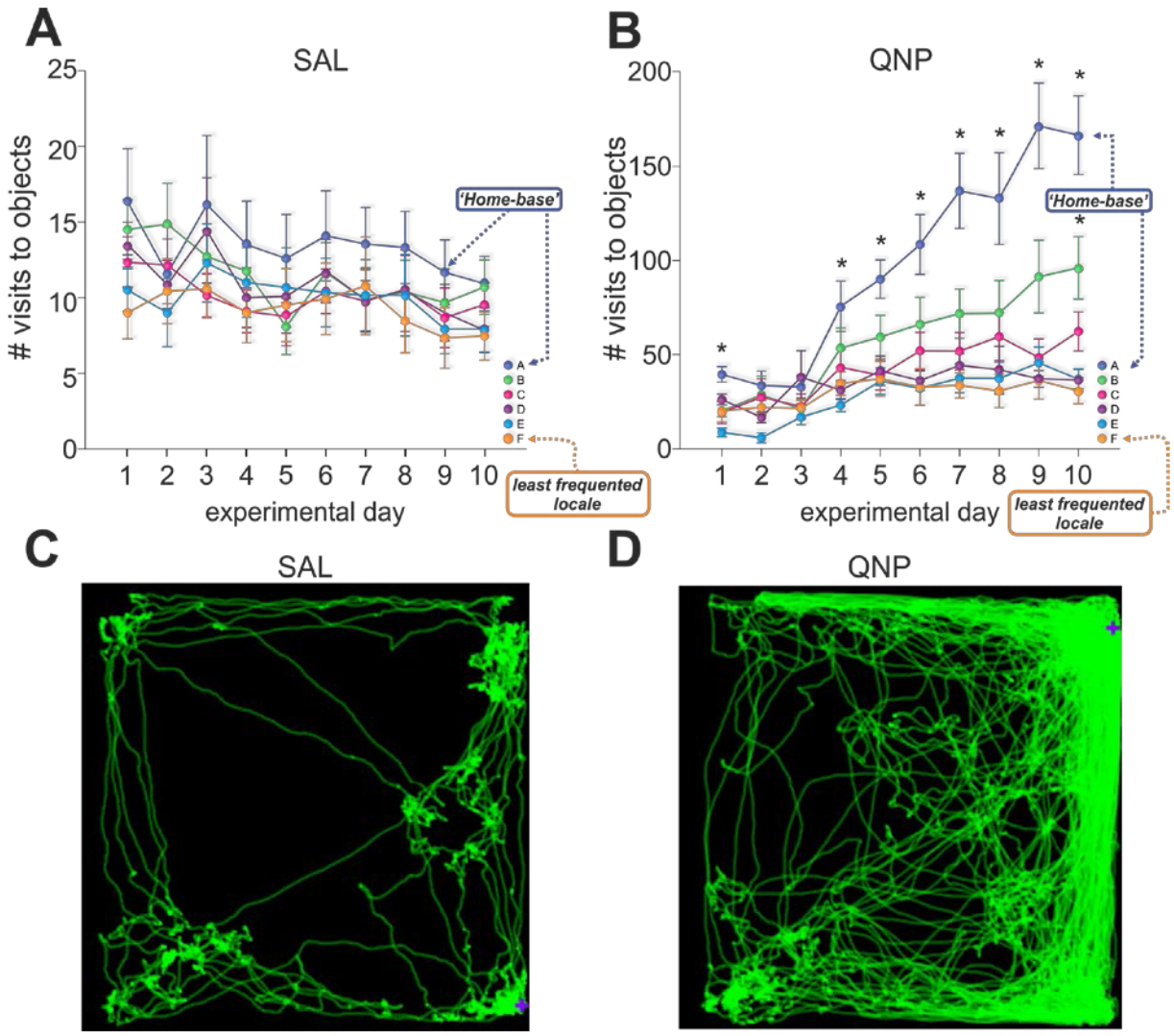

Figure 9 | Checking of zones in the enriched open-field over 10 sessions. Panel **A** shows visits to open-field re-coded zones in SAL treated rats, where no pattern of between-session checking developed. Panel **B** shows visits to open-field re-coded zones in QNP treated rats. Significant differences between visit frequencies are based on a Bonferroni post-hoc test with the level of accepted significance set to $p < 0.05$; denoted by an asterisk (*). Panels **C** and **D** show a typical trajectory of SAL treated rats (**C**) and QNP treated rats (**D**).

A

	GENERAL MIXED MODEL - checking frequency			
	df	error df	F	Sig.
intercept	1	622.347	1759.441	**<0.001**
session	9	140.488	24.311	**<0.001**
zone	5	339.372	42.267	**<0.001**
treatment	1	622.347	712.699	**<0.001**
session * zone	45	116.27	2.762	**<0.001**
session * treatment	9	140.488	30.972	**<0.001**
zone * treatment	5	339.372	34.522	**<0.001**
session * zone * treatment	45	116.27	2.686	**<0.001**

B

		ANOVA - zones A-F						
treatment	**session**	**df**	**mean square**	**F**	**Sig.**	**error df**	**# animals**	**Bonferroni post-hoc test, p < 0.05**
SAL	1	5	42.894	1.635	0.181	30	6	N/A
SAL	2	5	27.295	0.748	0.593	36	7	N/A
SAL	3	5	36.514	0.646	0.666	36	7	N/A
SAL	4	5	36.056	0.677	0.643	66	12	N/A
SAL	5	5	30.222	0.442	0.818	66	12	N/A
SAL	6	5	28.056	0.467	0.799	66	12	N/A
SAL	7	5	27.746	0.368	0.869	72	13	N/A
SAL	8	5	31.613	0.429	0.827	72	13	N/A
SAL	9	5	27.858	0.591	0.707	66	12	N/A
SAL	10	5	30.382	0.9	0.486	72	13	N/A

C

		ANOVA - zones A-F						
treatment	**session**	**df**	**mean square**	**F**	**Sig.**	**error df**	**# animals**	**Bonferroni post-hoc test, p < 0.05**
QNP	1	5	619.578	5.963	**0.001**	30	6	**A** vs. **B,C,E,F**
QNP	2	5	692.043	2.085	0.09	36	7	N/A
QNP	3	5	462.081	1.168	0.344	36	7	N/A
QNP	4	5	4622.628	4.645	**0.001**	72	13	**A** vs. **D,E,F**
QNP	5	5	5845.99	5.472	**<0.001**	72	13	**A** vs. **B,C,D,E,F**
QNP	6	5	11333.797	6.911	**<0.001**	72	13	**A** vs. **C,D,E,F**
QNP	7	5	19567.128	11.204	**<0.001**	72	13	**A** vs. **B,C,D,E,F**
QNP	8	5	18474.146	6.264	**<0.001**	72	13	**A** vs. **C,D,E,F**
QNP	9	5	36430.636	14.333	**<0.001**	72	13	**A** vs. **B,C,D,E,F**
QNP	10	5	35857.908	17.806	**<0.001**	72	13	**A** vs. **B,C,D,E,F**; **B** vs. **A, D, E, F**

Table 1 | Important outputs from the SPSS general mixed model (**A**) and subsequent analysis of variance (ANOVA, panels **B** and **C**).

6.2 Arc expression during checking behavior in the CA1 area of the hippocampus

In this experiment we visualized the activity of brain regions implicated in OCD during active checking behavior by utilizing fluorescent in situ hybridization (FISH). Namely, we were interested in *Arc* mRNA transcription in QNP treated and SAL treated rats after a 5min exposure to the enriched open-field arena. *Arc* mRNA is an established marker of neuronal plasticity-related activity (Gallo et al., 2018) and a reliable marker of heightened neuronal firing (Barth, 2007).

We used a factorial ANOVA to analyze the percent of *Arc* positive nuclei (*Arc*+) in the CA1 area of the hippocampus. Environment (OF or CC) and treatment (QNP or SAL) were used as between-subject measures. We found a significant effect of environment [$F(1,23) = 51.527$, $p < 0.001$], treatment [$F(1,23) = 17.115$, $p<0.001$], as well as a environment x treatment interaction [$F(1,23) = 16.828$, $p< 0.001$]. We used independent sample t-tests to compare the effect of treatment in each of the environments to dissect this interaction term. This analysis revealed that QNP treated animals had 11% *Arc*+ nuclei while SAL treated rats had 30% *Arc*+ nuclei [11.2% in QNP and 30.1% in SAL animals; $t(15) = 5.586$, $p<0.001$]. In baseline conditions (CC) both QNP and SAL treated animals had less than 5% Arc+ nuclei [4.1% in QNP and 4.2% in SAL animals; $t(8) = 0.062$, $p = 0.952$] (Figure 11B). This <5% baseline *Arc*+ fraction in cage-control animals is in line with previous results acquired in our and other laboratories (Guzowski and Worley, 2001; Buchtová et al., 2016).

Next, we assessed if checking behavior remained intact in a 5min session prior to sacrifice compared to the final 50min checking session. As, from the rats' perspective the beginning of the 5min session was indistinguishable from the previous 50min sessions, we expected that the pattern of zone checking would be very similar to the previous final 50min session in QNP treated rats. We found that despite the shorter length pattern of checking, the 5min session was indeed similar to the preceding 50min sensitization session (Table 2A and B, Figure 10C). To be able to compare checking in final 50min session and 5min session preceding sacrifice, the numbers of visits to each zone were standardized. Next, we conducted a repeated measure analysis of variance (RM-ANOVA). We then assessed a significant zone x treatment interaction using one-way ANOVAs for each session and treatment separately. In SAL treated animals there was no significant difference between zone visits in either the final 50min checking session or the 5min session prior to sacrifice (Table 2B, Figure 11C). In QNP treated rats there was a significant effect of zone in both the final 50min checking session and in the 5min session prior to sacrifice (Table 2B). Namely, the significance stemmed from significantly higher visits to zone 'A' compared to zones 'E' and 'F' in both the final 50min checking session and in 5min sessions, and significantly more visits to zone 'A' compared to zones 'B', 'C' and 'D' and 'B' compared to zones 'D', 'E' and 'F' in the 50min checking session.

To exclude the possibility that differences in the numbers of *Arc*+ nuclei were driven by differences in locomotion, we compared locomotion between QNP and SAL treated animals in the 5min session prior to sacrifice. Despite the fact that QNP treated rats displayed higher locomotion on average compared to SAL treated rats, the independent sample t-test revealed that this difference was not statistically significant (Figure 10D).

A

	RM-ANOVA - standarized score of checking			
	df	error df	F	Sig.
intercept	1	102	0	1
day	1	102	0	1
session	5	102	8.505	**<0.001**
treatment	1	102	0	1
session * zone	5	102	1.513	0.192
session * treatment	1	102	0	1
zone * treatment	5	102	3.25	**0.009**
session * zone * treatment	5	102	0.617	0.687

B

	ANOVA - standarized score checking						
	df	Mean Square	F	Sig.	error df	# of animals	Bonferonni post-hoc test, p<0.05
zone SAL session 10	5	1.289	1.325	0.268	54	10	N/A
zone QNP session 10	5	7.457	22.775	**<0.001**	48	9	**A** vs. **B,C,D,E,F; B** vs. **A,D,E,F**
zone SAL *Arc* session	5	0.389	0.369	0.868	54	10	N/A
zone QNP *Arc* session	5	3.216	4.181	**0.003**	48	9	**A** vs **E,F**

Table 2 | Repeated measured analysis of variance (RM-ANOVA, panel **A**) and subsequent analysis of variance (ANOVA, panel **B**), performed in SPSS.

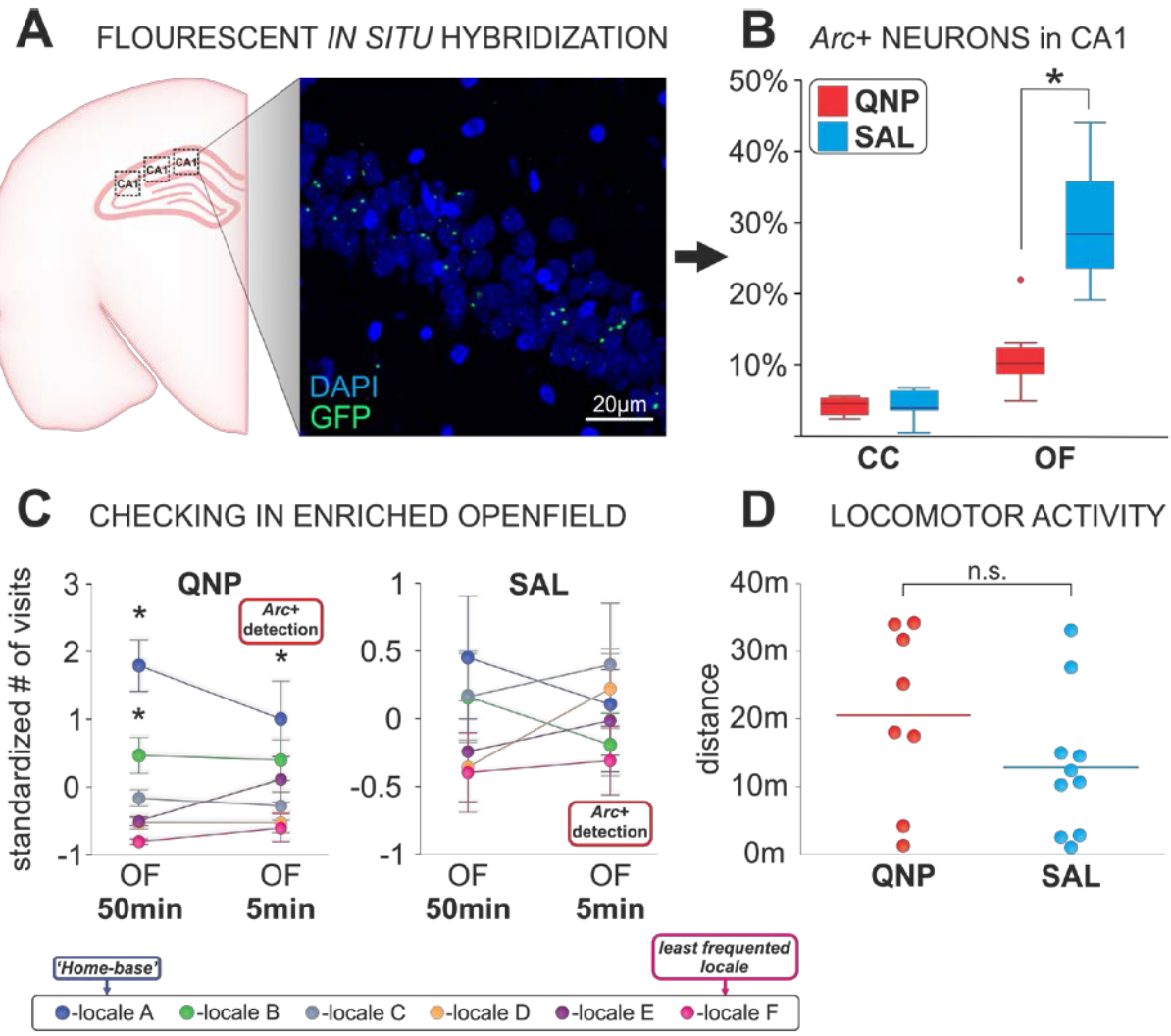

Figure 10 | Panel **A** depicts the location of sample collection (the CA1 arena of the hippocampus) and a representative acquired image. Panel **B** shows the percent of *Arc*+ neurons in the CA1 of the hippocampus. An asterisk (*) denotes significance at $p < 0.05$. QNP treated rats had three times lower numbers of *Arc*+ cells compared to SAL treated rats ($p < 0.001$). Panel **C** shows checking of zones during the final 50min checking session and the 5min session that preceded animal sacrifice, and *Arc*+ detection (mean ± standard error of mean). Panel **D** shows that in the 5min session prior to sacrifice and *Arc* detection there was no statistically significant difference in locomotion between QNP and SAL treated rats (data points and means shown).

6.3 Reversal learning on the Carousel arena in quinpirole treated rats

Since we found that QNP-treated rats has fewer *Arc*+ cells in the CA1 area of the hippocampus compared to SAL rats, we hypothesized that QNP-treated rats may be specifically impaired in a hippocampus dependent task such as the Carousel arena task. In this experiment we compared acquisition and reversal learning of the Carousel arena task between rats treated with QNP (QNP, n = 10) and control rats treated with saline (SAL, n = 11).

Two rats from each group did not reach the learning criterion of having less than 10 errors in the last acquisition session. These rats were not included in the reversal phase. Specifically, in the SAL group one of these rats froze throughout most of the session and the second did not find an effective avoidance strategy. In the QNP group neither of the two excluded rats appeared to seek an effective avoidance strategy (visual observation).

6.3.1 Locomotion

All QNP and SAL treated rats were included in the assessment of locomotor activity in four ACQ and four REV sessions by repeated measure two-way ANOVA (with the excluded animals included). ACQ and REV were analyzed together by repeated measures ANOVA. Because Mauchly's test indicated that the assumption of sphericity had been violated, (χ_2 (27) = 66.99, p < .001), degrees of freedom were corrected using the Greenhouse-Geisser estimate of sphericity (ε = .45) for tests that included a repeated measure. Importantly, QNP treated rats showed significantly higher overall locomotor activity compared to the control group [F(1,16) = 234.05 p < .001]. Moreover, overall locomotor activity varied throughout sessions [F(3.26, 52.15) = 2.95 p < .05]. Also, the analysis showed a significant effect of the sessions x groups interaction [F(3.26, 52.15) = 3.78 p < .05]. Subsequent analyses to dissect the effects of this interaction term showed a significant increase in locomotion between the third ACQ session (ACQ3) and fourth ACQ session (ACQ4) [session: F(1,16) = 9.69, p < .01; interaction: F(1,16) = 9.17, p<.01], and between acquisition day 4 (ACQ4) and the first day of reversal (REV1) [session: F(1,16) = 11.25, p < .01; interaction: F(1,16) = 9.49, p < .01]. Because a visual inspection of the control group did not show any fluctuations (Figure 11) in locomotor activity, the apparent up-regulation of activity in the QNP group accounts for both the significant sessions and the significant interaction effect.

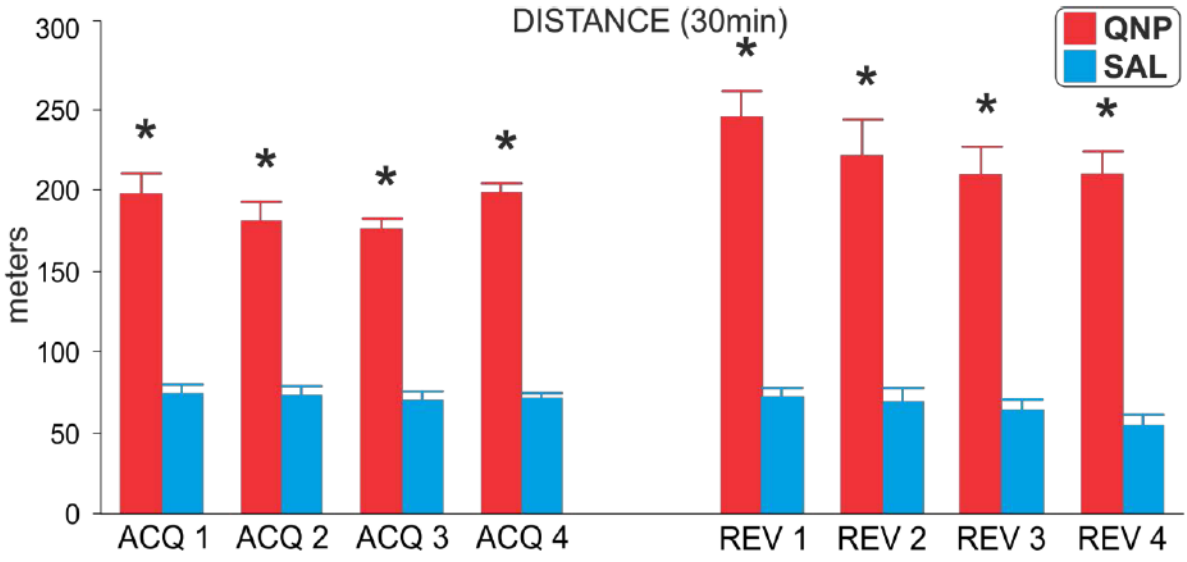

Figure 11 | Locomotion (meters/session) in quinpirole treated rats (QNP) compared to control rats treated with saline (SAL). There was significantly higher locomotion in QNP-rats compared to their controls during all sessions (ACQ1 – REV4). Data are presented as mean values ± SEM. Asterisks (*) denote a significant simple effect analysis that followed the significant group effect in ANOVA ($p < 0.05$).

6.3.2 Acquisition and reversal learning

6.3.2.1 Acquisition – number of errors

Learning behavior was analyzed for acquisition and reversal learning separately. Acquisition learning (ACQ1 – ACQ4) was analyzed with all cases included (to determine if there was a significant difference in overall learning capability between groups). In this case, the distribution of number of errors was not normal and no transformation was able to normalize them. Therefore, the Mann-Whitney test was used to compare the two experimental groups in each acquisition-learning day. The Bonferroni correction was applied to control for a family-wise error caused by the high number of comparisons (the new significance threshold was calculated to be $p< 0.013$). No significant differences in the number of errors were detected between groups in any of the acquisition sessions [ACQ1: $U = 42.00$, $z = -.92$, ns; ACQ2: $U = 54.50$, $z = -.04$, ns; ACQ3: $U = 41.50$, $z = -.97$, ns; ACQ4: $U = 42.50$, $z = -.90$, ns] (Figure 12A). The results show there was no difference between QNP and SAL groups in acquisition learning with all rats included in the study.

Next, acquisition learning was assessed with only rats that met the criteria to be included in reversal learning (less than 10 errors in the last acquisition session). After the exclusion of non-learners (> 10 errors), the data showed a normal distribution and equal variances after logarithmic transformations, which allowed a two-way repeated measure ANOVA to be conducted. 'Session' was considered a repeated measure and 'treatment group' was considered a between-subject factor. Because Mauchly's test indicated that the assumption of sphericity had been violated [$\chi2(5) = 42.39$, $p < .001$)], degrees of freedom were corrected using the Greenhouse-Geisser estimate of sphericity [$\varepsilon = .40$)] for tests that included repeated measures. Overall there was a significant decrease in the number of errors throughout sessions [sessions: $F(1.19,17.88) = 26.59$, $p < .001$], but there was no significant difference between QNP and saline treated groups [treatment: $F(1,15) = 39.95$, ns] or a sessions x treatment interaction [interaction: $F(1.19,17.88) = 0.93$, ns] (Figure 12B). Therefore, there was no significant difference between groups in acquisition learning with non-learning rats either included or excluded in the analysis.

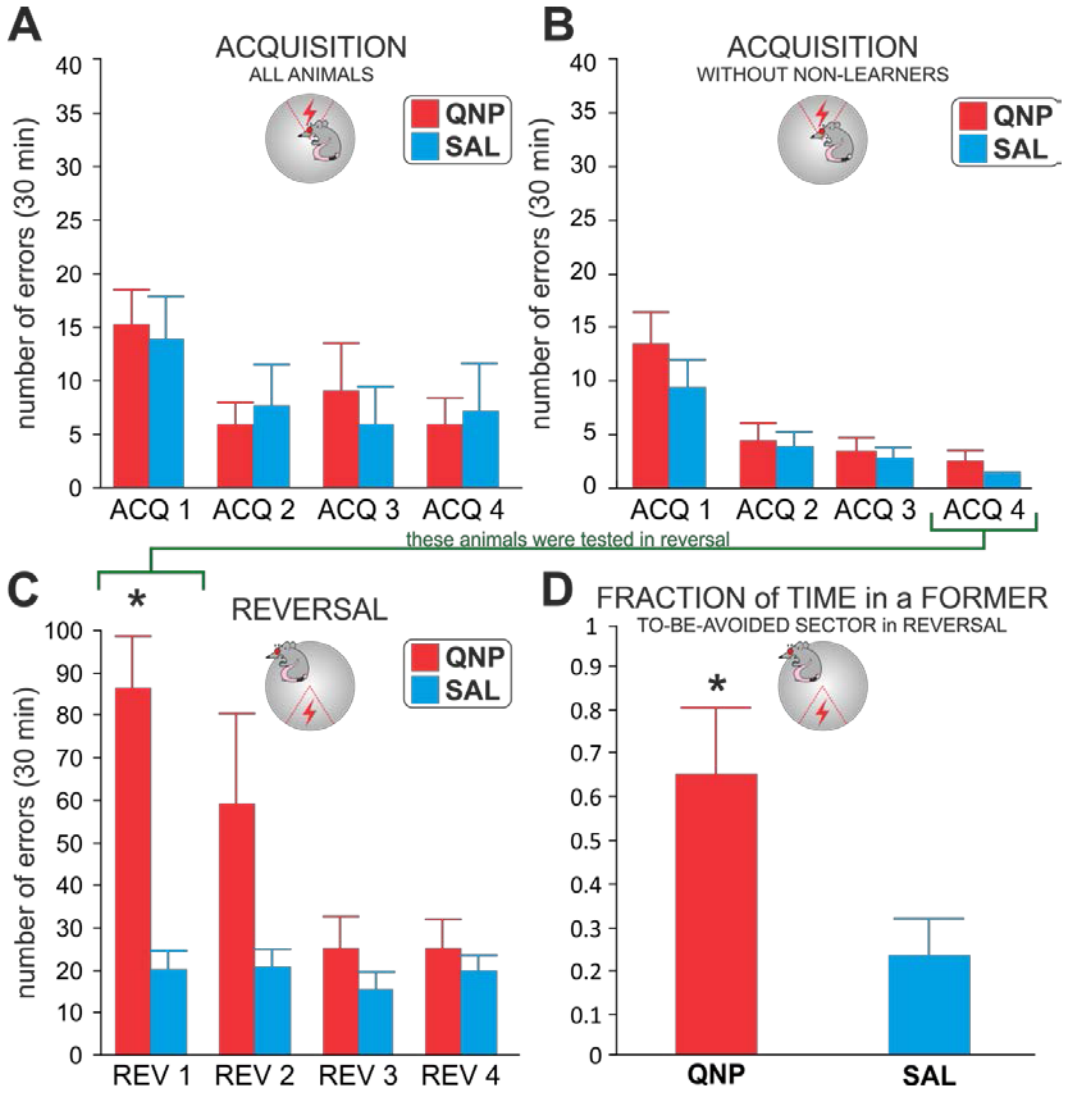

Figure 12 | **Number of errors** (entrances into the to-be-avoided sector during 30min) **in the Carousel arena** during acquisition sessions (ACQ1 – ACQ4) when all rats were included **(A)** and with non-learners excluded **(B)** and in four reversal sessions (REV1 – REV4) **(C)**. There was no difference in Carousel arena acquisition learning between QNP and SAL treated rats (**A**). Also, there was no difference between QNP and SAL treated rats that were tested in reversal (**B**). The post-Hoc test revealed a significantly more errors in QNP treated rats compared to Sal treated rats in first day of reversal testing (REV1, $p < 0.001$) **(C)**. **D** shows the percentage of time spent in the former shock sector compared to the mean time spent in always-safe sectors during first 10min of reversal (REV1). QNP-treated rats showed a significantly higher percentage of time spent in the former to-be-avoided sector, i.e. a lower rate of perseveration, compared to control rats (SAL).

6.3.2.2 Reversal – number of errors

Reversal learning is used as a proxy of cognitive flexibility in both humans and animal models. Since QNP treatment induces rigid behavior, we expected that QNP treated rats would be specifically impaired in the reversal part of the Carousel arena task.

Two-way repeated measure ANOVA was conducted on the number of errors in four reversal sessions (REV1-REV4, only rats which achieved less than 10 errors in ACQ4 were included) (Figure 13C). There was a significant decrease in errors throughout the sessions [$F(1.939, 25.204) = 5.444$ $p < .05$], even after degrees of freedom were corrected by the Greenhouse-Geisser estimate of sphericity [$\varepsilon = .65$] because the assumption of sphericity was significantly violated [$\chi2(5) = 18.673$, $p < .05$]. Importantly, QNP-treated animals made significantly more errors than SAL animals [$F(1,13) = 31.72$, $p < .001$]. The sessions x treatment interaction was also significant [$F(1.94, 25.20) = 4.61$, $p < .05$]. To further analyze this interaction term a simple effect analysis was conducted, which showed that QNP animals made significantly more errors only on the first day of reversal [REV1: $F(1,13) = 11.18$, $p < .01$; other sessions: REV2 $F(1,13) = 2.14$, ns; REV3 $F(1,13) = 0.73$, ns; REV4 $F(1,13) = 0.02$, ns].

6.3.2.3 Perseverative behavior

More details in the differences in reversal learning behavior can be seen by an analysis of the time spent in the former to-be-avoided sector after the change of shock location, defined by the ratio between the time spent in the former to-be-avoided sector and the time spent in always-safe sectors (all sectors but the reversed to-be-avoided sector). Spending less time in the former to-be-avoided sector indicates a high perseverative behavior, as the animal is still avoiding the previous to-be-avoided sector. We expected that QNP-treated animals would show increased perseveration, similarly as was shown in another task in QNP treated animals (Einat and Szechtman, 1995). Intriguingly, the t-test showed that control rats spent only $5.12 \pm 1.7\%$ of the time in the former shock zone while QNP-treated rats spent up to $13.32 \pm 2.5\%$ in the former shock zone in the first 10min of the first reversal session [REV1: $t(15) = -2.76$, $p < .05$] (Figure 13D). Therefore, counter-intuitively, control animals showed more perseveration than QNP treated animals.

6.3.2.4 Time to the first error

'Time to the first error' describes the latency (in seconds) of the rat to enter the to-be-avoided sector, and is considered to be an indicator of long-term between-sessions memory (Stuchlík et al., 2013). Values of 'time to the first error' were non-parametrically distributed in both QNP and SAL groups and no transformation was capable of normalizing them. Therefore, the non-parametric Mann-Whitney test had to be used to analyze the data for each day separately (both in acquisition and in reversal). The Bonferroni correction was applied to control for the family-wise error caused by the high number of comparisons (the new significance threshold was calculated to be $p< 0.008$). Despite an apparent trend towards better long-term memory retention in control animals compared to QNP animals (Figure 13), it was not statistically significant [ACQ2: $U = 53.00$, $z = -.14$, ns; ACQ3: $U = 37.00$, $z = -1.27$, ns; ACQ4: $U = 27.00$, $z = -1.97$, $p = 0.049$; REV2: $U = 52.00$, $z = -.21$, ns; REV3: $U = 44.50$, $z= -.42$, ns; REV4: $U = 49.00$, $z = -.42$, ns]. In summary, after the family-wise correction of significance threshold, there was no significant difference in between-session memory between saline-treated rats and rats treated with quinpirole.

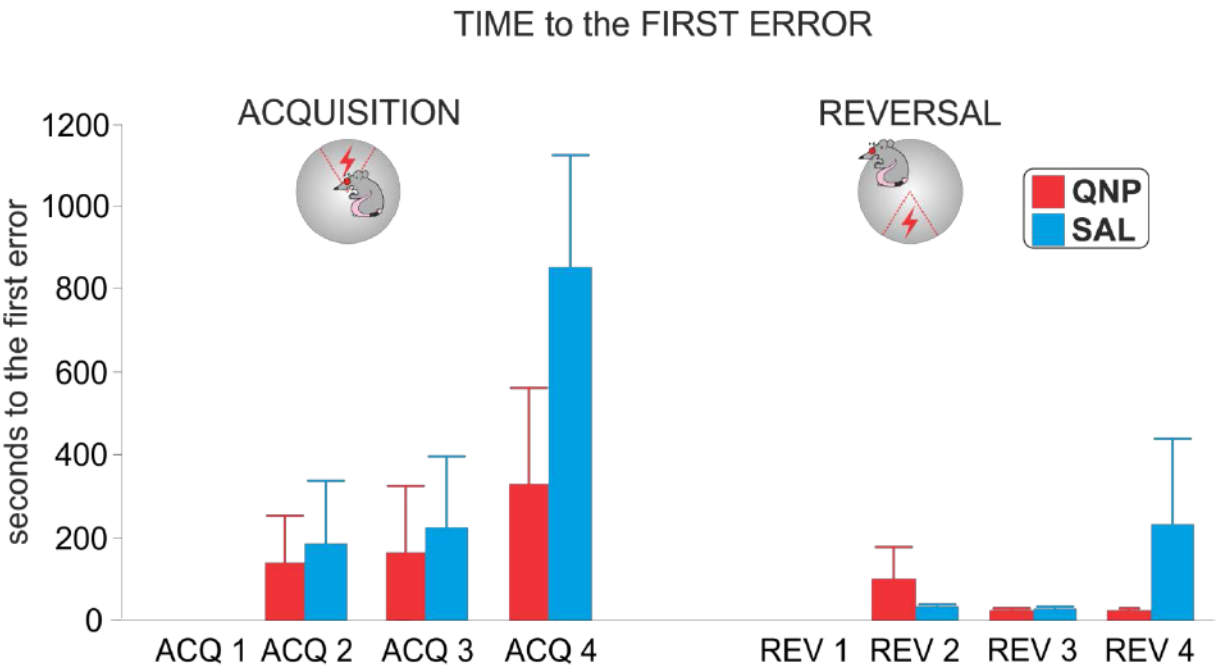

Figure 13 | Time to the first error (mean seconds ± S.E.M.) in experiment 1 (ACQ2–ACQ4; REV2–REV4). There was no significant difference between QNP and SAL treated rats at $p < 0.05$.

6.4 Effects of clomipramine and risperidone on Carousel arena performance in quinpirole treated rats

This experiment was conducted to assess if the most commonly used OCD treatments improve the cognitive flexibility deficit in reversal learning that was observed in the previous experiment. CMI had already been shown to reduce checking behavior in QNP treated rats (Szechtman et al., 1998); therefore we hypothesized that it may be also effective in improving Carousel arena task reversal. We included a combination of CMI and RIS as an augmentation to QNP, as a combination of antidepressants and antipsychotics was shown to be effective in improving symptoms in treatment resistant OCD patients (Dold et al., 2013). To test the effects of these 'treatments' on the QNP-induced reversal-learning deficit, we used a slightly modified Carousel arena task protocol (5 acquisition sessions and 3 reversal sessions), with the following treatment groups: QNP treated rats (QNP; n =15), QNP treated rats augmented with CMI (QNP+CMI, n = 11); QNP treated rats augmented with RIS (QNP+RIS; n = 11); QNP treated rats augmented with CMI and RIS together (QNP+CMI+RIS; n = 11), and saline treated control animals (SAL; n = 10).

6.4.1 Acquisition – number of errors

The number of errors to the to-be-avoided sector was used as the main output parameter. The numbers of errors were not normally distributed in either QNP or SAL groups, so logarithmic transformation was used to achieve normality. The two-way ANOVA showed a significant effect of treatment [$F(1,59) = 941.880$, $p = 0.019$]. Hochberg's post hoc test showed that only the quinpirole treated with clomipramine group made significantly more errors compared to the control group [QNP+CMI: $p = 0.038$]. Also, there was a significant overall decrease in errors during acquisition learning [$F(4,236) = 101.599$, $p < 0.001$]. A comparison of performance across acquisition sessions showed that between each set of sessions there was a significant improvement [ACQ1/ACQ2: $F(1,59) = 92.862$, $p < 0.001$, ACQ2/ACQ3: $F(1,59) = 14.513$, $p < 0.001$, ACQ3/ACQ4: $F(1,59) = 5.899$, $p = 0.018$, ACQ4/ACQ5: $F(1,59) = 20.530$, $p < 0.001$].

Moreover, there was a significant group x session interaction [$F(20.236) = 1.741$, $p = 0.028$]. Simple effects analysis explained this interaction as arising from significant differences between groups in the first acquisition session [$F(5,59) = 2.25$, $p = 0.061$] and in the fifth acquisition session [$F(5,59) = 4.81$, $p = 0.001$]. Hochberg's post hoc test of the first acquisition session revealed worse performance of the QNP+CMI group compared with the QNP+CMI+RIS group ($p = 0.039$), the group with the least number of errors. On the fifth session, the last day of acquisition, the post-hoc test showed worse performance of the QNP+RIS group [$p = 0.038$] and QNP+CMI group [$p = 0.001$] compared to controls (SAL). Results are displayed in Figure 15A.

In the group of rats receiving a combination of QNP+CMI, only four animals met the criterion of less than ten errors (n = 4); therefore the whole group was excluded from the statistical analysis of reversal learning. Other groups had only slightly decreased sample sizes due to the exclusion of animals based on this criterion (QNP, n = 10; QNP+CMI+RIS n = 9; QNP+RIS n = 8; SAL, n = 9). Analysis of the number of animals excluded for reversal did not show any significant difference between groups $\chi2(5) = 8.735$, $p = 0.122$ (the QNP+CMI group was omitted from this analysis).

6.4.2 Reversal – number of errors

The numbers of errors in each group in reversal learning were not normally distributed, and so were log transformed to achieve normality. The two-way ANOVA showed a significant effect of session [$F(2,66) = 46.081$, $p < 0.001$], and a significant decrease in errors between the REV1 and REV2 sessions [$F(1,33) = 38.297$, $p < 0.001$] as well as between the REV2 and REV3 sessions [$F(1,33) = 19.774$, $p < 0.001$]. Surprisingly, the effect of treatment was not significant [$F(3,33) = 0.296$, $p = 0.828$], suggesting that there was no difference in reversal performance between groups. However, the group x session interaction was significant [$F(6,66) = 2.817$, $p = 0.027$]. Simple effect analysis explained this interaction as arising from the difference between the tested animal groups during the first reversal session [REV1; $F(3,33) = 3.89$, $p = 0.013$]. Hochberg's post hoc test showed that the significant difference in the first reversal session was caused by the higher number of errors made by the QNP treated rats compared to the control group [$p = 0.010$]. This experiment replicates our previous findings where we found that QNP treated rats made more errors compared to the control group specifically in the first reversal session. Results are displayed in Figure 14.

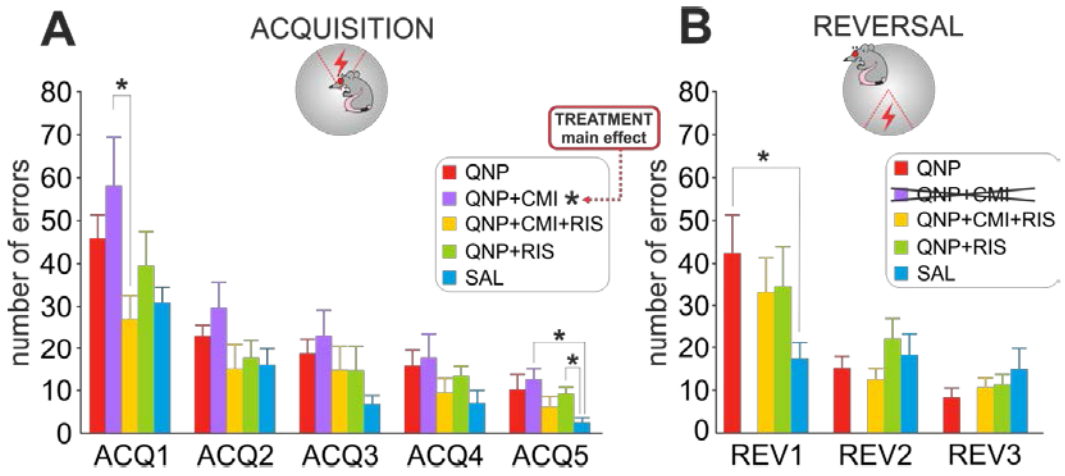

Figure 14 | Number of errors in Carousel arena acquisition (panel A; ACQ1-ACQ5) and reversal (panel B; REV1-REV3). A. Comparisons of numbers of errors (entrances to the to-be-avoided sector) in all treatment groups during acquisition. Throughout all sessions, the group receiving the combination QNP+CMI made significantly more errors than the control group. Moreover, in the first session the QNP+CMI group made significantly more errors than the group treated with a combination of QNP+CMI+RIS. On the last day of acquisition, the groups treated with QNP+RIS and QNP+CMI made significantly more errors than the SAL group. The average number of errors is displayed ±SEM. * denotes a significant difference at $p < 0.05$. **B.** Comparisons of number of errors (entrances to the to-be-avoided sector) between all treated groups during reversal. In the reversal phase of the experiment there was significant impairment in the QNP group compared to the SAL group. The average number of errors is displayed ±SEM. * denotes a significant difference from the control group at $p < 0.05$.

6.5 Hippocampus-independent two-way active avoidance in shuttle boxes in quinpirole treated rats and augmentation with clomipramine

Since we had discovered impaired acquisition learning in animals treated with a combination of QNP and CMI, we decided to test the effects of CMI and QNP in a different task. We were interested if cognitive impairment was general or specific for the hippocampus-dependent Carousel arena task. To this aim, we used two-way active avoidance conditioning in shuttle boxes, a task in which successful performance is facilitated by a lesion of the hippocampus (Wang et al., 2015). We hypothesized that QNP treated rats and QNP treated rats augmented with CMI would show superior performance compared to control rats, because our previous data showed that hippocampal function was likely compromised in QNP, CMI and QNP+CMI groups of animals.
We used rats treated with QNP (QNP; n = 6), rats treated with CMI alone (CMI; n = 6), animals treated with a combination of QNP and CMI (QNP+CMI; n = 6) and saline treated controls (SAL, n = 7).

Three-way ANOVA was used to analyze results of the active avoidance shuttle box test. Between-subject measures were QNP and CMI and the within-subject measure was the session number (session 1 – session 5). All data was logarithmically transformed to meet parametric assumptions.

The most important parameter was the number of conditioned escapes (CSE). A CSE occurs when an animal escapes after the sound is presented (before the foot shock). There was an overall increase in the number of escapes with training [sessions: $F(4,84) = 15.661$, $p < 0.001$]. Importantly, there was a significant increase in conditioned escapes (CSE) when QNP was used [$F(1,21) = 35.722$, $p < 0.001$], while CMI did not significantly affect the number of CSEs [$F(1,21) = 1.080$, $p = 0.310$]. As Figure 15A shows, both groups receiving QNP (QNP and QNP+CMI) had higher numbers of CSE. These results indicate better performance in this hippocampus-independent task when animals were treated with QNP. In line with the observed decrease in hippocampal function, QNP appears to produce a similar effect as a hippocampal lesion in this task.

The number of unconditioned escapes (USE) was assessed in the same manner. A USE occurs when an animal escapes to the next chamber after it is foot shocked. There was no change in the number of USE throughout the training [sessions: $F(4,84) = 1.611$, $p = 0.179$]. Also, neither QNP nor CMI affected the number of unconditioned escapes [QNP: $F(1,21) = 2.704$, $p = 0.115$; CMI: $F(1,21) = 2.786$, $p = 0.110$] (Figure 15B).

Analysis of the ratio of USE to CSE (which we considered a true indicator of learning) showed that there were significantly more conditioned escapes than unconditioned escapes with time [$F(1,84) = 14.640$, $p < 0.001$]. While CMI did not have an effect on the USE to CSE ratio [$F(1,21) = 0.832$, $p = 0.372$], QNP significantly increased the number of conditioned escapes in favor of unconditioned escapes [$F(1,21) = 16.781$, $p = 0.001$]. Both groups treated with QNP (QNP and QNP+CMI) had a lower USE to CSE ratio, indicating better performance in this task (Figure 15C).

Lastly, the number of escape failures was also analyzed. There was a significant reduction in the number of times when an animal did not react to either a conditioned or unconditioned stimulus [session: $F(1,84) = 4.082$, $p = 0.005$]. Although there was no significant effect of treatment on the number of escape failures [QNP: $F(1,21) = 3.215$, $p = 0.087$; CMI: $F(1,21) = 2.399$, $p = 0.136$], there was a significant CMI x QNP interaction [$F(1,21) = 5.855$, $p = 0.025$].

When CMI, QNP or both were present, animals displayed equivalently low levels of escape failures (Figure 15D), while saline treated controls (SAL) showed a higher incidence of non-responding.

In summary, clomipramine decreased non-responding behavior without affecting conditioned escapes, while QNP both decreased non-responding behavior and increased the number of conditioned escapes.

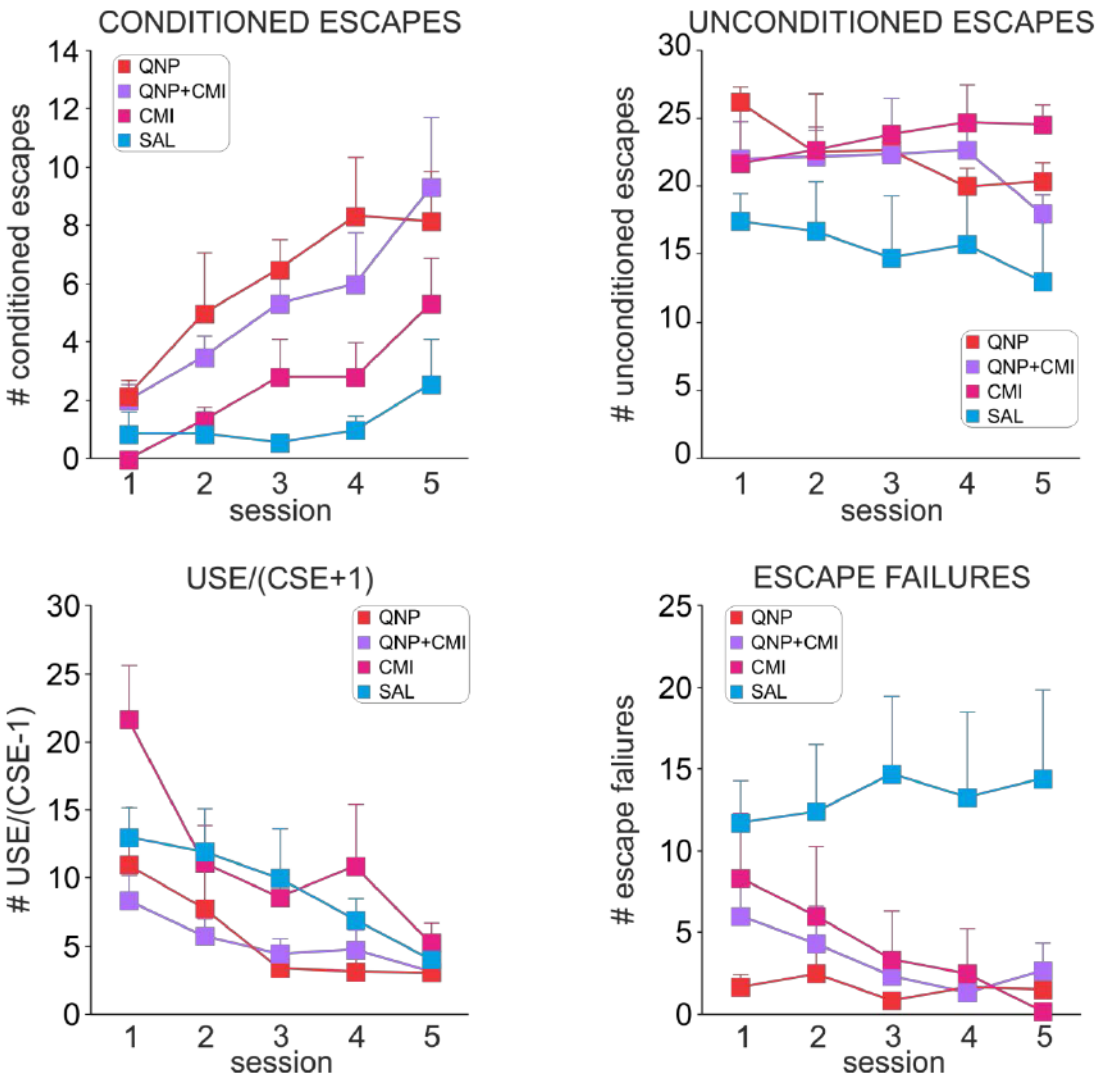

Figure 15 | Two-way active avoidance in shuttle boxes. A. The number of conditioned escapes (CSE). **B.** The number of unconditioned escapes (USE). **C.** The ratio of unconditioned escapes to conditioned escapes (USE / (CSE + 1)). **D.** The number of escape failures. There was a significant increase in the number of conditioned escapes when quinpirole was included, and significantly reduced freezing when quinpirole, clomipramine, or both were included. The average number of errors is displayed ± SEM. Significances are not included, because data was analyzed by three-way ANOVA.

7 GENERAL DISCUSSION

This dissertation thesis has focused on a detailed analysis of inflexible behavior displayed by the quinpirole sensitization model (QSM) of Obsessive Compulsive Disorder (OCD). We showed that rats treated with QNP display stable checking behavior in an enriched open-field arena, and these checks were not limited to one particular zone. Unprecedentedly, we observed that during stereotypical checking behavior the hippocampal CA1 region is less active compared to the CA1 region of control animals during exploration of the same enriched open-field arena. Supporting the deficiency of hippocampal function in QNP sensitized rats, we shown that QNP treated rats are impaired in the hippocampus-dependent Carousel arena task. One established OCD treatment – a combination of clomipramine and risperidone – reversed this deficit. Moreover, we confirmed the presence of a selective hippocampal deficit by showing that QNP treated rats are superior in a hippocampus-independent active avoidance task. Based on these results, we suggest that hippocampal dysfunction may be central to the induction of stereotypical behavior and relevant to OCD.

7.1 Between-session stability of checking behavior

Here we confirmed that rats treated with QNP display stereotypical checking as described by the inventor of this OCD animal model – prof. Szechtman (Szechtman et al., 1998). Stereotypical checking, typical of QNP sensitized animals, is traditionally observed in an open-field arena enriched with 2 – 3 objects. Animals displaying checking behavior usually repeatedly visit ('check') these objects and also repeatedly visit corners of the arena (together called zones). We reproduced this checking behavior in a smaller arena (1 x 1 m compared to 2 x 2 m in original study). In many studies, visits to the 'home base' – the most frequently visited zone – is considered the main measure of stereotypical checking. However, OCD rituals are usually complex (Swedo et al., 1989). To determine if a more complex pattern of checking activity was present we extended the analysis and examined the stability of preferences for all zones between all consecutive sessions. We found that QNP treated rats displayed the same pattern of preferences for zones in all sessions, not only for the 'home base'. QNP treated rats also displayed the same pattern of checking when the session in the enriched open field was much shorter: i.e. during the 5min sessions that preceded sacrifice for IEG *Arc* expression analysis. This is important, as CA1 hypoactivity emerges only during stereotypical checking and not during baseline conditions (cage controls or saline-treated rats in the enriched open-field arena).

7.2 Decreased hippocampal *Arc* expression during checking behavior in the enriched open-field arena in QSM

To measure the activity of the hippocampal CA1 region in the QNP sensitization model of OCD we used *in situ* hybridization of *Arc* mRNA. Although using IEG expression restricts output to a binary characterization of previously active or inactive neurons (pattern, rate and spatial specificity of firing cannot be captured), IEG expression profiles reveal important information about which neurons were recruited during a given behavioral experience. In the present study we observed a robust decrease of *Arc* expressing neurons in the CA1 of the hippocampus (a 60% decrease of *Arc*+ nuclei compared to controls) during stereotypic checking in the enriched open-

field arena in QNP treated rats. In rats that were repeatedly treated with QNP, but did not execute checking behavior, CA1 activity was comparable to control animals. This finding stresses the importance of the environmental dependency of QNP sensitization (Szechtman et al., 1993). As mentioned above, although the activity of neurons was measured during a shorter period than the 'standard' sensitization session (5min compared to 50min), the pattern of stereotypical checking remained very similar. Assessing IEG expression following 5min short sessions had an important advantage in equalizing locomotor activity between groups, as control animals were usually hypoactive later into the session. In other studies that measured regional brain activity using local cerebral glucose utilization, the possible effect of locomotion on study outcome has not been accounted for (Carpenter et al., 2003). Yet, our findings are in line with findings of studies that examined brain activity in QNP treated rats without checking activity. The [18F]-FDG uptake was decreased by 19% in the hippocampus in anesthetized QNP treated rats measured by MicroPET/CT imaging (Servaes et al., 2016), and a statistically insignificant decrease of hippocampal cerebral glucose utilization was observed in QNP treated rats when rats were sensitized in locomotor activity boxes (Carpenter et al., 2003).
We suggest three possible mechanisms by which QNP could induce decreased hippocampal activity:

1. Decreasing the overall dopamine tone induced by the QNP-mediated inhibition of dopamine release from the VTA.
2. Directly altering hippocampal function by the activation of D_2 receptors in the hippocampus.
3. QNP-mediated decreasing of the activity of regions projecting to the hippocampus.

The first possibility is that the decrease of *Arc*-expressing neurons in the CA1 results from **decreased overall dopamine tone**. It has repeatedly been described that QNP has a biphasic effect on an animal's brain. First, D_2 autoreceptors expressed by VTA neurons are activated by QNP. Activation of VTA D_2 autoreceptors inhibits dopamine release from dopaminergic terminals (Sulzer et al., 2016). Hypo-locomotion observed 5 – 30min following QNP injection has been attributed to the activation of D_2 autoreceptors (Skirboll et al., 1979). Conversely, hyper-locomotion, which occurs during time frame when our experiments were conducted (30min and longer), has been attributed to the stimulation of postsynaptic D_2 receptors (Wu et al., 1993). However, the inhibition of dopamine release from VTA persists alongside the activation of postsynaptic auto-receptors (Sesia et al., 2013). While the overall effect of QNP is complex, a reduction of dopamine tone by itself could reduce *Arc* expression, and it has been shown that tonic dopamine is important for both the encoding and persistence of memory traces (Lisman and Grace, 2005; Bethus et al., 2010; Shohamy and Adcock, 2010). A lack of dopamine induced by D_2 autoreceptors activation in the VTA could thus result in an overall decrease of encoding in the hippocampus and thus reduce the expression of plasticity-related genes such as *Arc*.

The second possibility is that QNP decreases *Arc* expression **directly by acting on D2 receptors expressed in the hippocampus**. D_2 receptors are widely expressed in the hippocampus in two specific neuronal subtypes – glutamatergic mossy cells (Etter and Krezel, 2014) and inhibitory GABAergic cells (Gangarossa et al., 2012; Puighermanal et al., 2015). Activation of D_2 receptors by quinpirole atypically induces an increase in the excitability of mossy cells in the

hippocampal *hilus* (Etter and Krezel, 2014). Although the exact function of mossy cells is currently unknown, it was shown that they send excitatory projections to both granule cells in the dentate gyrus and to inhibitory interneurons mediating feed-forward inhibition (Scharfman, 2018). The net effect of the activation of hilar cells is a decrease of dentate gyrus output (Hsu et al., 2016). Since the dentate gyrus is an important input structure to the CA1 via CA3 hippocampal regions, it is likely that CA1 activity could be decreased in this manner. Therefore, a QNP-mediated effect on mossy cells and inhibitory cells in the hippocampal *hilus* may also explain the reduction of *Arc* expression in the CA1 region.

The last possibility is that reduced *Arc* expression in the CA1 area is a result of **decreased excitatory input from upstream structures**. In this study we did not look at *Arc* expression in any of the upstream structures of the hippocampus (most notably the entorhinal cortex and subiculum). However, the effect of D_2 receptor activation in the cortex is difficult to detangle. D_2 activation has been described as both decreasing (Tseng and O'Donnell, 2007) and increasing cortical pyramidal excitability (Vitrac et al., 2014; Robinson and Sohal, 2017), and to increase cortical inhibition (Xu and Yao, 2010) by different mechanisms. It is therefore possible that CA1 *Arc* expression is reduced as a result of decreased input to the hippocampus, but it is as likely that cortical input is increased following QNP administration. Regardless of the exact mechanism, the decrease of CA1 *Arc* expression suggests that the overall hippocampal output to structures downstream of the hippocampus is reduced.

7.3 Carousel arena task learning in QSM

Next, we observed a reversal learning deficit in QNP treated rats, further supporting the notion of functionally-relevant hippocampal hypo-activity in QNP treated rats. Specifically, QNP treated rats showed Carousel arena task acquisition learning comparable with control rats, but displayed impaired reversal learning that was not associated with perseverative responding.

7.3.1 Acquisition learning on the Carousel arena in QSM

QNP treated rats acquired the Carousel maze task at a similar rate as the control group, despite their characteristic hyper-locomotion observed following QNP treatment. Importantly, the same number of rats per group reached the threshold of 10 errors in a 30min session by the fourth session. This indicates that chronic sensitization of dopamine D_2-like receptors by QNP (and their ongoing stimulation) does not affect cognitive coordination (perceptual segregation). Our results replicate a study conducted in our laboratory that showed that quinpirole does not impair Carousel arena task acquisition (Stuchlik et al., 2007). Yet, performance on some other spatial tasks is impaired by systemic D_2 receptor activation. For example, quinpirole was shown to induce cognitive deficits in the Morris Water maze (Cardoso-Cruz et al., 2014). Although D_2 availability was linked to episodic memory capability in humans (Nyberg et al., 2016), activating D_2 receptors by QNP did not alter long-term memory retention in rats (de Lima et al., 2011).

7.3.2 Reversal learning of the Carousel arena task in QSM

In the reversal learning, QNP treated rats showed a significant, albeit transient, reversal-learning deficit. This reversal deficit was manifested as an increased number of errors during the first session after reversal of to-be-avoided sector compared to the control group. It should be noted that this deficit was specific only for the beginning of reversal training, since by the third and fourth reversal sessions QNP treated rats displayed a comparable number of errors as the control group. The next section discusses cognitive processes that may have been impaired during reversal in QSM.

7.3.2.1 Dissecting reversal learning deficit in QSM

It has been proposed that there are three parallel processes that occur during successful reversal (Klanker et al., 2013):

1. Extinction of a response that is no longer rewarded
2. Response maintenance
3. Behavioral switch to the new reward/punishment

If a **deficit in extinction** causally impaired reversal in QNP treated rats, a perseverative response is expected. However, in our study we actually observed lower perseveration in rats treated with QNP. However, this lack of perseveration is in contrast with repeated findings that chronic administration of quinpirole induces perseverative behavior in alternation tasks (Einat and Szechtman, 1995; Kontis et al., 2008). Similarly, perseverance is indicated by studies documenting enhanced 'compulsive' lever pressing after repeated administration of QNP (Joel et al., 2001). Moreover, in lever pressing task a marked reversal learning deficit was associated with a high incidence of perseverative responding following QNP administration (Boulougouris et al., 2009). Despite the oft-reported perseverative behavior, non-perseverative behavior in reversal was also observed following D_2-like manipulation. For example, non-perseverative errors in reversal were demonstrated when quinpirole was infused locally into the nucleus accumbens (Haluk and Floresco, 2009). Non-perseverative errors were also observed in spatial reversal learning in humans after systemic administration of another D2-like agonist – bromocriptine (Mehta et al., 2001). Still, the low perseveration in QNP treated rats in our study is intriguing in light of previous studies that have reported high perseverative behavior specifically in chronically QNP treated rats (Kontis et al., 2008; Boulougouris et al., 2009).

Reversal impairment due to a **defect in a behavioral switch** is associated with disorganized behavior (Klanker et al., 2013). Behavioral switches are related to responses to new reward/punishment contingencies. Indeed, it was found that D_2 antagonists facilitate task switching while D_2 agonists impair behavioral switching (van der Schaaf et al., 2014).

A **defect in response maintenance** is associated with an inability to improve in both acquisition and reversal tasks (both between sessions and within one session) (Klanker et al., 2013). Response maintenance refers to the persistence of responding despite the high effort needed to acquire a reward. Although it was shown that mesolimbic dopamine is needed to

maintain instrumental responding (Fischbach-Weiss et al., 2018), in the light of intact acquisition in QNP treated rats this possibility is unlikely.

The absence of perseverative errors and intact acquisition suggest that in QNP treated rats **deficient behavioral switching** drives the impaired reversal learning. Since both the tonic and phasic release of dopamine mediates the valence of reward and punishment (Bromberg-Martin et al., 2010), the deregulation of dopaminergic signaling could easily induce the reversal learning deficit that was observed in the Carousel arena task.

One important limitation is that QNP treated animals could be less sensitive to electric shocks. This possibility was not directly tested in our study. However, since acquisition learning in both QNP treated and control rats were similar, we assume that their sensitivity to electrical stimulation is unaltered by QNP treatment. Studies testing a QNP effect on pain sensitivity offer contradictory results, with some studies proposing analgesic (Roane and Paul, 1992) and some hypo-analgesic effects (Magnusson and Fisher, 2000; Munro, 2007). Lastly, a lesion of dopaminergic projections to the striatum had no effect on escape learning or responses to electric shocks, suggesting that intact dopaminergic transmission is not necessary for successful avoidance learning (Price and Fibiger, 1975).

7.4 Clomipramine and clomipramine augmented by risperidone in QNP treated rats

In the last two experiments, we investigated the effects of commonly used pharmacological treatments used in OCD on cognitive flexibility performance in QNP treated rats. First, we showed a cognitive flexibility deficit in QNP treated rats. Second, we observed effects of the tricyclic antidepressant clomipramine (CMI), the antipsychotic risperidone (RIS), and a combination thereof on cognitive coordination and flexibility. We discovered that CMI impaired acquisition learning in QNP treated rats. Moreover, we showed that RIS does not ameliorate the learning impairment of QNP treated rats, but the administration of CMI and RIS together – a combination of pharmaceuticals used in SRI-resistant OCD patients – ameliorates the reversal learning deficit induced by QNP treatment. Last, using a behavioral test that does not require the hippocampus, we discovered that CMI augmentation in QNP treated rats is specific for the hippocampus-dependent Carousel arena task.

7.4.1 A proposed mechanisms of action of the CMI on hippocampal function

QNP and CMI both inhibit dopamine release from the ventral tegmental area (VTA) in a direct and indirect manner, respectively. Therefore, CMI may potentiate the effects of QNP. Dopamine D_2 receptors are present in the VTA as auto-receptors (Li et al., 1996), and it was shown that stimulation of D_2 receptors by quinpirole reduces the firing of dopamine (DA) neurons (Koulchitsky et al., 2012; Sesia et al., 2013). Moreover, serotonin neurons exert an inhibitory effect on dopaminergic neurons in the VTA (Ugedo et al., 1989), and systemic SSRI administration was shown to decrease VTA dopamine release (Prisco and Esposito, 1995). Thus, the addition of CMI to QNP treatment may have decreased dopaminergic tone even further. Since the transfer of information from the hippocampus to the PFC is dependent on D_1 activity (Goto

and Grace, 2008), a dramatic reduction of DA tone could manifest as deficient acquisition learning in hippocampus-dependent tasks. A reduction of hippocampus–PFC communication, on the other hand, could be beneficial in solving a hippocampus-independent two-way active avoidance task since irrelevant spatial information supplied by the hippocampus does not interfere with the task. Indeed, it has been shown that lesions of the hippocampus increase the number of conditioned escapes, probably by enforcing only a tone-shock association while place information (which is irrelevant in this task version) is disregarded (Wang et al., 2015).

7.4.2 RIS and CMI co-administration in QNP treated rats rescues reversal learning

Despite the detrimental effect of CMI alone and no positive effect of RIS alone when administered to QNP treated rats, when CMI and RIS are co-administered, the reversal deficit is decreased in QNP treated rats. Offering a potential mechanism of action would be pure conjecture given the complexity of the molecular targets of both CMI and RIS. Yet, our findings still have important implications for OCD treatment. Risperidone is the most effective antipsychotic prescribed to augment the SRI treatment of OCD (Dold et al., 2013), and it is intriguing that risperidone augmentation improved reversal learning in an animal model of OCD. It has been proposed that antipsychotics benefit patients treated with antidepressants because they decrease dopaminergic tone (Chernoloz et al., 2009). This is in line with our proposed mechanism of an acquisition learning deficit after clomipramine augmentation (which could have aggravated a dopamine neurotransmission reduction). The effectiveness of clomipramine and risperidone in rescuing the reversal deficit in QNP treated rats opens up a possibility that a combination of SRI with an antipsychotic may be the best treatment option for OCD patients with cognitive flexibility deficits. Based on the consistent checking behavior observed in the enriched open-field arena and the reversal deficit in the Carousel arena task we propose that QNP treatment-induced checking behavior possibly models a subgroup of OCD patients with a cognitive flexibility deficit.

7.5 Hippocampal dysfunction in OCD?

Cognitive flexibility deficits have been associated with obsessive-compulsive disorder for a long time (reviewed in (Benzina et al., 2016)), and the rigid and inflexible behavior characteristic for OCD rightly alludes to deficiencies in overall cognitive flexibility. Both the over-activity and abnormal functioning of the orbitofrontal cortex (OFC) has often been regarded as a common neural substrate of OCD and accompanying cognitive deficits (Remijnse et al., 2006b). However, the hippocampus may contribute to both cognitive inflexibility and OCD as well.

Hippocampal aberrations are implicated in OCD by several studies. Reduced hippocampal volume has repeatedly been observed in patients with OCD (Kang et al., 2003; Atmaca et al., 2008; Reess et al., 2018). Shape deformity of the hippocampus was also observed in OCD patients (Hong et al., 2007). Moreover, OCD patients show abnormal activation of the hippocampus during reward-based learning (Marsh et al., 2015) and during implicit sequence learning (Rauch et al., 1997, 2001). Importantly, activity of the right hippocampus was decreased after successful SSRI treatment (Kang et al., 2003) and, conversely, was elevated after symptom provocation (Adler et al., 2000). This could indicate that the hippocampus may be directly

involved in the OCD pathology. Yet, the implications of these findings to OCD are generally poorly discussed in the literature (Atmaca, 2011). A single theory that considers the hippocampus in OCD pathology gives it a role of compensating for deficiencies in procedural memory (Ullman and Pullman, 2015). However, given that the hippocampus is overactive even during symptom provocation (Adler et al., 2000), and that OCD has developed in several cases following traumatic brain injury localized to the temporal lobe (Max et al., 1995), it is possible that the hippocampus is involved more directly in OCD than is currently thought.

Although hippocampal aberrations observed in OCD have been proposed to play a compensatory role in OCD (Ullman and Pullman, 2015), we propose that the role of the hippocampus may be causal. CSTC circuits and the hippocampus are involved, in parallel, in the generation of responses. The caudate that is most often implicated in OCD, is involved in habitual response-based learning, while the nucleus accumbens (ventral striatum) and hippocampus are implied in flexible goal-directed behaviors. In a seminal study by Packard and McGaugh, a lesion to the caudate resulted in a goal directed response while a lesion to the hippocampus resulted in a stereotypical response in a T-maze task (Packard and McGaugh, 1996). In this light, hippocampal deficiency could lead to the overt activity of CSTC circuits involving the caudate – such as the orbitofrontal circuit. In line with this, hippocampal insufficiency in QNP treated rats could also result in stereotypical behavior. Hyperactivity in the orbitofrontal CSTC circuit could thus be a compensation of hippocampal deficiency – not a cause. Studies exploring a causal role of the hippocampus in the generation of stereotypical checking in QSM are currently under way in our laboratory, and results will be published along together with the results presented here.

8 CONCLUSIONS

One goal of this work is to point to new potential directions in the research on Obsessive Compulsive Disorder. Alterations in hippocampal shape and function have often been reported in literature, but were not given much attention. In our research, we may have stumbled onto an important aspect of OCD. Although our findings are solely correlational, we have discovered that an impaired hippocampal state is associated with stereotypical checking and cognitive inflexibility – both hallmarks of OCD. Namely, we found the reduced expression of the immediate-early gene Arc (a marker of plasticity-related neuronal activity) in the main hippocampal output region – the CA1. Also, at the level of behavior, we found impairment in a hippocampus-dependent task and superior performance in a hippocampus-independent task in the QNP sensitization model of OCD. These complementary findings warrant further research to show causality and the implementation of more complex reversal tasks to challenge cognitive inflexibility in OCD patients. Research along both of these paths is currently underway in our laboratory. We will apply QNP directly to the hippocampus in QNP-treated animals and assess stereotypical checking in the enriched open-field arena. Also, OCD patients are being tested in a virtual analog of the Carousel arena task (by Iveta Fajnerova, Ph.D. from National Institute of Mental Health). The present study also assessed the effects of common drugs prescribed to OCD patients – clomipramine, risperidone and the combination thereof – on cognitive flexibility in the QNP sensitization animal model of OCD. We found that the combination of clomipramine and risperidone may be the most effective in rescuing QNP induced cognitive deficits, as acquisition and reversal performance were spared in animals receiving this treatment. We propose that QNP sensitization could model a subgroup of OCD patients with cognitive flexibility deficits stemming from hippocampal dysfunction. We further suggest that this subgroup of patients may benefit most from the augmentation of SRI treatment with antipsychotics to improve cognitive flexibility.

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10 PUBLICATIONS

Publications relevant to the thesis:

2017 **Detrimental effect of clomipramine on hippocampus-dependent learning in an animal model of obsessive-compulsive disorder induced by sensitization with d2/d3 agonist quinpirole.** **Hatalova H**, Radostova D, Pistikova A, Vales K, Stuchlik A. Behav Brain Res. 2017 Jan 15;317:210-217.

2016 **Validity of Quinpirole Sensitization Rat Model of OCD: Linking Evidence from Animal and Clinical Studies.** Stuchlik A, Radostová D, **Hatalova H**, Vales K, Nekovarova T, Koprivova J, Svoboda J, Horacek J. Front Behav Neurosci. 2016 Oct 26;10:209.

2014 **Spatial reversal learning in chronically sensitized rats and in undrugged sensitized rats with dopamine D2-like receptor agonist quinpirole.** **Hatalova H**, Radostova D, Pistikova A, Vales K, Stuchlik A. Front Behav Neurosci. 2014 Apr 11;8:122. doi: 10.3389/fnbeh.2014.00122.

Other publication:

2018 **The McGill Transgenic Rat Model of Alzheimer's Disease Displays Cognitive and Motor Impairments, Changes in Anxiety and Social Behavior, and Altered Circadian Activity.** Petrasek T, Vojtechova I, Lobellova V, Popelikova A, Janikova M, **Brozka H**, Houdek P, Sladek M, Sumova A, Kristofikova Z, Vales K, Stuchlík A.Front Aging Neurosci. 2018;10:250. doi: 10.3389/fnagi.2018.00250.

2018 **Neonatal immune activation by lipopolysaccharide causes inadequate emotional responses to novel situations but no changes in anxiety or cognitive behavior in Wistar rats.** Vojtechova I, Petrasek T, Maleninska K, **Brozka H**, Tejkalova H, Horacek J, Stuchlik A, Vales K. Behav Brain Res. 2018 Sep 3;349:42-53. doi: 10.1016/j.bbr.2018.05.001.

2017 **Adult neurogenesis in the hippocampus from a perspective of discrimination and generalization: a hypothesis.** Pistikova A, **Brozka H**, Stuchlik A. Physiol Res. 2017 Jul 18;66(3):441-448. Review.

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2013 **Place avoidance tasks as tools in the behavioral neuroscience of learning and memory.** Stuchlik A, Petrasek T, Prokopova I, Holubova K, **Hatalova H**, Valea K, Kubík S, Dockery C, Wesierska M. Physiol Res. 2013;62 Suppl 1:S1-S19. Review.

2013 **Comparison of Long-Evans and Wistar rats in sensitivity to central cholinergic blockade with scopolamine in two spatial tasks: an active place avoidance and the Morris water maze.** Entlerova M, Lobellova V, **Hatalova H**, Zemanova A, Vales K, Stuchlik A. Physiol Behav. 2013;120:11-8. doi: 10.1016/j.physbeh.2013.06.024.

2013 **Two learning tasks provide evidence for disrupted behavioural flexibility in an animal model of schizophrenia-like behaviour induced by acute MK-801: a dose-response study.** Lobellova V, Entlerova M, Svojanovska B, **Hatalova H**, Prokopova I, Petrasek T, Vales K, Kubik S, Fajnerova I, Stuchlik A. Behav Brain Res. 2013 Jun 1;246:55-62. doi: 10.1016/j.bbr.2013.03.006