DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Dimensions of compulsivity – on the relation between compulsive grooming, anxiety and cognitive flexibility, and the effect of deep brain stimulation

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Matthijs Feenstra (Netherland Institute for Neuroscience) e do Professor Doutor Carlos Duarte (Universidade de Coimbra).

Cindy Pinhal

2014

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Abstract:

Obsessive-compulsive disorder (OCD) is a neuropsychiatric disorder characterized by alternation between moments of recurrent, intrusive, and unwanted thoughts (obsessions) and repetitive ritualistic behaviour (compulsions). Besides these two main features, researchers believe that patients have problems in cognitive flexibility, i.e. ability to adapt behaviours in response to changes in the environment.

A number of patients do not respond effectively to the current treatments, pharmacotherapy and psychotherapy thus, new treatments have been investigated. A novel and promising therapy is deep brain stimulation (DBS). In DBS, electrical stimulation is released through small electrodes that are implanted in specific targets in the brain. As stimulation indifferent areas can have different effects, the choice of the right target is of utmost importance. In particular, stimulation in the anterior internal capsule (IC) close to the nucleus accumbens has shown to be effective in the reduction of OCD symptoms.

Animal models have been developed to study the neurobiology of OCD and its symptoms. In fact, *Sapap3*$^{-/-}$ mice are used because these mice present both an abnormal grooming activity (compulsion) and increased levels of anxiety, two main symptoms of OCD.

This project focused on two main goals: further assess the validity of *Sapap3*$^{-/-}$mice as a multidimensional model of OCD and unveil the involved neurobiology as well as optimize treatment for OCD.

In order to address the first question we assessed OCD-like behaviour in *Sapap3*$^{-/-}$ mice, by investigating their cognitive flexibility. Unfortunately, our

results were inconclusive, since both *Sapap3*$^{-/-}$ and wild-type C57BL/6 mice were not able to reach learning plateau.

For the second goal, we electrically stimulated *Sapap3*$^{-/-}$ mice in the IC and examined the effect that DBS has in some of the typical symptoms of OCD, such as compulsive behaviour and anxiety. Our results suggest that stimulation in the IC is effective in compulsive behaviour, reducing the percentage of grooming in both male and female *Sapap3*$^{-/-}$ mice. Regarding unconditioned anxiety, the conclusions are mixed: there is a clear lack of effect of DBS in *Sapap3*$^{-/-}$ male mice, however clear conclusions were not possible to be drawn from *Sapap3*$^{-/-}$ female mice due to reduced number of animals included. Finally, in what concerns conditioned anxiety and fear extinction, DBS seems to be inefficient in *Sapap3*$^{-/-}$ mice. Furthermore, our results suggest that DBS acts in an acute way but is not able to induce chronic changes.

In conclusion, although DBS has shown to be effective for some OCD-like symptoms it was not for all. In fact, more work is needed to elucidate the results that were not so clear. Furthermore, other targets could be assessed with this approach.

Key-words: Obsessive-compulsive disorder (OCD), Deep Brain Stimulation (DBS), Internal Capsule (IC), *Sapap3*$^{-/-}$ *mice*.

Resumo

O distúrbio obsessivo-compulsivo (DOC) é um distúrbio neuropsiquiátrico caracterizado por alternações entre momentos de pensamentos recorrentes, intrusivos e indesejados (obsessões) e comportamentos repetitivos e ritualísticos (compulsões). Para além destas duas características, investigadores acreditam que os pacientes têm problemas na flexibilidade cognitiva, isto é, na capacidade de adaptar o comportamento às mudanças do ambiente.

Alguns pacientes não respondem de forma efectiva aos tratamentos actuais, farmacoterapia e psicoterapia, portanto, novos tratamentos têm sido investigados. Uma nova e promissora terapia é a estimulação cerebral profunda (ECP). Em ECP uma estimulação eléctrica é aplicada através de pequenos eléctrodos que são implantados em zonas específicas do cérebro. Uma vez que a estimulação em diferentes áreas pode ter efeitos diferentes, a escolha da área é de maior importância. Em particular, a estimulação na capsula interna (CI), junto ao nucleus accumbens tem-se mostrado eficaz na redução dos sintomas de DOC.

Modelos animais têm sido desenvolvidos para estudar a neurobiologia e os sintomas de DOC. De facto, o modelo de ratinho *Sapap3*$^{-/-}$ é usado porque estes ratos apresentam ambos comportamentos compulsivos e aumento de ansiedade, dois dos sintomas mais frequentes em DOC.

O projecto focou-se em dois objectivos principais: atestar a validez dos ratinhos *Sapap3*$^{-/-}$ como modelo multidimensional para DOC, e desvendar a neurobiologia bem como optimizar o tratamento para DOC.

Para abordar a primeira questão nós avaliámos aflexibilidade cognitiva, outro comportamento típico de DOC, em ratinhos *Sapap3*$^{-/-}$. Infelizmente, os nossos resultados foram inconclusivos uma vez que ambos os *Sapap3*$^{-/-}$ e os C57BL/6 não foram capazes de aprender esta tarefa.

Para o segundo objectivo, nós estimulámos electricamente os ratinhos*Sapap3*$^{-/-}$ na CI e examinámos o seu efeito em sintomas típicos de DOC, tais como comportamento compulsivo e ansiedade. Os nossos resultados sugerem que a estimulação na CI é efectiva para comportamento compulsivo, reduzindo a percentagem de *grooming* em *Sapap3*$^{-/-}$ machos e fêmeas. No que diz respeito à ansiedade incondicionada as conclusões são mistas: existe uma clara falta de efeito da ECP nos ratinhos*Sapap3*$^{-/-}$ machos, contudo conclusões claras não se puderam retirar dos ratinhos*Sapap3*$^{-/-}$ fêmeas devido ao reduzido número de animais incluídos. Por fim, no que diz respeito à ansiedade condicionada e extinção do medo, ECP parece ser ineficiente para os camundongos *Sapap3*$^{-/-}$. Para além disto, os nossos resultados sugerem que a acção da ECP é aguda não sendo capaz contudo de induzir mudanças de forma crónica.

Em conclusão, apesar da ECP se ter mostrado eficiente para alguns sintomas típicos de DOC não o foi para todos. De facto, mais trabalho é necessário para elucidar os resultados que não são claros. Para além disso, esta abordagem poderia ser usada para estudar outras áreas do cérebro.

Palavras-chave:Distúrbio obsessivo-compulsivo (DOC), Estimulação cerebral profunda (ECP), Capsula Interna (CI), ratinhos*Sapap3*$^{-/-}$.

- Chapter 1 -

Introduction

1.1. Clinical aspects of OCD

Obsessive-compulsive disorder (OCD) is a psychiatric condition characterized by the presence of obsessions and compulsions. Formerly, OCD was considered to be a rare disorder as it was poorly understood. However, research done in the past two decades helped doctors to recognize symptoms and diagnose people with OCD, being nowadays the fourth most common mental disorder with a lifetime prevalence of 1-3%, affecting both men and women equally (Denys et al., 2010; Schilman et al., 2010).

In 80% of the cases the onset lies in a young age, with an initial peak incidence occurring in pre-puberty, achieving a chronic and debilitating course if not treated (Abramowitz et al., 2009; Merlo et al., 2006).

OCD is associated with the emergence of other diseases. Indeed, an epidemiological study revealed that 84% of youth diagnosed with OCD had comorbid disorders such as major depression (62%), social phobia (38%), alcohol dependence (24%) and dysthymia (22%) (Keeley et al., 2008; Overbeek et al., 2002).

In the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), OCD was classified as an anxiety disorder once anxiety is the core symptom of this syndrome. However, neurobiological similarities with other disorders as hoarding, hair pulling disorder and skin picking, took it apart from anxiety disorder and clustered it together with those disorders in DSM-V (Pallanti et al., 2014).

1.1.1. Symptoms' presentation

OCD patients generally present an alternation between moments of obsessions and moments of compulsions. Obsessions are defined as recurrent and persistent thoughts, impulses or images that continue despite attempts to ignore, suppress or neutralize them. They are experienced in an intrusive and inappropriate way and cause high levels of anxiety and distress. Compulsions are defined as repetitive behaviours or mental acts that a person feels driven to perform in response to an obsession or according to rigid rules. This behaviour is always unrealistic or excessive (Marazziti et al., 2010). Tables I and II summarize the most common obsessions and compulsions, respectively, presented in OCD (Melro et al., 2006).

Table I: Common obsessions in OCD

Categories	Obsessive concerns
Contamination	Dirt; germs; animals/insects; illnesses; bodily waste; contaminants; household cleaners; "sticky" substances; spreading contamination…
Aggression	Causing harm to self or others due to thoughts or behaviours; acting upon aggressive impulses; saying inappropriate words/phrases; stealing or breaking things; frightening/violent images.
Sexual	Forbidden/perverse sexual thoughts, images; homosexuality; molestation; sexual acts toward others.
Hoarding/saving	Losing things; throwing away objects that might be important.
Magical thinking	Lucky/unlucky numbers, colors, names…
Health/body	Contracting illness (especially if fatal or rare); appearance; physical abnormalities (real or imagined).
Mortality/religion	Dying and not going to heaven; offending God; being sinful; morality/perfection; right/wrong.
Miscellaneous	Knowing/remembering certain things; saying things exactly right; not saying certain words/phrases; intrusive images sounds, words, music, numbers…

Table II: Common compulsions in OCD

Categories	Compulsive rituals
Washing/cleaning	Excessive/ritualized hand washing, showering, bathing, tooth brushing, grooming, toileting; cleaning clothing/personal items; avoiding "contaminated" objects/places
Checking	Checking locks, alarms, school supplies, homework, toys, books; checking associated with washing, dressing, undressing, somatic concerns; checking that did/will not harm self or others; checking for mistakes.
Repeating	Rewriting; rereading; recopying; erasing; going in/out door; getting up/down from seat; repeating words/phrases.
Counting	Counting objects; mental counting (especially up to a "magic" number); counting steps, chewing, hair-brushing.
Ordering/arranging	Lining up objects in a certain way; arranging things in specific patterns; making things symmetrical; "balancing" actions.
Hoarding and saving	Keeping unimportant/unnecessary items and/ or trash.
Superstitions	Touching/tapping routines to prevent bad things from happening; avoiding stepping on cracks, lines, etc; avoiding "unlucky" objects/places.
Reassurance-seeking	Asking a parent to repeatedly answer the same questions; asking parents to describe what they are doing/planning to do; forcing family members to do things in a certain way or at a certain time; forcing family members to avoid certain things/activities.
Miscellaneous	Mental rituals; needing to tell/ask/confess; ritualized eating behaviors; excessive list-making; needing to touch/tap/rub; needing to do things until it feels "just right"; hair-pulling; measures to prevent something bad from happening.

Some authors describe that symptoms appear in a cyclic sight, with obsession thought to promote anxiety followed by compulsions that lead to a temporarily relief of anxiety until new obsessions begin (Heyman et al., 2006). However, other authors suggest that compulsion, rather than goal directed action, are a response to habit formation that fail to extinguish (Robbins et al., 2012)

Although patients generally recognize obsessions or compulsions as a product of their imagination, they are not able to prevent this behaviour (Goodman et al., 2007).

Nevertheless, obsessions and compulsions can be presented in people without OCD (Crye et al., 2010). Therefore, in order to distinguish OCD from this last situation, obsessions and/or compulsions must be time consuming (more than one hour per day) and interfere with daily functioning (Franklin et al., 2011).

Other symptoms are usually found in patients, such as impaired cognitive flexibility, i.e. the ability that someone has to change its behaviour in accordance with the environment (Fineberg et al., 2010).

1.1.2. Diagnosis

According to DSM-V criteria, in order to be diagnosed with OCD, a person must have either obsession or compulsion and recognize this behaviour as excessive or unreasonable. Children are an exception as they might not have sufficient cognitive awareness to make this judgment (Abramowitz et al., 2009). These obsessions and compulsions must be “time consuming”, “interfere with routine” and cannot be “due to direct physiological effects of a substance (e.g., drug of abuse or medication) or a general medical condition”.

There is no laboratory test to diagnose OCD; therefore it may be hard to identify it. Indeed, studies have demonstrated that in general, it takes 8 years for someone to be diagnosed as having OCD. One of the reasons for this is that OCD’s symptoms are often confused with other neuropsychopathies such as depression (Fullana et al., 2009). Due to the lack of tools to identify OCD,

diagnosis is made by interviews, clinician-rated measures, and self-report measures (Grabill et al., 2008).

1.2. Neurobiology of OCD

Despite the fact that OCD's causes remain elusive; researchers have identified molecules and specific circuitries that might be involved in this disorder.

1.2.1. Neuroanatomy

Specific areas of the brain seem to be involved in the neurobiology of OCD as obsessions and compulsions are the main characteristics of this disorder. In particular the prefrontal cortex seems to be a good candidate as is involved in the inhibition of responses as well as planning and verification of previous actions (Rauch et al., 1997). Indeed, imaging studies have identified alterations not only in the size, but also in the activity of this and other areas such as dorso-lateral prefrontal cortex, inferior parietal cortex, anterior cingulate, thalamus, medial orbital gyrus, inferior frontal gyrus, anterior cerebellum, inferior frontal gyrus, caudate nucleus, nucleus accumbens, amygdala, ventral putamen and globus pallidus (Fig. 1) (Whiteside et al., 2004; MacMaster et al., 2008; Figee et al., 2013).

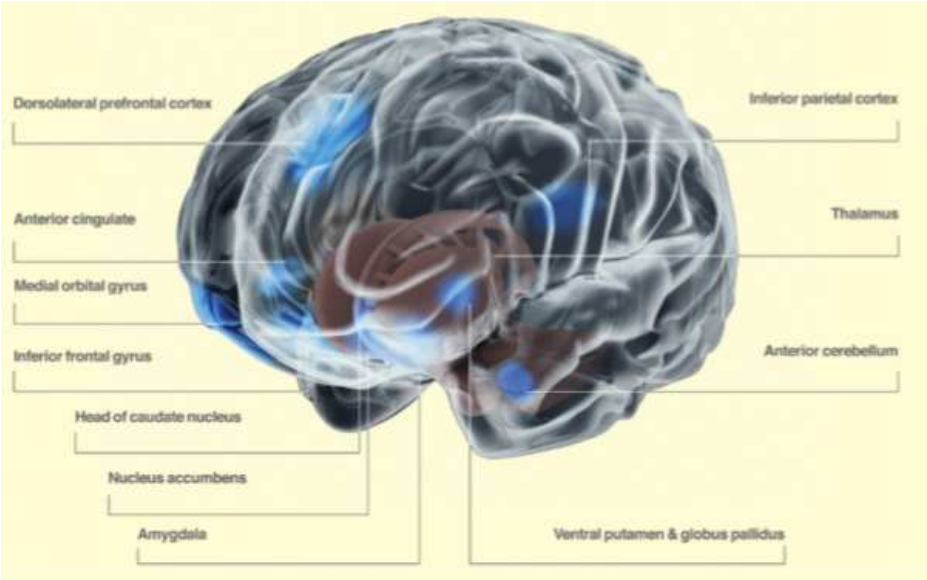

Fig.1: Brain regions presenting alteration in size and/or activity in OCD patients through structural and functional imaging studies.

As a matter of fact, an idea that seems to be well accepted is that OCD patients are impaired in the cortico-striatal-thalamic-cortical (CSTC) circuit. The first evidence resulted from the fact that OCD patients present symptoms' similarities with disorders involving striatal impairments, such as Tourette's syndrome, Sydenham's chorea, Huntington's disorder, and Parkinson's disorder (Stein, 2002). Conversely, OCD patients frequently present abnormalities in measures and paradigms used in neuropsychiatry, (e.g. neurological soft signs, olfactory identification) and neuropsychology (e.g. executive functions, visual memory function), which are consistent with CSTC dysfunction (Stein, 2002).

In the CSTC the cerebral cortex projects to the striatum. Then, the striatum projects either to the globus pallidus pars externa (GPe) - indirect pathway - or to the globus pallidus pars interna (GPi) and substantia nigra pars reticulata (SNr) - direct pathway. Ultimately, the firing of GPi and SNrGABAergic cells is modulated, which in turn inhibits glutamatergic neurons of the anterior

thalamus. In the end, thalamic neurons project back to frontal neo-cortex to both anterior cingulate and the orbito-frontal cortex (Fig. 2) (Marchand, 2010).

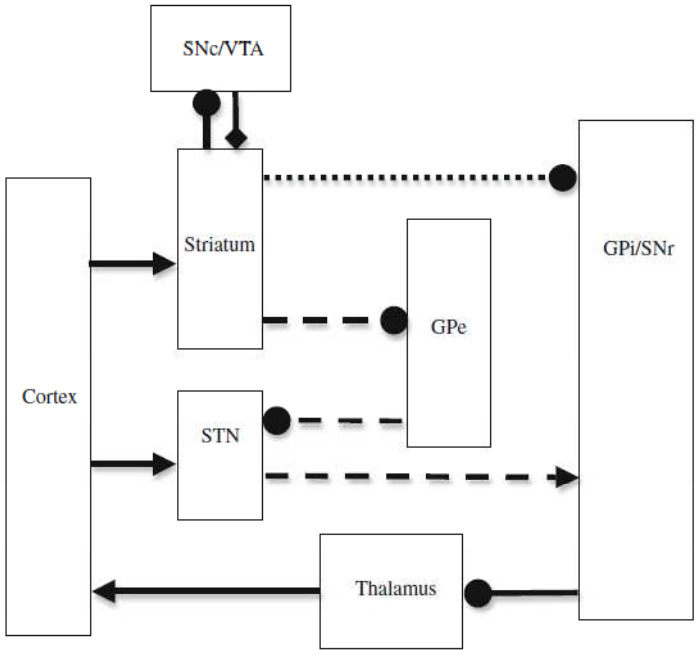

Fig.2: Schematic representation of the Cortico-Striatal-Thalamic-Cortical (CSTC) circuit.

In fact, the involvement of CSTC circuitry was confirmed with brain imaging studies. Abnormalities such as decreased volume or increased grey matter density in cortico-striatal-thalamic-cortical circuits were found, as well as an increase of activity in orbitofrontal and cingulate cortices and striatum at rest, especially when subjects were exposed to a feared stimulus. Moreover, pharmacotherapy, behavioural therapy and neurosurgical interruption currently used in the clinical treatment for OCD, can normalize the activity of the CSTC circuit (Stein, 2002).

1.2.2. Neurochemistry

Obsessive-compulsive disorder has been linked to a disruption in serotonin, dopamine and glutamatergic systems.

The first evidence of an involvement of serotonin system came from the use of clomipramine, a tricyclic antidepressant, which has been effectively used to treat OCD symptoms (Thorénet et al., 1980). Nowadays, selective serotonin reuptake inhibitors (SSRIs) are used as the first line treatment in OCD, reducing effectively its symptoms. Nonetheless, no specific abnormality in the serotonin system has been identified to date (van Dijk et al, 2010).

Dopamine, on the other hand, has been suggested to be involved in the pathology of OCD as administration of dopamine antagonists leads to stereotypic behaviour or exacerbation of symptoms in OCD (Goodman et al., 1990).

Moreover, recent studies have proposed abnormalities in glutamate neurotransmission and homeostasis. Indeed, while no agent is yet being used in the clinic, increasing evidence supports the potential utility of riluzole, memantine, N-acetylcysteine, D-cycloserine, and other glutamate-modulating agents in the treatment of this disorder. Furthermore, magnetic resonance spectroscopy (MRS) studies have produced some evidence of glutamate deregulation in patients with OCD (Pittenger et al., 2011).

1.2.3. Neurogenetic

Studies have shown that the risk for OCD is higher in first-degree relatives than for second and second-degree relatives' higher than third-degree. This effect is independent of the environment, suggesting a genetic component.

However, and due to its high heterogeneity, a single gene is not likely to be responsible for OCD. Candidate genes for OCD include genes affecting serotonin, dopamine and glutamate systems (Nestadt et al., 2010; Stewart et al., 2010).

Serotonergic genes such as 5-H*TTLPR* serotonin transporter, 5*HT1D* beta receptor gene, 5*HT2a* serotonin receptor, and serotonin *5HT2c* receptor have been suggested to be involved in OCD pathology (Greenberg et al, 1998; Meira-Lima et al., 2004).Dopamine genes have included the dopamine receptor type 4 (*DRD4*) gene and the *D2* receptor gene (Billett et al., 1998; Millet et al., 2003; Denys et al., 2004).Lastly, glutamatergic genes have also been strongly implicated in OCD pathology including *SLC1A1*glutamate transporter, *SAPAP3* gene that encodes a scaffolding protein, and *GRIN2B*glutamate receptor gene (Chakrabarty et al., 2005; Welch et al., 2007; Arnold et al., 2006; Stewart et al., 2007; Alonso et al., 2012).

1.2.4. Neuroinflammation

OCD in children might also be caused by a neuroinflammation process through the exposure to a group A beta haemolytic Streptococcus (GABHS). This group has been identified by the name PANDAS (paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections). OCD's pathogenesis in these patients is thought to be caused by an autoimmune process. The hypothesis is that the body is exposed to Streptococcus, developing antibodies that react with nerve tissue, damaging it. In such cases, if the intervention occurs early in the course of the disorder, symptoms are

sometimes successfully treated with antibiotics (Trifiletti et al., 1999; da Rocha et al., 2008).

Although some evidences suggest the existence of an inflammatory component in OCD, no reliable biomarkers are currently available.

1.3. Treatment options for OCD

OCD remains poorly recognized and undertreated. Although the time between the onset of symptoms and diagnosis may be decreasing, the treatment starts generally just after the emergence of depressive symptoms. Thus, accurate and timely assessment of clinical presentation is critical to limit impairment and improve prognosis. Moreover, it is important to note that the way patients respond to treatment is highly variable, so the choice of a specific therapeutic approach is merely empirical. Therefore, it would be of great importance to homogenously group patients in order to develop more focused treatment strategies. With that in mind, several studies have been investigating predictive factors, i.e. factors that influence the way patients respond to treatment. For example, earlier onset of treatment is usually a good indicator of the treatment's success and vice-versa (Erzegovesi et al., 2001, Skoog et al., 1999).

Presently, there are two main supported treatments available for OCD in children and adults, namely pharmacotherapy and cognitive-behavioural therapy. In addition, Deep Brain Stimulation (DBS) is now emerging as a novel and promising therapeutic approach for OCD patients.

1.3.1. Pharmacotherapy

1.3.1.1. Serotonin Reuptake Inhibitors (SRIs) and Selective Serotonin Reuptake Inhibitors (SSRIs)

The use of serotonergic medications in the treatment of OCD has received a lot of attention in the past two decades.

Clomipramine, a tricyclic antidepressant (TCA) with a prevalent inhibitory activity on serotonin (5-HT) reuptake, was the first agent approved by the Food and Drug Administration (FDA) for the treatment of OCD. In fact, studies done in adults have shown a clear improvement of symptoms when patients were administrated with clomipramine or selective serotonin reuptake inhibitors as compared with placebo (Foa et al., 2005; Greist et al., 1995; Zohar et al., 1996). Thereafter, each of these medications has been approved by the FDA as treatments for adult OCD. However, the mechanisms by which SSRIs exert its effect remain poorly understood.

Currently, many different SSRIs are used in the treatment of OCD. In fact, the choice between one SSRI and another depends mainly on personal preference, once these substances are very similar. It is described that female patients seem to respond better to SSRI treatment that male (Mundo et al., 1999; Stein et al., 2001). Furthermore, although the mechanistic relationship is unknown, it is widely described that OCD patients with previous treatments have less chance to respond to SSRIs (Stein et al., 2001; Ackerman et al., 1998).

Among others, it is possible to find presently being used sertraline, citalopram, fluoxetine, paroxetine and fluvoxamine (Marazziti et al., 2010).

1.3.1.2. Antipsychotics and other drugs

Due to the high number of OCD subjects not responding to a switch to another SSRI, the evaluation of additive therapeutic options has been highly studied.

Many studies have examined the action of antipsychotic compounds in this disorder. Indeed, the combination of antipsychotics such as risperidone, haloperidol, olanzapine or quetiapine with an SSRI was shown to be more effective than SSRI monotherapy in treatment-resistant cases (Skapinakis et al., 2007; Bloch et al., 2006).

Several other drugs such as antagonist/agonist of glutamate receptors and antidepressants others than SSRIs have been studied in the treatment of OCD, but so far none of these approaches has reached sufficient empirical evidence to become recommended in treatment guidelines (Bandelow et al., 2008).

1.3.2. Cognitive-Behavioural Therapy (CBT)

Evidence supports the usefulness of CBT as a non-pharmacological treatment for OCD (Foa, 2010). The exposure and response prevention (EX/RP) is the psychosocial intervention most used is OCD with a high efficacy (Abramowitz et al., 2005; Rosa-Alcazar et al., 2008). In this intervention, patients are exposed to obsessional cues using real life situations, and then forced to contact with the stimuli reported as distressing, preventing the compulsion and discussing mistaken beliefs. The exposure is usually done in a gradual way, with situations provoking moderate distress confronted before more upsetting ones. It is believed that repeated and prolonged exposure to

feared thoughts and situations will elucidate mistaken associations held by patients and thereby promote habituation (Foa et al., 1986). The optimal frequency by which patients should be exposed is not well established yet, being this dependent on the motivation of the patients to daily expose themselves to stressful situations. However, good results have been achieved using one session per week, two sessions per week or intensive treatment format, this last involving daily sessions over the course of approximately one month (Foa et al., 2005; Franklin et al., 2000).

1.3.3. Deep Brain Stimulation

Despite the advances done in the last years in pharmacotherapy and psychotherapy, about 40 to 60% of patients show no or just partial symptom improvement. Therefore, the search for other effective strategies is mandatory (Pallanti et al., 2006; Gabriels, 2003). Bearing this in mind, new approaches started to be developed and improved, with Deep Brain Stimulation (DBS) showing remarkable results (Lakhan, 2010).

DBS was accepted for the first time as a treatment for refractory OCD by the FDA in 2009. It involves the delivery of electrical stimulation to specific brain regions, using permanently placed small electrodes, implanted through a neurosurgery that relies on stereotactic techniques. Electrodes are placed in specific areas bellow the skull and connected to a pulse generator generally placed under the collarbone (Fig. 3). This pulse generator can be externally accessed and settings can therefore be optimized to each patient individually to achieve better treatment and reduced side effects (Lozano et al., 2013).

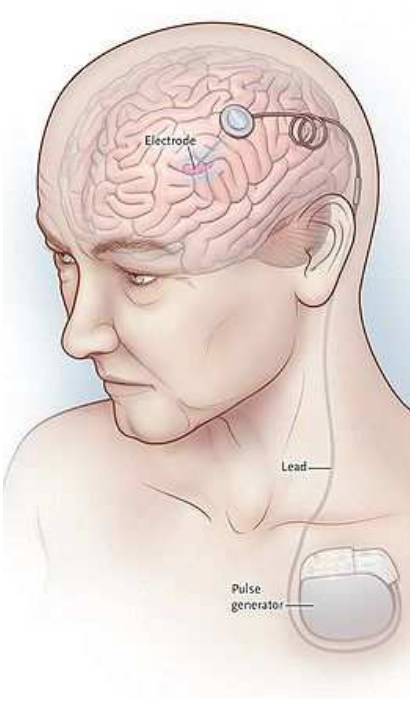

Fig.3: Schematic representation of an implanted equipment of deep brain stimulation. Electrodes are placed in the brain, in a specific neuroanatomical target, and connected to the pulse generator through a lead. The pulse generator is usually situated bellow the collarbone.

It is well accepted that DBS affects neuronal discharge patterns not only locally but also in distant brain areas, by conveying bioelectric impulses along projections (Carron et al., 2013). Despite the considerable effort to understand the mechanisms behind DBS-driven clinical improvements, the precise mechanism by which DBS works remains unclear. Nevertheless, researchers have hypothesized that DBS may act by one of the next four different ways: i) by inhibiting the target; ii) by activating the target; iii) by inhibiting and activating the target in a combined way; and iv) by disrupting the pathological oscillations, restoring the rhythmic activity and synchronization – noisy signal hypothesis (Karas et al., 2013).

Before performing DBS surgery several variables are considered. In the case of OCD, patients have to be treatment-resistant both with pharmacotherapy and psychotherapy. Furthermore, patients need to be screened for comorbid psychiatric disease, as certain conditions predispose patients to worse DBS outcomes. Additionally, patients need to be neurologically stable and be able to understand the dangers associated with this therapy (Williams et al., 2013).

Treatment efficacy is greatly dependent on the choice of the target. As the brain is a complex organ, the choice of the area to stimulate should be a careful and rigorous process. The selection of the targets is done empirically and/or by the result of an understanding of the presumed pathophysiology of OCD. Several targets have been proposed, and the differential therapeutic success of these targets suggests that each may have a distinct role in OCD. However, only few targets are used nowadays in the clinic for the treatment of OCD (Fig. 4) (Figee et al., 2013).

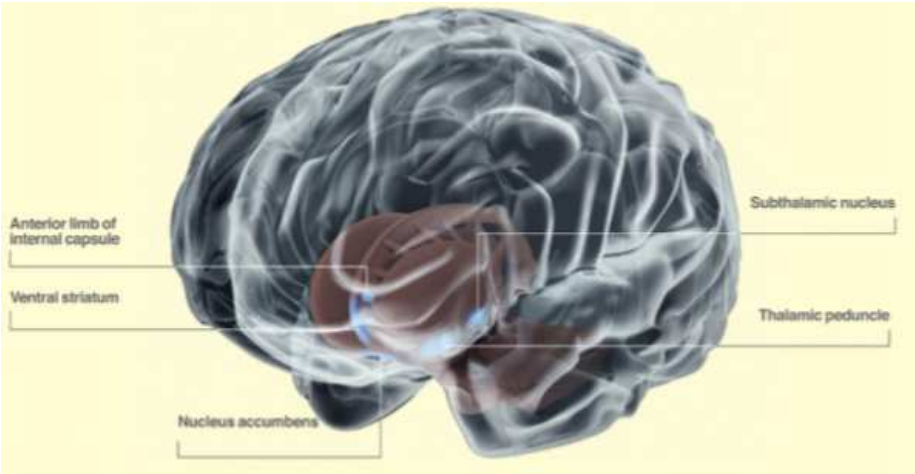

Fig. 4: Neuroanatomical targets used currently in the clinic for the treatment of OCD patients.

In particular, due to its efficacy, the stimulation of the internal capsule (IC) has been frequently used as brain target for DBS. The internal capsule is a structure composed mainly of glial cells and myelinated axons that are both ascending and descending. Localized in the frontal part of the brain, this region separates the caudate nucleus and the thalamus from the putamen and the globus pallidus. As the axons that run through it are part of the cortico-striatal-thalamo-cortical (CSTC) networks (Lehman et al., 2011), this region has been highly used as a target for DBS. Indeed, clinical studies have shown a positive effect in the reduction of OCD symptoms when the IC was targeted with DBS (Greenberg et al, 2010).

Studies performed in humans have demonstrated that DBS can successfully decrease OCD symptoms in a sequential order with mood improving first (after a few seconds), followed by anxiety (in minutes), obsessions (within days) and finally compulsions (can take weeks or even months) (Denys et al., 2010).

Nevertheless, DBS also has some risks associated, namely related to the surgical procedures, application of stimulation or long-term consequences of the implanted hardware. Life threatening events like brain haemorrhage are rare, occurring only in 1-2% of patients while less severe events such as infection and stimulation-related side effects present in 9% of patients (Lozano et al., 2013).

1.4. Animal models of OCD

"If you are going to study a human disease you cannot, for ethical reasons, perform the initial work in humans; you have to develop a model. Some models may be *in vitro* - literally, in glass tubes – but as you learn more and more, you must eventually test ideas *in vivo*- in living animals. That means you have to have a way of producing the disease that allows you to study it." (The Animal Research War by P. Michael Conn and James V. Parker)

The use of animals in research is essential to develop new and more effective methods for diagnosing and treating diseases that affect both humans and animals. Over the past century, the mouse has been used as the major mammalian model system due to its genetic and physiological similarities with humans as well as the ease with which its genome can be manipulated and analysed.

Even though it is impossible to develop an animal model that mimics a psychiatric condition in its full, some criteria must be satisfied depending on the purpose of the model (Joel et al., 2006). Although there is no consensus among authors, three criteria have been highly accepted to validate a specific strain of mice as a disease model: face validity, construct validity and predictive validity. The face validity concerns the similarity in symptoms and behaviour between mice and humans which in the case of OCD would be the repetitive and excessive behaviour, compulsion and the perseveration. The construct validity describes that there must exist the involvement of the same areas or molecules affected in humans and in the animal model. In the case of OCD models, animals should present impairment in areas such as orbito-frontal cortex, cingulated cortex and basal ganglia as well as deregulation in serotonergic,

dopaminergic and glutamatergic systems. Finally, the predictive validity compares the effect of the treatment used in humans with their response in animals. Therefore, a relief of the symptoms through the use of SRIs and SSRIs should be observed in an animal model of OCD. Nevertheless, these criteria are not always fulfilled and researchers need to adapt the criteria with the disorder being studied (Albelda et al., 2012).

In what concerns OCD specifically, there are three different types of animal models used: genetic models, pharmacological models and behavioural models. Table IV summarizes the findings obtained until now in the different models (Wang et al., 2009; Albelda et al., 2012).

Table III: Current mouse models for OCD and main findings

	Face validity	Predictive validity	Construct validity
Genetic models			
Hoxb8	▪ Compulsive grooming	-	▪ Hoxb8 is expressed in brain regions involved in OCD
5-HT2c KO	▪ Increase of compulsive behaviours (chewing and head-digging)	-	▪ Evidences of the involvement of 5-HT2c receptors in OCD; ▪ Abnormalities in the mesolimbic dopaminergic system; ▪ Dentate gyrus-specific deficit
DAT KD	▪ Increase of grooming with more difficulty to be interrupted	-	▪ Implication of the basal ganglia
D1CT-7	▪ Compulsive behaviour; repetitive leaping behaviour; increase of anxiety-related behaviours; ▪ Tourette's syndrome-like behaviours (tics, complexity, flurries); limbic seizures	▪ Positive effect of clonidrine - effective in Tourette's syndrome but not in OCD	▪ Overlap of the cholera toxin transgene is expressed and neural circuitry implicated in OCD; ▪ Evidences of glutamatergic involvement
Estrogen-deficient mice	▪ Compulsive behaviour; ▪ Decrease in inhibition (a form of plasticity)	-	
Sapap3 KO	▪ Compulsive grooming; increased anxiety-related behaviours	▪ SSRI	▪ Evidences of cortico-striatum defects

Pharmacological models

8-OHDPAT	▪ Excessive checking; ▪ Motor preservation	▪ SSRI	▪ Evidences of the involvement of 5-HT1a receptors in OCD
Quinpirol	▪ Excessive checking	▪ SSRI	▪ Evidences of the involvement of 5-HT receptors in OCD; ▪ Involvement of Nucleus Accumbens

Behavioural models

Marble burying	▪ Barbering behaviours	▪ SSRI	-
Stereotypic behaviour in Deer mice	▪ Stereotypic behaviours	▪ Effectiveness other SSRI	▪ Involvement of frontal cortex and striatum
Signal attenuation	▪ Compulsive behaviour	▪ SSRI	▪ Involvement of OFC and striatum; ▪ Evidences of the involvement of 5-HT receptors, dopamine and glutamate.

1.4.1. *Sapap3*$^{-/-}$ mice

Synapse-associated protein 90/postsynaptic density-95-associated protein *(SAPAP)* is a family of proteins that interacts with two other proteins, PSD95 and Shank, working together as postsynaptic scaffolding proteins at excitatory synapses (Takeuchi et al., 1997). These proteins are localized in the postsynaptic density (PSD) where they are responsible for controlling the trafficking, anchoring, and clustering of glutamate receptors and adhesion molecules (Fig. 5). Besides, they link postsynaptic receptors with their downstream signalling proteins and regulate the dynamics of cytoskeletal structures (Verpelli et al., 2012). Given their central role, it is not surprising that deletion or mutations in these genes cause severe neuropsychiatric disorders such as autism, mental retardation, and schizophrenia (Mameza et al., 2013; Yerabhamet al., 2013).

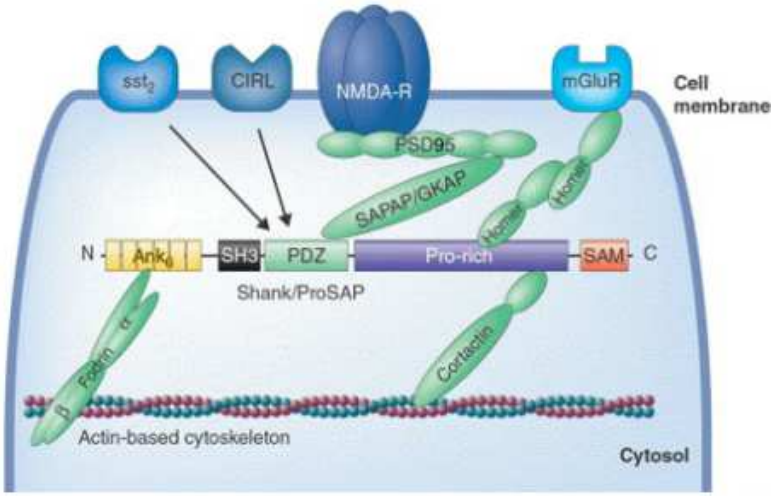

Fig. 5: Schematic representation of the PSD. SAPAP/GKAP proteins bind directly to Shank protein and PSD95 and together they are responsible for the clustering and right placement of all the molecules present in the PSD.

SAPAPs are encoded by four genes that are widely expressed throughout the nervous system; however *SAPAP3* is the only *SAPAP* that is highly expressed in the striatum.

Genetic deletion of *Sapap3* in mice leads to behavioural abnormalities consistent with OCD. In fact, this model fulfils the three criteria used to assess the validity of a model. In what concerns the face validity, *Sapap3*$^{-/-}$ mice present increased anxiety and compulsive grooming, that in the end lead to facial hair loss and skin lesions (Fig. 6).

Fig. 6: Comparison of *Sapap3*$^{+/-}$ mice and *Sapap3*$^{-/-}$ mice. *Sapap3*$^{-/-}$ mice show skin lesions, both in face and neck, due to the excessive grooming behaviour.

The construct validity criterion is fulfilled once mice were found to have defects in glutamatergic transmission at cortico-striatal synapses. Indeed, reintroduction of *Sapap3* specifically into the striatum can rescue both synaptic and behavioural defects, confirming the critical role of the cortico-striatal circuitry (Welch et al., 2007). Finally, this model is strengthened when the repeated administration of fluoxetine, mentioned earlier as used in OCD treatment, successfully alleviated compulsive grooming and anxiety– predicative validity (Welch et al, 2007; Ting et al, 2012).

Furthermore, recent studies have identified abnormalities in human gene that encode the *SAPAP3* gene corroborating the hypothesis that it might be involved in OCD (Züchner et al., 2009).

In summary, OCD is a non-genetic psychiatric condition that lacks a cure. OCD patients have very variable phenotype, being extremely challenging to identify the underlying neurobiological causes. Due to this lack of scientific knowledge about OCD, considerable effort has been put into fundamental studies. With that in mind, some mouse models have been developed (such as the *Sapap3*$^{-/-}$ mouse model) and more studies should be directed into validating these models for the study of this disorder. It is important to consider, though, that it is highly unlikely that a mouse model would ever reasonably resemble such complex disorder. Moreover, the testing of novel therapeutic approaches (as DBS) is mandatory in order to help the patients and further unravel the neurobiological circuitry involved.

Following these ideas, in this project we propose to use the *Sapap3*$^{-/-}$ mouse model to study the neurobiology of OCD and the effect of DBS as a treatment option. Thus, this project has two main goals.

1. Further assess the validity of the *Sapap3*$^{-/-}$ mouse model as an OCD model: In order to do that, we will investigate other symptoms that are present in OCD patients which, to the best of our knowledge, were never assessed in *Sapap3*$^{-/-}$ mice before. Specifically, we intend to investigate cognitive flexibility, i.e., the ability one has to change its behaviour in accordance to the environment. Indeed, the verification of impaired cognitive flexibility in these mice would strengthen their validity as a model of OCD and enable further studies.

2. Study the effect of DBS in the internal capsule (IC) upon OCD-like behaviours present in the *Sapap3*$^{-/-}$ mouse model. For that, we will look at the effect of DBS versus Sham treated mice in compulsion, unconditioned and conditioned anxiety, as well as fear extinction. Interestingly, both acute and chronic effects of DBS will be evaluated using this approach. Moreover, *post-mortem* analysis of these brains by histology techniques with c-Fos (a marker of neuronal activity) will allow us to identify the cells or circuits involved in the effect observed behaviourally.

Altogether, this set of experiments will allow us to further understand the validity of the *Sapap3*$^{-/-}$ mouse model, the neurobiology of OCD and provide hints on the functioning mechanism of DBS.

- Chapter 2 -

Material and Methods

2.1. Subjects

The study was conducted in accordance with governmental guidelines for care of laboratory animals and approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands.

Sapap3$^{-/-}$ mice (20-40g, NIN, the Netherlands) (Welch et al., 2007) and C57BL/6 mice (20-40g, Harlan, the Netherlands) were housed individually in standard housing conditions (8:00 to 20:00 dark phase, controlled temperature and humidity). Water and food were provided according to the experiments' requirements. For cognitive flexibility assessment, *Sapap3*$^{-/-}$ mice had about 10 months and C57BL/6 mice 4 months. In DBS experiments *Sapap3*$^{-/-}$ mice were older than 5 months. One week before the surgical procedure (mentioned later), mice were handled daily with a plastic tube (4.5cm of diameter) for 30 seconds. All experiments were performed in the dark period.

2.2. Electrode construction

Bipolar electrodes consisted of two twisted Teflon-coated platinum/ iridium wires with a diameter of 0.07 mm. On one side, tips were cut off straight, forming an exposed surface area. On the other side, the insulation layer was removed, tips were soldered in two connecting pins and epoxy glue was used to keep the pins parallel. Electrodes were then bent twice in an angle of 90 degrees (around 5mm) to prevent its displacement once implanted in the brain (Fig. 7).

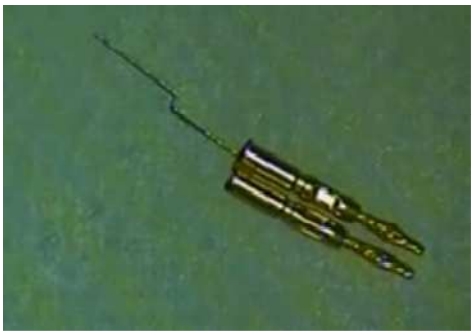

Fig. 7: Bipolar electrode used to electrically stimulate the brain of mice.

2.3. Stereotaxic surgery

Animals were deeply anaesthetized with 2% isoflurane and placed in a stereotaxic frame. After skin incision and exposure of the skull surface, lidocaine, a local anaesthesia, was applied. Then, electrodes were placed in the correct place according to the stereotaxic coordinates of the internal capsule: Antero-posterior axis: +3.34; Medio-lateral axis: ±1.3 and Dorso-ventral axis: -4.6 (Paxinos & Franklin, 2007). Five holes were then drilled, two for the electrodes and three to support the head cap (Fig. 8a). After positioning and holding the electrodes, dental acrylic was overlaid such that the electrodes were completely fixed in the correct position (Fig. 8b). After the dental acrylic had hardened, the electrodes were released from the micromanipulators and the head cap fixed with dental acrylic (Fig. 8c). The animals were then released from the stereotaxic frame and injected with saline to prevent dehydration, and with Meloxicam to alleviate pain (1mg/kg). Animals were allowed to recover for at least one week before further experimentation.

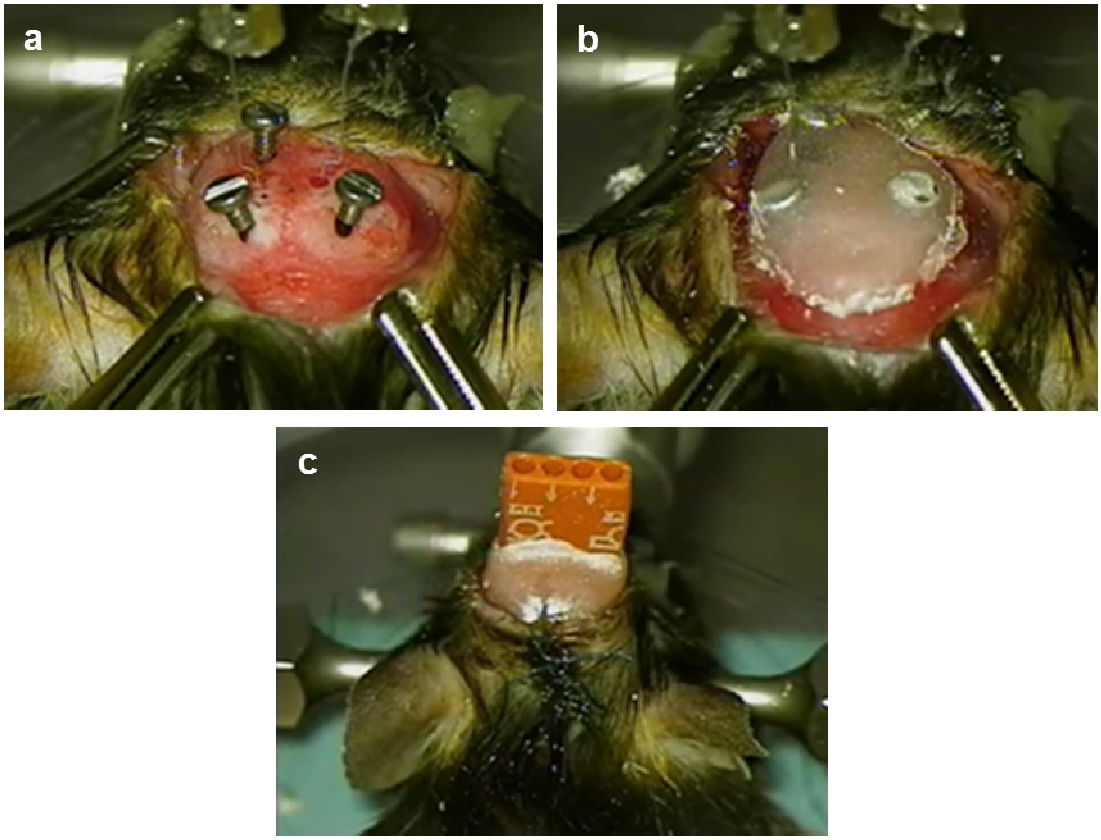

Fig. 8: Stereotaxic surgery.a) Five holes were drilled in the skull; three forscrews to fixate the head-cape, and two centrally localized, for the electrodes; **b)** dental acrylic is placed in the skull to fixate the electrodes and prevent their displacement; **c)** head cape is fixed with dental acrylic to protect the skull.

2.4. Behavioural tests

2.4.1. Compulsion evaluation: quantification of grooming

One week after surgery, grooming behaviour was accessed in an open field. The open field consists of a box (25cm x 25cm), equipped with a motion sensor to analyse the amount of time spent moving. Mice were connected to the Deep Brain Stimulation (DBS) cable through a commutator allowing free unrestricted movement (WPI Digital Stimulator, model DLS8000, World

Precision Instruments, Sarasota, FL). All mice were connected but not stimulated for 20 minutes, allowing them to become familiarized with the setup, and then stimulated either with 0µA (Sham) or 300µA (DBS). The administered DBS pulses were biphasic, with a width of 80µs, at a frequency of 120 Hz. Mouse behaviour was recorded and analysed through Ethovision XT7 (Noldus Information Technology, The Netherlands) and grooming activity was assessed manually for 110 minutes. In short, animals were connected but not stimulated for 20 minutes and then stimulated (sham or DBS) for 90 minutes.

2.4.2. Unconditioned anxiety evaluation: Elevated Plus Maze

The unconditioned anxiety was assessed through an elevated plus maze task. Mice were connected to the DBS cable and stimulated for 20 minutes, as previously described, in their home cages. After that time, mice were placed on the centre section of the elevated plus maze being allowed to freely explore the maze for 10 minutes, while being stimulated (Sham or DBS). The maze consisted of four arms, two closed arms (4.5cm wide, 30cm long and 15cm high) and two open arms (4.5cm wide and 30cm long). Number of entries and time spent in each arm, latency to the first entrance, velocity and distance covered were calculated through Ethovision XT7 (Noldus Information Technology, The Netherlands) software.

2.4.3. Conditioned anxiety and fear extinction: Vogel Conflict Test

2.4.3.1. Unconditioned anxiety

The conditioned anxiety was assessed through a Vogel conflict test. Animals were kept on water restriction and allowed to drink water for 30 minutes each day, for 3 days. Daily water intake was recorded by weighing the bottles. In case of too low intake the bottles were placed more time, until water intake reach normal levels (±1.0g). On the day of the experiments, animals were connected to the DBS cable for 20 minutes in their home cages, either stimulated with 300µA or sham. On the first day, (adaptation 1) mice were placed in the skinner-box (with no levers/nosepoke holes and with a water bottle freely accessible) (19cm x 21,5cm) and allowed to drink water for 10 minutes after the first lick. On the second day (adaptation 2), mice were placed in the skinner-box and allowed to drink water for 5 minutes after the start of the test. On the third day (test 1), mice were placed in the same skinner-box under the same conditions; however a footshock (0.25 mA for *Sapap3*$^{-/-}$ males) was released in the grid at each 20 licks. The skinner-box was equipped with a sensor which allowed the assessment of the number of licks, number of bouts and latency to the first lick through MED-PC software (MED-PC IV). All results were normalized with the time of the task in each day.

2.4.3.2. Extinction

The extinction of aversive memory was assessed by placing the mice in the same skinnerbox used previously for the Vogel Conflict Test. The day after the VCT (extinction), mice were connected to the DBS cable and stimulated for 20 minutes, as previously. Then, mice were placed in the skinner-box and

allowed to explore and drink water for 15 minutes. In order to consolidate the memory, animals were kept 30 more minutes in the skinnerbox but without the bottle of water. The day after (test 2), mice were directly placed in the skinnerbox without being connected, and water intake was allowed for 5 minutes. As in VCT, number of licks, number of bouts and latency to the first lick was assessed.

2.4.4. Cognitive flexibility

2.4.4.1. Apparatus

Training and testing was conducted in two identical triangular operant conditioning chambers (19cm x 19cm x 25cm) (Lafayette-Campden). The front wall of each chamber consisted of an infrared touch screen. The rear wall consisted of (1) a centrally mounted liquid dipper which provided access to 150 µL of a 20% sucrose solution as reward, (2) a trial initiation stimulus light located above the food receptacle, and (3) a house light centrally mounted at the top of the chamber. Operant conditioning chambers were controlled by a Lafayette Instruments control unit running ABET II and Whisker software.

2.4.4.2. General procedures

Mice were trained and tested in the same chamber between 9:00 and 14:00 daily, five days a week, until they completed the experiment. During this time mice were maintained at 85% of baseline weight, which was taken at the beginning of the experiment.

2.4.4.3. Training

Before the acquisition task, mice were trained in five different stages. Training stages lasted for 60min, with the exception of the first stage of habituation that lasted 40 min, or until mice reached criterion, whichever occurred first. In the first stage, the house light was turned off and stimuli were not shown on the touch screen. A tone of 3 KHz was played and the tray-light was illuminated with the food-tray being primed with 150 µL of 20% sucrose. The reward tray light was turned off after the mouse left the reward tray and a 10s delay was applied before the starting of a new trial. In the second stage, stimuli (6.5 cm × 6.5 cm frame with 0.2 cm wide) were displayed randomly on the right or left of the screen, one at a time. After a delay of 30s the image was removed, the tray light illuminated, a tone presented and the sucrose solution delivered. Entry to collect the food turns off the tray light and an inter-trial interval (ITI) of 20s starts. Mice reached criterion on this stage after having collected at least 30 rewards in 60 min. The third stage is similar to the previous with the exception that mice are now required to nose-poke the stimulus on the screen in order to receive the reward. The fourth stage is similar to the last one with the exception that the mice are required to initiate the trial by nose-poking and leaving the reward tray before the stimulus is being displayed on the screen. Finally, in the last stage of training, a punishment is added after an incorrect nose poke in the black side of the screen with lights inverting for a time out period of 5s. Mice reached criterion after nose-poking at least 23/30 times the stimulus in 60 min for two following days.

2.4.4.4. Acquisition of the visual discrimination

The acquisition stage was identical to the last stage of training with the exception that, for the first time, two visual stimuli were randomly presented on the right or left side of the screen (Fig. 9). Nose-poking the correct stimulus resulted in food delivery while nose-poking the incorrect stimulus resulted in a timeout, signalled by the changing of the house light. Immediately following a nose-poke to either stimulus, the visual stimuli were removed from the screen. A 20s ITI (after reward delivery) or a timeout (after an incorrect response) started, followed by the illumination of the light located above the food receptacle, signalling that the mouse could initiate another trial.

During each session, the second and all subsequent trials were considered either “correction” or “non-correction” trials depending on the correctness of the previous trial. Specifically, a correction trial followed an incorrect trial and a non-correction trial followed a correct trial. During correction trials, stimulus presentation was not randomized. Rather, the correct and incorrect stimuli were presented on the same side as in the previous trial. The purpose of this was to prevent the development of a strategy in which mice ignored the visual stimuli, choosing always the same side, and therefore rewarded on 50% of the trials. A non-correction trial followed a correct trial, and stimulus presentation was randomized.

Mice reached criterion when they achieved 80% of correctness (calculated using non-correction trials) in 60 min for two days in a row.

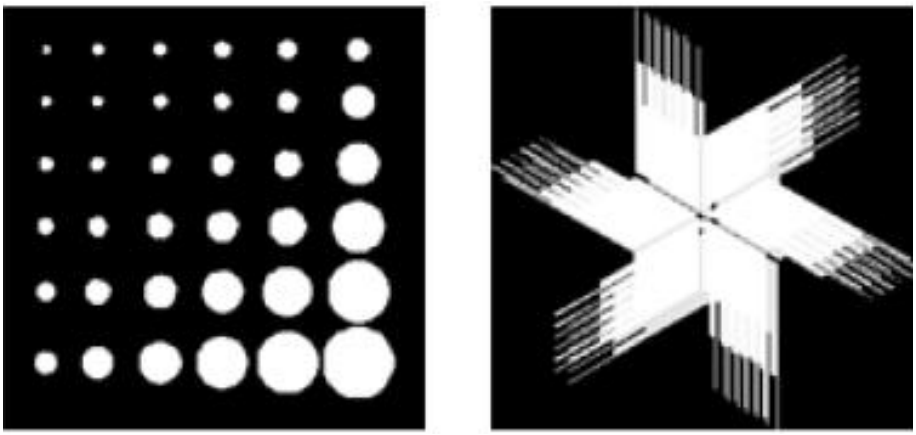

Fig. 9: Stimulus pair used in visual discrimination and reversal learning

2.4.4.5. Reversal

Once mice reached criterion on the acquisition stage, they were tested on a reversal stage. Serial reversal stages were identical to the acquisition stage with the exception that the response contingencies were reversed relative to the previous stage. Specifically, mice that were rewarded for nose-poking stimulus A during the acquisition stage were rewarded for nose-poking stimulus B during reversal and vice-versa.

2.5. Histology:

After the completion of all the experiments, mice received a stimulation of 20min and kept in the homecage for 1h30. After this period, mice were deeply anesthetized with pentobarbital and perfused transcardially, initially with 0.1M PBS and then with cold 4 % paraformaldehyde fixative in 0.1M PBS. Brains were then removed and post-fixed for 24 h and submerged in 30% sucrose in 0.1 M PBS for approximately 48 h. Brains were then frozen and kept at -80ºC.

In order to undergo the staining protocol, brains were cut with a cryostat into 30µm coronal sections and kept in TBS.

2.5.1. Cresyl violet staining

Electrode placement was confirmed using a cresyl violet staining. Briefly, slices were placed in coated glasses and allowed to dry for 24h. Tissue sections were hydrated with 95%, 70% and 50% Ethanol, washed with tap water and soaked into cresyl violet solution for 10 min. Then, slices were washed with flowing tap water, dehydrated with 50%, 70%, 95%, and 100% Ethanol and soaked into xylene. Finally, slides were mounted with entellan and analysed under a microscope. Electrode placement was determined by comparison with a Paxinos and Franklin atlas.

2.5.2. c-Fos staining

Endogenous peroxidase was first suppressed using a solution of 10% methanol in TBS with 3% hydrogen peroxide (H_2O_2). Free-floating sections were rinsed briefly with TBS and blocked with gelatine. Then, sections were incubated in the anti-Fos primary antibody for 2h at room temperature (Ab-6 goat anti-serum, Santa Cruz Biotechnology; final dilution 1:1500). Sections were rinsed three times in TBS and incubated in the secondary biotinylated antibody for 1h at room temperature (BA-9500goat antiserum, Vector Laboratories, Burlingame, CA; final dilution 1:400). After 3 rinses in TBS, sections were incubated in an avidin-biotin complex (Vectastain ABC Elite Kit, Vector Laboratories, final dilution 1:800) and rinsed again three times. Slices were then incubated in 7% 3,3′-diaminobenzidine with 7% ammonium nickel sulfate and

0.03% hydrogen peroxide in TBS for 10 minutes. The reaction was stopped and slices washed with TBS. Finally, slices were mounted on gelatinized slides and dehydrated through soaking them in increased concentrations of ethanol solutions. Slices were analysed under a microscope.

2.6. Statistical analysis

Data is reported as mean ± SEM. Statistical analyses of the percentage of time spent grooming, percentage of time and frequency spent in each arm, the amount of licks, licks/bouts and latency for the first lick were performed with repeated-measures ANOVA, with stimulation parameters (ON/OFF) as between subjects' factor. Latency to the open arm, distance, velocity and number of shocks were determined with Student's two-tailed t tests. Male and female data were combined in the % grooming results, since sex differences were not observed. A p value of <0.05 was considered statistically significant.

- Chapter 3 -

Results

3.1. Characterization of *Sapap3*$^{-/-}$ mice

Sapap3$^{-/-}$ mice have been highly used as a model of OCD. In fact, previous studies have demonstrated that *Sapap3*$^{-/-}$ present OCD-like behaviour such as excessive time spent in repetitive behaviour, to the point of being self-injurious, and increased anxiety-like behaviours (Welch et al, 2007). However, other typical symptoms of OCD were not assessed.

It is described that OCD patients present impairment in cognitive flexibility – the ability to change response strategies upon alteration of the environment (Fineberg et al, 2010). To the best of our knowledge, whether this feature is present or not in *Sapap3*$^{-/-}$ mice was never assessed and its existence would strengthen the value of *Sapap3*$^{-/-}$ mice as a model of OCD.

In rodents, cognitive flexibility has been investigated through reversal-learning tasks. In these tasks, animals learn to discriminate between two stimuli, one as being the correct and the other as incorrect. In order to receive a reward (generally a food reward), animals must choose the correct stimulus. Once the rule is learned in a high level, the response contingencies are reversed such that the previous correct response is now the incorrect and vice-versa.

We conducted a pilot experiment aiming to assess cognitive flexibility behaviour in *Sapap3*$^{-/-}$ mice. In order to do that, *Sapap3*$^{-/-}$ mice were tested in a touchscreen chamber under a discrimination/reversal task.

Until now, conclusions were not possible to be obtained from this study. Indeed, *Sapap3*$^{-/-}$ mice failed to learn this task. However, this was not due to a problem in reversing the previously acquired rule, but rather impairment in learning the discrimination task. In fact, mice failed to achieve the discrimination phase, staying only in the initial training phase (Table IV).

Table IV: Number of *Sapap3*$^{-/-}$ mice that achieved the different phase of discrimination/reversal task

Initial Training	Discrimination phase	Reversal phase
		
5/5	2/5	0/5

Moreover, animals who achieved discrimination phase did not show improvement in this task within time (Fig. 10). Of note that in figure 10 only one of the animals that achieved discrimination phase is shown, since the behaviour is similar between the two.

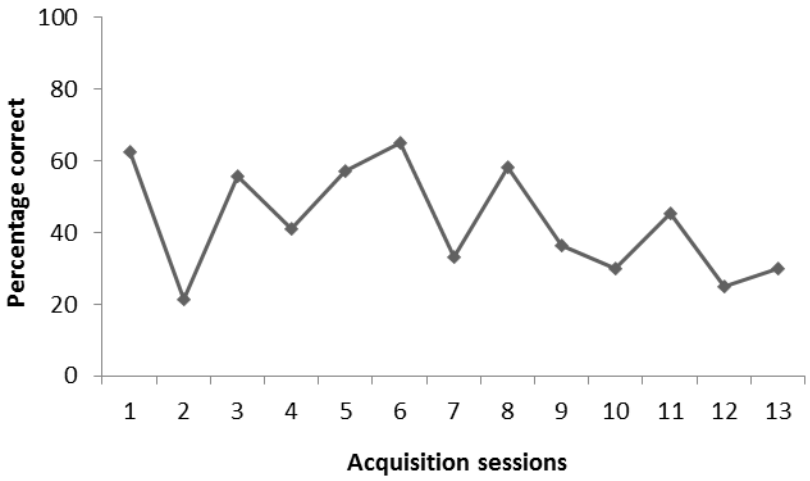

Fig. 10: Percentage of correct answers by a *Sapap3*$^{-/-}$ mouse in a discrimination task. Mouse shows no improvement with acquisition

Animals were not able to continue in training for a longer period because they were they developed serious lesions (self-inflicted damage due to excessive grooming).

These results could suggest that, rather than having deficits in cognitive flexibility, *Sapap3*$^{-/-}$ mice would have a learning problem. With this in mind, we tested wild type C57Bl/6 mice in the same task, under the same conditions.

Contrary to what is described in the literature (Horner et al, 2013) our wild-type C57Bl/6 mice were not able to learn the task with the documented success rate. Indeed, not all the animals were able to learn the task and achieve discrimination phase (Table V). Once again, two out of our five animals achieved the discrimination phase but showed no improvement with time (Fig. 11). Curiously, the only animal that was able to complete this step (80% correct answers, two days in a row) did it within 8 days, the same number of session described as normal in literature (Fig.12) (Homer et al, 2013).

Table V: Number of C57BL/6 mice that achieved the different phase of discrimination/reversal task

Initial Training	**Discrimination phase**	**Reversal phase**
		
5/5	3/5	1/5

The animals used in this task were not naïve; on the contrary, they were used previously in a 5-Choice Serial Reaction Time (5CSRT) (see Mar et al, 2013 for more details). We found that animals were not able to learn the 5CSRT task; however, the performance was not similar for all the animals, with some animals performing better than the others. Interestingly, the animals that presented more difficulties in the 5CSRT task were also the ones with the worst performance in the discrimination task.

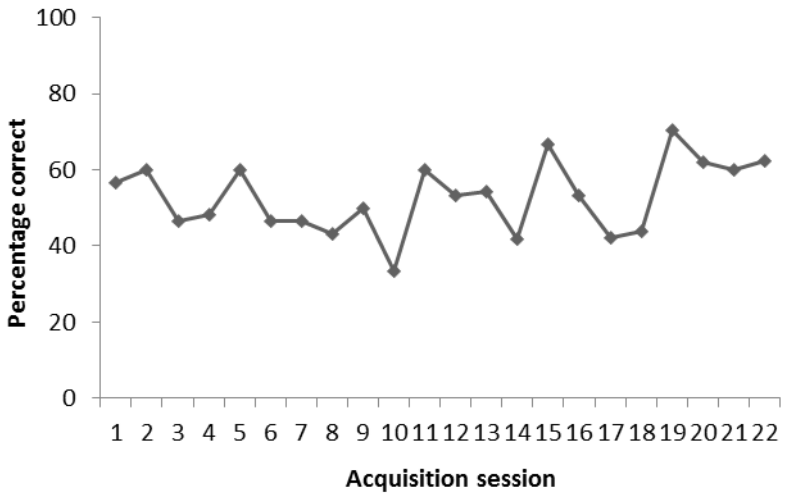

Fig. 11: Percentage of correct answer by a C57BL/6 mouse in a discrimination task. Mouse shows no improvement with acquisition.

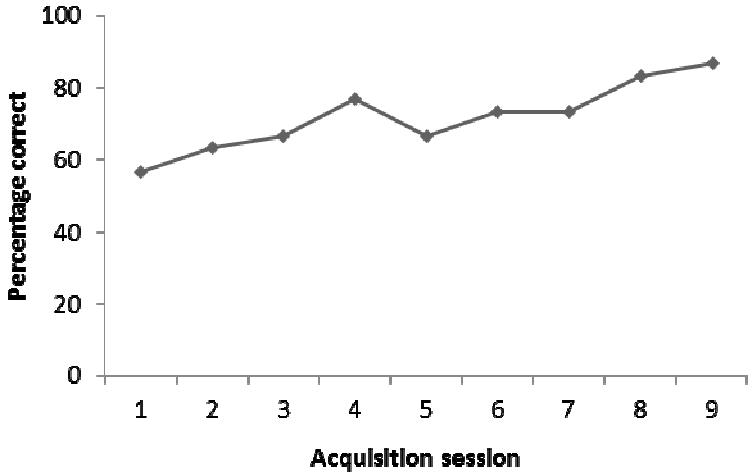

Fig. 12: Percentage of correct answer by a WT C57BL/6 mouse in a discrimination task. Results of the only mouse that learned the discrimination task. Criterion was achieved in the days 8 and 9.

No conclusions could be taken from these results; however, work is still ongoing. Future studies might help clarifying if *Sapap3*$^{-/-}$mice have an impaired cognitive flexibility.

3.2. Reduction of compulsive behaviour in DBS stimulated mice

One of the most frequent symptoms present in OCD is the urge to behave compulsively. Previous studies have shown that *Sapap3*$^{-/-}$ have an increased compulsive behaviour, namely grooming (Welch et al, 2007).

To determine whether DBS would have an effect on compulsive behaviour, animals were placed in an open field and grooming behaviour was manually assessed for 90 minutes. Of note that during the entire procedure (90 min) mice were being stimulated (either DBS or Sham).

Results show that DBS stimulated mice have a significant decrease in the percentage of time spent grooming when compared with Sham stimulated mice (DBS: 9.7 ±1.9; Sham: 20.3 ± 3.5; $p<0.05$)(Fig. 13).

When time points where analysed individually, differences between the two stimulation conditions were not found at all-time points (Fig. 14). A repeated measures ANOVA analysis of the percentage of grooming during the 110 minutes (20 minutes of habituation plus 90 minutes of stimulation) have shown that no significant effect was seen within time ($p=0.238$), a significant effect was seen in time versus stimulation ($p=0.001$) and a significant effect seen in stimulation ($p=0.027$)

In more detail, at the beginning of the experiment, before the stimulation was turned on, groups seem to be homogeneous as the percentage of grooming in the two first 10-minute bins is the same for both groups. Indeed, a repeated measures ANOVA analysis over the 20 min baseline has showed no significant effect within time ($p=0.351$) neither in time versus stimulation ($p=0.091$).

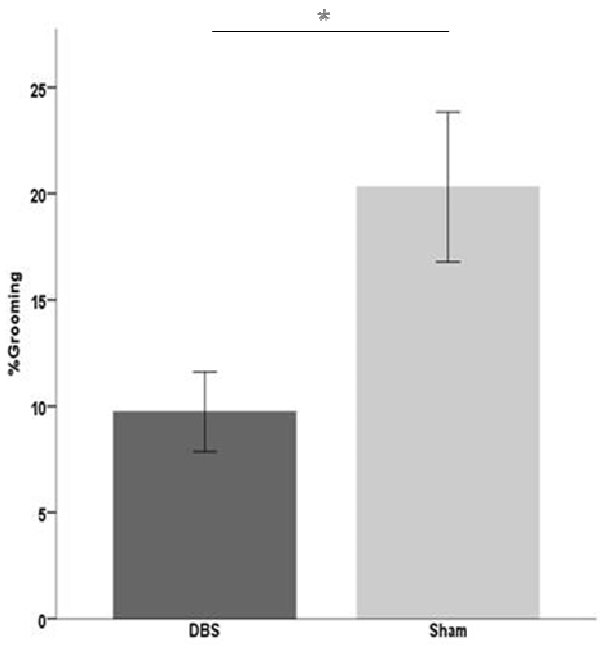

Fig. 13: Percentage of grooming in DBS and Sham stimulated mice. Dark grey, DBS stimulated mice (n=10); light grey, Sham stimulated mice (n=13). DBS stimulated mice spent significantly less time grooming. *p<0.05, two-tailed t-test. Data is presented as means ± s.e.m

For this reason, the significant effect seen previously was due to an effect after the stimulation was turned on. Indeed, repeated measures ANOVA analysis performed in the 90 minutes after the stimulation was turned on has shown a significant effect in time versus stimulation ($p<0.05$) while this effect did not change with time ($p=0.660$). We then analysed in more detail where the difference existed. Indeed, a significant difference between the two stimulation condition was seen only in the data points T=30 (DBS: 5.4 ± 2.7; Sham: 27.9 ± 4.9; $p<0.05$) and T=40 (DBS: 7.2 ±1.4; Sham: 21.1± 5.1; $p<0.05$). Although there is no significant difference in the remaining time-points, the DBS stimulated mice spend in average less time grooming than the Sham stimulated mice.

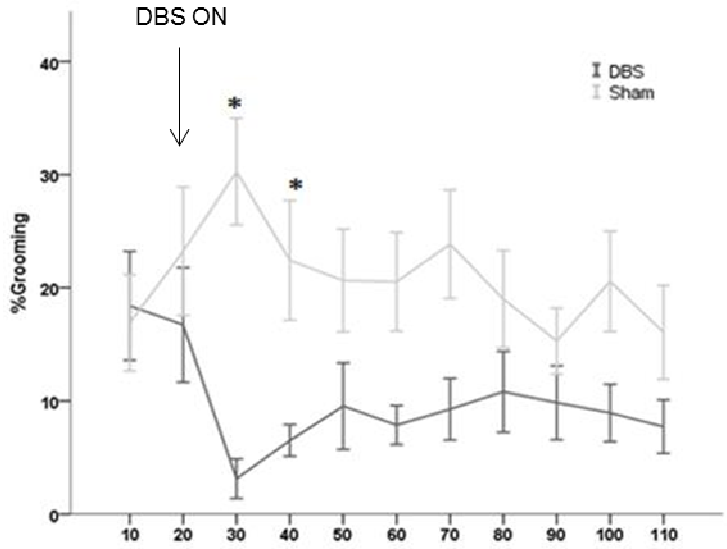

Fig. 14: Percentage of grooming within time-course, in DBS and Sham stimulated mice. Dark grey, DBS stimulated mice (n=10); light grey, Sham stimulated mice (n=13). Time is presented in minutes. Significant differences were found only for T=10 (average % grooming of the first 10 minutes) and T=20 (average % grooming of 10 to 20 minutes). *p<0.05, repeated measures ANOVA. Data is presented as means ± s.e.m

These results suggest that DBS is capable of reducing compulsive grooming.

3.3. Effect of DBS in unconditioned anxiety

Another typical symptom of OCD is the increase of anxiety behaviours.

Sapap3$^{-/-}$ mice have revealed increased levels of unconditioned anxiety when compared with wild type mice through an open field, dark-light emergence and elevated zero maze tests (Welch et al., 2007). With this in mind, we aimed to investigate whether DBS would have an effect in unconditioned anxiety. In order to do that, we conducted an elevated plus maze task. Here, animals that

are more anxious will tend to spend more time in the closed arms while less anxious animals will tend to explore both types of arms of the maze in an equal way.

This test was performed both in male and female mice with results differing between sexes, and therefore analysed separately.

3.3.1. Males

In what concerns males, DBS does not seem to have an effect in the unconditioned anxiety. Indeed, repeated measures ANOVA analysis showed no significant difference in the percentage of time spent in each arm influenced by the stimulation ($p=0.244$) (Fig. 15a). Both DBS and Sham stimulated mice spent more time in the closed arm than in the open arm or middle part, a signal of anxiety. In particular, a two-tailed t-test analyses has shown that DBS stimulated mice differed significantly in the time spent between the closed arm and the middle ($p<0.01$) and between the closed arm and the open arm ($p<0.001$), being the difference between the middle and the open arm not significant ($p=0.05$). On the other hand, a two-tailed t-test analysis performed in Sham stimulated mice showed a significant effect between the closed arm than in the middle ($p<0.01$), however no difference was seen between the closed arm and the open arm ($p=0.115$) and the middle and the open arm ($p=0.251$).

Another way to look at the results from the elevated plus maze is through the number of entries that the animals do in each arm. Indeed, these results are in accordance with the ones obtained from the percentage of time spent in each arm (Fig. 15b). A repeated measures ANOVA analysis showed no difference between the number of entries in each arm depending on the DBS or Sham

stimulation (p=0.921). In particular, a two-tailed t-test showed that DBS stimulated mice entered significantly less often in the open arm than in the closed arm ($p<0.05$), being the difference between the closed arm and the middle (p=0.215) and the middle and the open arm (p=0.157) not significant. In what regards Sham stimulated mice, although it seems that the number of entries is higher in the closed arm, a two-tailed t-test showed no significant difference between the closed arm and the open arm (p=0.174), the closed and the middle arm (p=0.09) or middle and open arm (p=0.933).

The latency to explore the open arm for the first time can also be used as a measure to assess the anxiety. As described above, anxious animals will tend to avoid open arms, and therefore, the latency to the open arm will be higher. Results obtained are once again in accordance with the percentage of time and frequency spent in each time, with a two-tailed t-test showing no significant difference between stimulation conditions (DBS: 107.9s ± 38.9; Sham: 77.7s ± 45.5; p=0.639) (Fig. 15c).

One could argue that the results above described would be dependent of a motor activity difference between the two stimulation conditions, so we assessed distance travelled and the velocity of these mice. Indeed, a two-tailed t-test showed no differences neither in the distance (Fig.15d) (DBS: 2200.0cm ± 303.1; Sham: 1820.2cm ± 492.2; p=0.558) or in the velocity (DBS: 3.7cm/s± 0.5; Sham: 3.1cm/s ± 0.8; p=0.557) (Fig.15e)

All together, these results suggest that DBS does not have an effect in unconditioned anxiety in male mice.

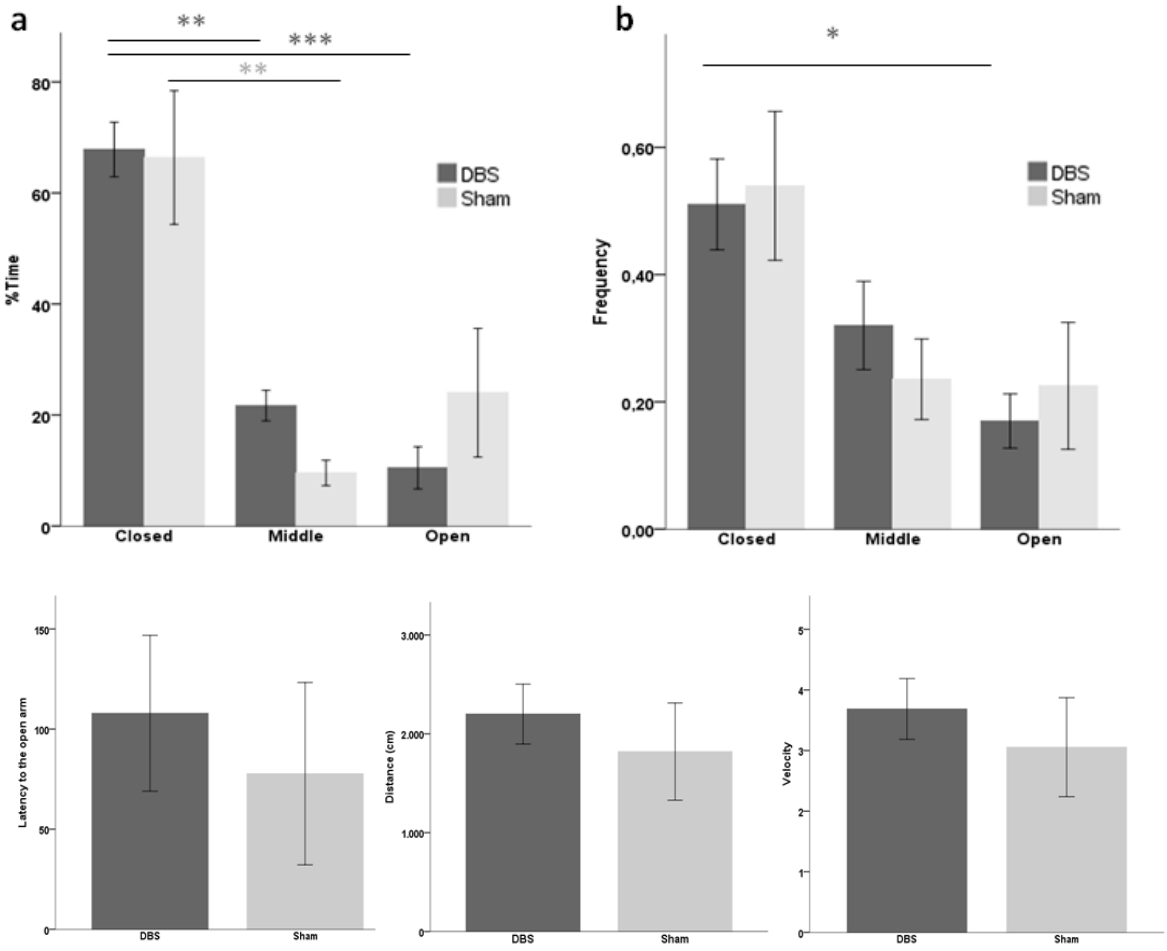

Fig. 15: Effect of DBS in unconditioned anxiety in DBS and Sham stimulated male mice. Dark grey, DBS stimulated mice (n=6); light grey, Sham stimulated mice (n=8).**a**, no effect of stimulation in the percentage of time spent in each arm, being this results confirmed with **b**, the frequency of entries in each arm; **c**, both DBS and Sham stimulated mice took about the same time (in seconds) to enter the open arm for the first time; this difference was no due to a motor effect once no difference was seen in **d**, the distance (in centimetres) and **e**, in the velocity (centimetres/seconds); *p<0.05, **p<0.01 and ***p<0.001 Repeated measures ANOVA for **a** and **b**, and two-tailed t-test for **c, d** and **e**. Data is presented as means ± s.e.m

3.3.2. Females

The same experiment was performed in female *Sapap3*$^{-/-}$ mice. A repeated measures ANOVA analysis was performed to assess the effect of stimulation in the percentage of time spent in each arm. Indeed, no significant effect was seen (p=0.347)(Fig. 16a). However, the pattern is different from the one found in males. In particular, Sham stimulated mice seem to spend more time in the closed arm than in the open arm, however, this value was not significant as analysed through a two-tailed t-test (p=0.144). Similarly, a two-tailed t-test showed no differences between time spent in the closed arm and in the middle part (p=0.027) and middle and open arm (p=0.594). This pattern resembles the results obtained previously for the male mice. Contrarily, female DBS stimulated mice seem to present a different trend. A closer analyses performed with a two-tailed t-test showed that mice seem to equally explore the closed and the open arm (p=0.959) with no significant differences seen between the closed arm and middle (p=0.209) or middle and open arm (p=0.453).

This idea was confirmed with the number of entries in the different arms (fig. 16b). A repeated measure ANOVA analysis performed in the number of entries in each arm showed no significant effect of the stimulation. Indeed, a two-tailed t-test showed that there is no difference in Sham stimulated mice in the number of entries in the closed arm and open (p=0.450), no significant difference between the closed and the middle arm despite the trend to spent more time in the closed arm than the open (p=0.053) and no difference between the middle and open arm (p=0.369). On the other hand, a two-tailed t-test showed that DBS enter nearly the same time in the closed arm and the open

arm (p=0.818), with no significant effect seen between closed and middle arm (p=0.106) and middle and open (p=0.250).

The strongest evidence of a different effect in females comes from the analysis of the latency to explore the open arm for the first time (Fig. 16c). Indeed, even if no significant difference between DBS and Sham stimulated mice was seen through a two-tailed t-test analysis (DBS: 6.61s ± 1.9; Sham: 60.2s ± 41.3; p=0.062), a trend is visible, with Sham stimulated mice taking longer to enter the open arm for the first time.

As previously seen in males, the results obtained were not influenced by a motor difference between the two groups. Indeed, a two-tailed t-test analysis showed no significant differences between stimulation groups in distance (DBS: 1426.4cm ± 387.8; Sham: 967.9cm ±; 107.1; p=0.208)(Fig. 16d) and in the velocity(DBS: 2.43cm/s ± 0.6; Sham: 1.6cm/s ±; 0.16 p=0.166)(Fig.16e).

Although we cannot conclude that DBS had an anxiolytic effect in female mice, once the results are not significantly different, this might be due to a high variability seen in both groups. We would have to increase the number of mice per group in order to decrease this variability, and therefore clarify this effect. Indeed, a power analysis was performed and a minimum of 16 animals would be necessary to see a significant difference.

Interestingly, these results suggest that the unconditioned anxiety in males and females might be modulated by different neurobiological mechanisms.

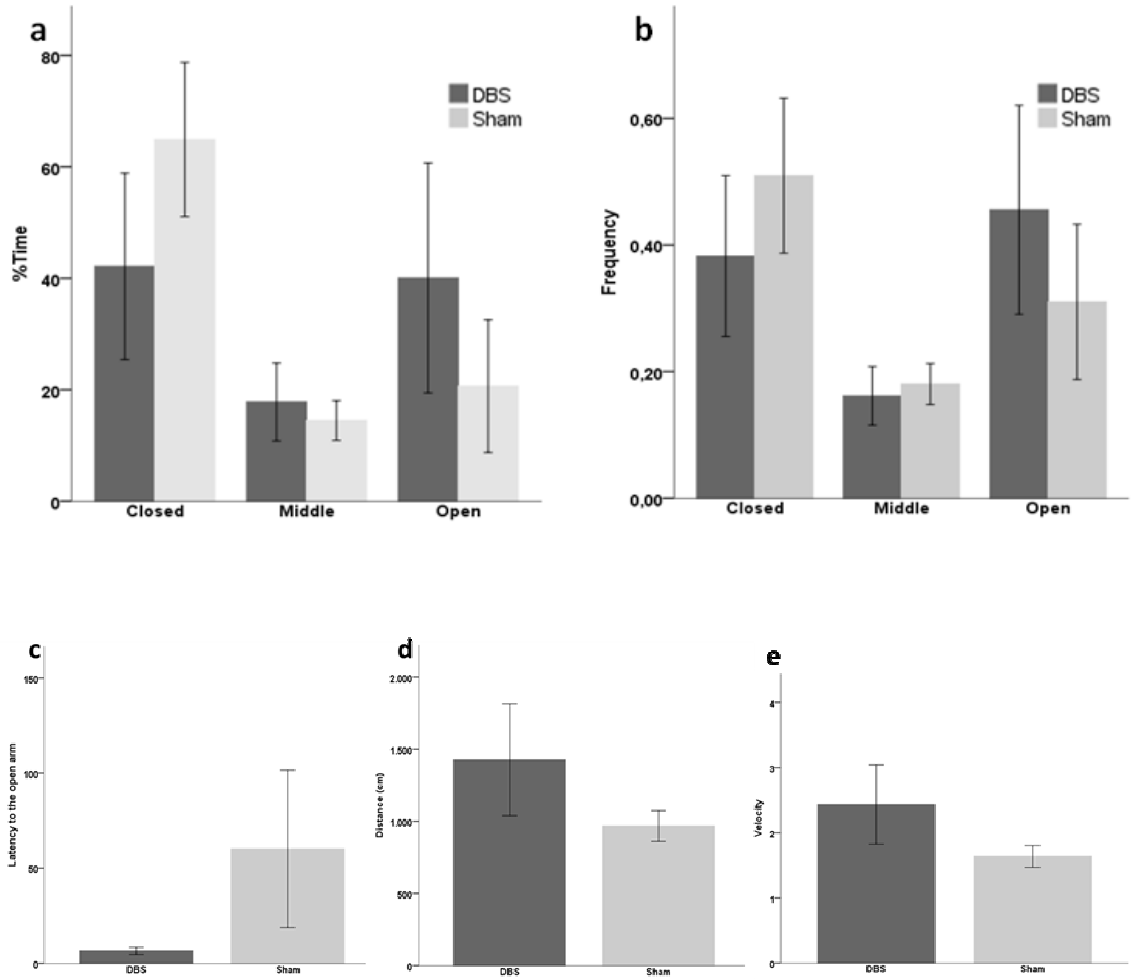

Fig. 16: Effect of DBS in unconditioned anxiety in DBS and Sham stimulated female mice. Dark grey, DBS stimulated mice (n=4); light grey, Sham stimulated mice (n=6).**a**, no significant effect of stimulation in the percentage of time spent in each arm, confirmed with **b**, the frequency of entries in each arm; **c**, DBS stimulated mice entered in the open arm faster than Sham stimulated and these results seems to not being dependent of a motor activity as no difference was seen in **d**, the distance and **e**, the velocity (time is presented in seconds); Repeated measures ANOVA for **a** and **b**, and two-tailed t-test for **c, d** and **e**. Data is presented as means ± s.e.m

3.4. Effect of DBS in conditioned anxiety, fear extinction and memory consolidation

Clinical studies have shown that DBS is capable of reducing anxiety symptoms. However, this reduction seems to be restricted to conditioned anxiety – anxiety acquired by a specific and traumatic event – and not affect the unconditioned anxiety – innate (Denys et al., 2010; Greenberg et al., 2010). On the other hand, previous studies suggested that DBS was effective in extinguish the fear conditioning in rats (Rodriguez-Romaguera et al., 2012)

In order to assess the effect of DBS in conditioned anxiety and fear extinction, we tested *Sapap3*$^{-/-}$ DBS stimulated mice and *Sapap3*$^{-/-}$ Sham stimulated mice in a Vogel Conflict test. In this test, animals receive a punishment (mild electric shock) leading to conditioned (learned) suppression of response for reinforcement (water). Thus, less anxious animals will continue drinking water regardless of the shock. Our test consisted of 5 experimental sessions in 5 days (Fig. 17). In the two first days (adap1 and adap2) mice were allowed to freely explore and drink water; on the third day (test1), the conditioned anxiety was tested, and mice received a footshock at every20th lick; on the fourth day (extinction) the animals were allowed to freely drink water without being shocked (fear extinction); finally, on the fifth day (test2), the animals were again allowed to freely drink water as the consolidation of the memory was assessed.

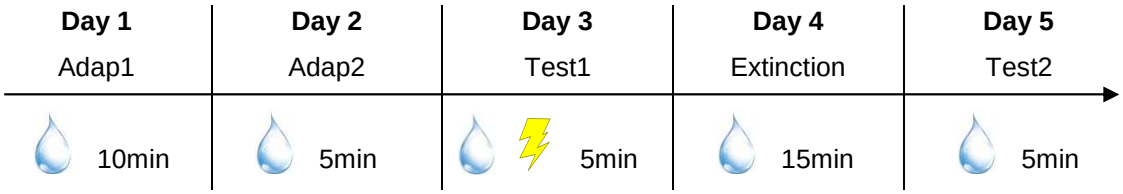

Fig. 17: Schematic representation of the schedule used in unconditioned anxiety and fear extinction assessment. Animals were tested 5 days in a row. In all days animals were allowed to drink water for a certain time, depending the day. On the third day, at every 20th lick the animal received a footshock (0.25mA *Sapap3*$^{-/-}$ male). During the experiments, all animals were connected and received DBS or Sham treatment.

A two-tailed t-test analysis showed no effect of DBS in the number of shocks taken by each group (DBS: 18 ± 4.1; Sham: 18.6 ±5.5; p=0.930) (Fig.18a).

When analysed the effect of stimulation in the number of licks (during adap1, adap2 and test1) through a repeated measures ANOVA analysis, no significant effect was seen (p=0.209) (Fig. 18b). Specifically, when analysed the effect of stimulation in number of licks Test1/number of licks Adap2 with a two-tailed t-test, no significant effect was found (DBS: 0.73 ± 0.19; Sham: 0.68 ± 0.21; p=0.872).

Thus, results suggest that DBS was not capable to reduce the conditioned anxiety in DBS stimulated mice.

A prominent feature observed in most OCD patients is repetitive avoidance behaviours that fail to extinguish. This feature suggests impairment in the circuits that regulate fear extinction (Rodriguez-Romaguera et al., 2012). With this in mind, we hypothesized that DBS could facilitate the extinction of

fear. To study this we tested the mice in the same conditions, the day after the Vogel Conflict test.

In the first 3 days of the experiment DBS stimulated mice tend to take more time to drink for the first time when compared with Sham stimulated mice. However, this difference was not significant as confirmed with a repeated measures ANOVA analysis ($p=0.209$). However, this pattern changed in the extinction day, where the latency for the first lick in DBS stimulated mice seems to be lower than Sham stimulated mice (fig. 18c). However, this value was not significant as analysed through a two-tailed t-test (DBS: 76.4s ± 68.9; Sham: 153.4s ± 109.2; $p=0.544$). Moreover, a repeated measures ANOVA analysis was done to analyse the effect of the stimulation in the latency for the first lick between Test1 and Extinction, and no significant difference was seen ($p=0.642$).

These results were confirmed with the number of licks. In fact, when analysed the effect of stimulation in "number of licks Extinction/number of licks Test1" with a two-tailed t-test, no significant effect was found (DBS: 1.7 ± 0.72; Sham: 1.6 ± 0.67; $p=0.892$).

Altogether, these results suggest DBS had no effect in the fear conditioning.

Finally, the day after extinction, mice were tested again under the same conditions, to assess memory consolidation. Our results show that both DBS and Sham stimulated mice seem to behave in the same way. Both DBS and Sham stimulated mice had an increase of licks when comparing "number licks test2/number of licks extinction" (35% and 33% respectively)(fig.18b). However, these values were not significantly different from each other as analysed

through a two-tailed t-test (DBS: 3.08 ± 0.45; Sham: 3.46 ± 0.69; p=0.641). On the other hand, both groups took approximately the same time to drink for the first time as analysed by a two-tailed t-test (DBS: 21.4 ± 9.9; Sham: 21.3 ± 9.5; p=0.994) (Fig. 18c).

To make sure that the lack of effect of DBS observed in conditioning anxiety, fear conditioning and memory consolidation was not due to a difference in the number of times the animals started to drink, i.e. the number of bouts, we analyzed the number of licks/bouts. A repeated measures ANOVA analysis showed that there is no significant difference in the number of licks/bouts (p=0.564)(Fig.18d).

In conclusion, these results suggest than DBS has no effect in conditioned anxiety, fear extinction or memory consolidation.

3.5. Long term effect of DBS

The results shown above suggest that DBS decreases some of OCD-like behaviour in this mouse model. However, whether this effect is acute or chronic is still not known. In order to assess this question, we analysed the compulsive behaviour in both DBS and Sham stimulated mice in the end of all experiments. Here, none of the groups was stimulated. The aim was to evaluate whether DBS permanently rescued the impairment *Sapap3*$^{-/-}$ mice, or if this effect was only evident when DBS was ongoing.

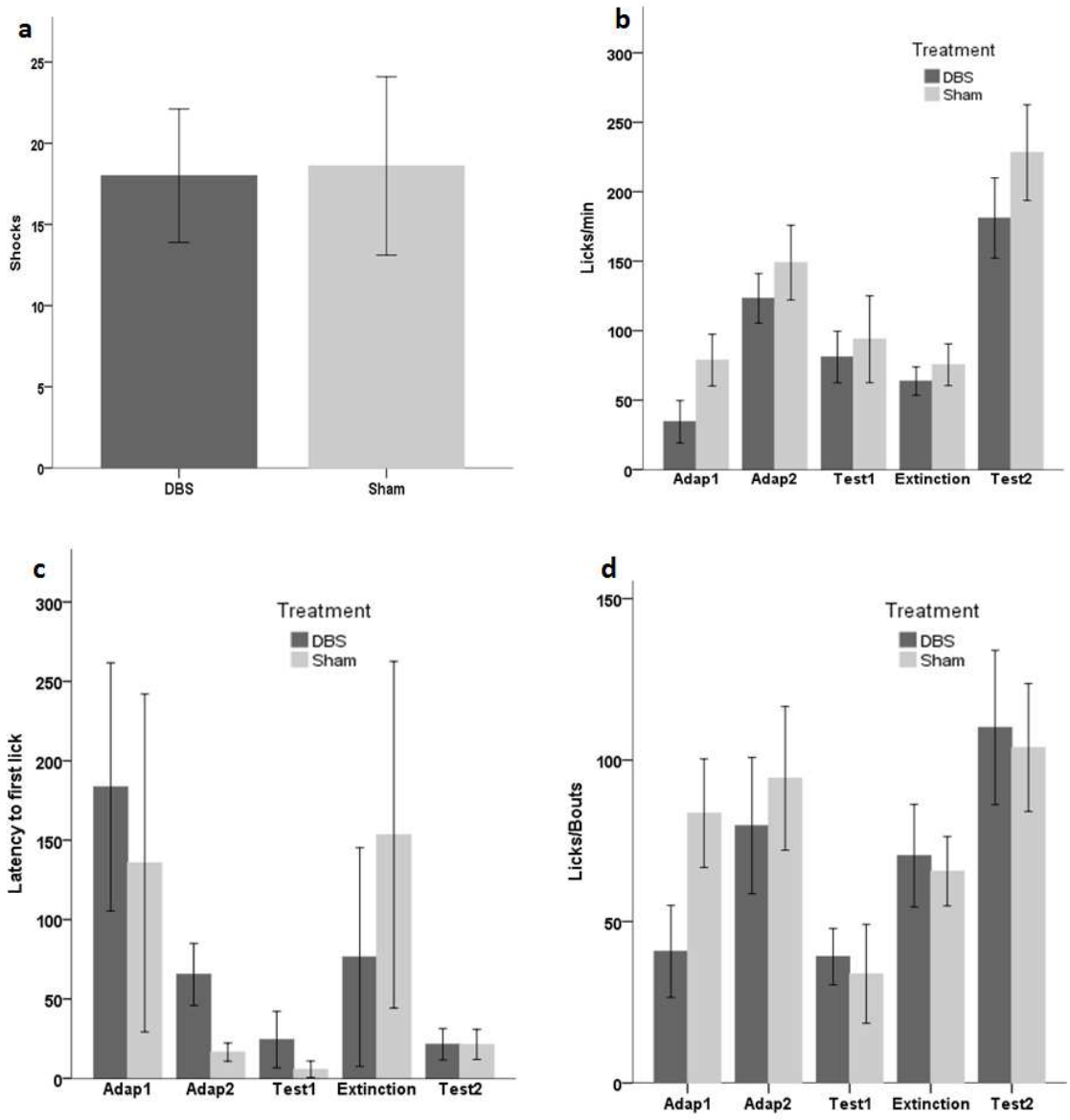

Fig. 18: Conditioned anxiety, fear extinction and memory consolidation in DBS and Sham stimulated mice. Dark grey, DBS stimulated mice (n=7); light grey, Sham stimulated mice (n=5). **a**, no significant difference in the number of shocks, **b**, number of licks **c,** latency to the first lick; or **d**, number of licks/bouts; Two-tailed t-test for **a**, and repeated measures ANOVA for **b, c** and **d**. Data is presented as means ± s.e.m.

It is important to note that the animals used here are also the ones used for the first experiment, when stimulation was turned ON for DBS stimulated mice. However, some animals did not achieve this last stage. This was mainly due to two different reasons: animals were too injured and therefore had to be sacrificed (generally animals which the percentage of grooming was higher); or because the animals lost the head cap during the course of the experiments. In order to establish a comparison between initial and final stage, we excluded also from the initial experiment the animals that failed to achieve the final stage.

Our results show that DBS and Sham stimulated mice have similar levels of grooming in these conditions. In fact, a two-tailed t-test analysis in the final experiment showed no significant difference in the percentage of grooming depending of the group (DBS: 12.7 ±2.1; Sham: 12.6 ± 2.1; $p=0.979$).

In the first experiment we reported a significant difference in grooming behaviour, with DBS stimulated mice spending significantly less time grooming than Sham stimulated mice ($p<0.05$). However, we see that this effect in not present in the final experiment, when the stimulation was turned off (Fig. 19).

Although there seems to be a difference between the levels of percentage grooming in the initial stage and the final stage, the SEMs largely overlap and statistical analysis shows that there is no difference.

These results suggest that DBS acts in an acute way, i.e., when it is ongoing; however, it is not capable to induce chronic changes. These results are in accordance with the results previously described in the clinic and within our laboratory (Denys et al, 2010).

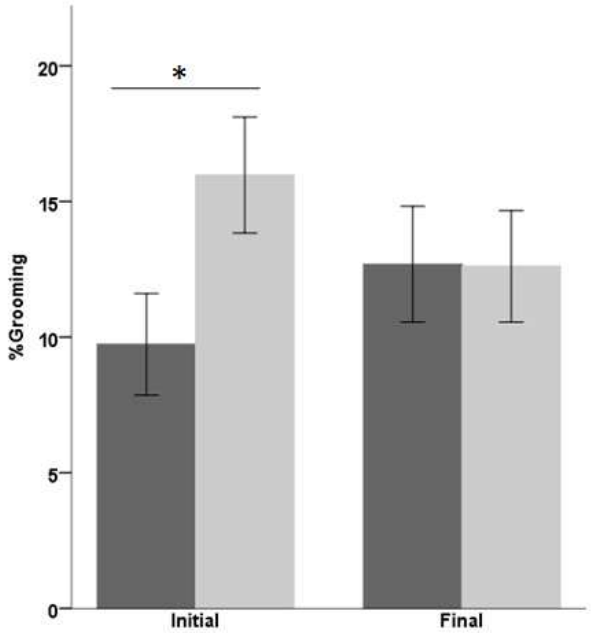

Fig. 19: Long term effect of DBS in the absence of DBS in previously DBS and Sham stimulated mice. Dark grey, DBS stimulated mice (n=4); light grey, Sham stimulated mice (n=5). In the initial stage, mice were either stimulated or not depending on whether they were DBS or Sham mice respectively. In this condition DBS stimulated mice spent significantly less time grooming. In the final stage both DBS and Sham animals were not stimulated. Under these conditions no significant difference was seen between DBS and Sham group. *p<0.05, two-tailed t-test. Data is presented as means ± s.e.m.

3.6. Electrode position

Electrodes were implanted in the IC using the coordinates referred in the Paxinos and Franklin atlas.

After, histology was performed and the slices stained with cresyl violet. The position where the electrodes were implanted was checked by overlapping the picture of the slice, with of the same region taken from the Paxinos and Franklin atlas (fig. 20).

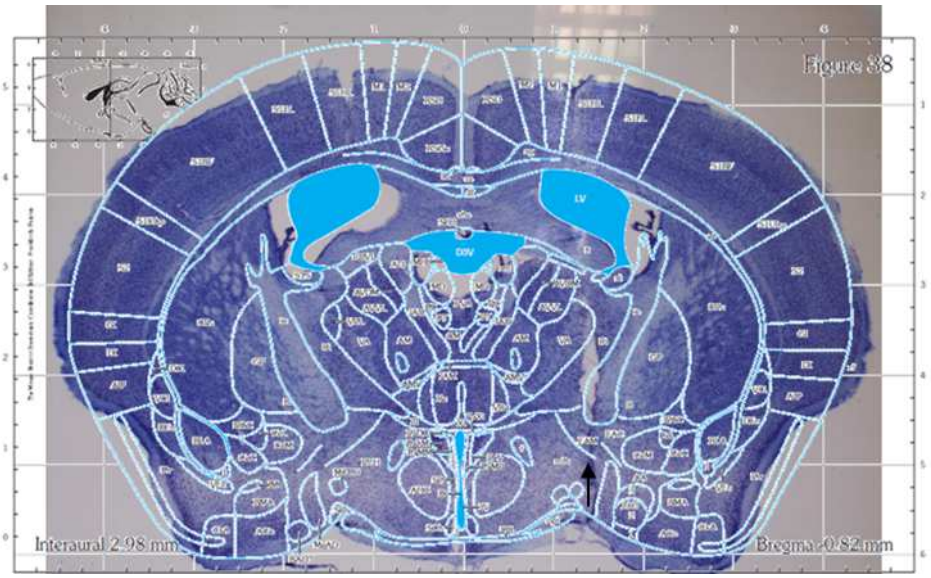

Fig. 20: Overlap of a slice from *Sapap3*$^{-/-}$ brain stained with image taken from a mouse atlas. Overview of coronal section of the brain stained with a cresyl-violet technique. Arrow indicates the tract of the electrode.

The IC is of reduced size and we have learned that its targeting is highly technically demanding. In fact, not all the animals used in these experiments had both electrodes implanted in the IC. Thus, we had three different groups relative to the position of the electrode: animals with both electrodes implanted in the IC, animals with only one electrode implanted in the correct position, and animals with both electrodes nearby the IC. We are planning to include this information in our analysis in the future, in order to verify if this affects the behaviour of the animals.

3.7. Brain areas activated due to DBS

c-Fos is a cellular proto-oncogene belonging to the immediate early gene family of transcription factors. Expression of c-Fos is an indirect marker of neuronal activity once c-Fos is often expressed when neurons fire action

potentials. Thus, up-regulation of c-Fos mRNA in a neuron indicates recent activity.

With this in mind, we used c-Fos staining to underlie the anatomical mapping of neuronal circuits induced by DBS. This approach would allow us to clarify the anatomical areas in the brain that have been changed through DBS, and consequently, involved in the pathology of OCD. DBS and Sham stimulated mice were perfused after the last experiment and their brains were stained against the c-Fos, allowing the expression of c-Fos to be visible through black dots (fig. 21).

Previous studies in our lab showed that also around the electrodes in the IC an increase in Fos immunoreactivity was visible. Our initial goal was therefore to quantify the expression of c-Fos around the electrode placement and see whether DBS would have activated the surrounding cells in addition to the passing fibres. Moreover, we were interested in evaluating which other brain areas could have been affected by DBS. However, in some of the animals, the placement of the electrode was not clear since an obvious tract of the electrodes was not visible. In this way, we were not able to do the quantification of c-Fos around the electrode as we could be inducing error by comparing slices that were differently far from the implantation place, and therefore, places where the intensity of the signal would be different.

This work, however, is still ongoing and we are trying to optimize the process in order to clearly visualize the tips of the electrodes without affecting the expression of c-Fos. In fact, through expression of c-Fos we could have a better idea of which areas are being changed by DBS and therefore, which areas are possibly involved in the pathology of OCD.

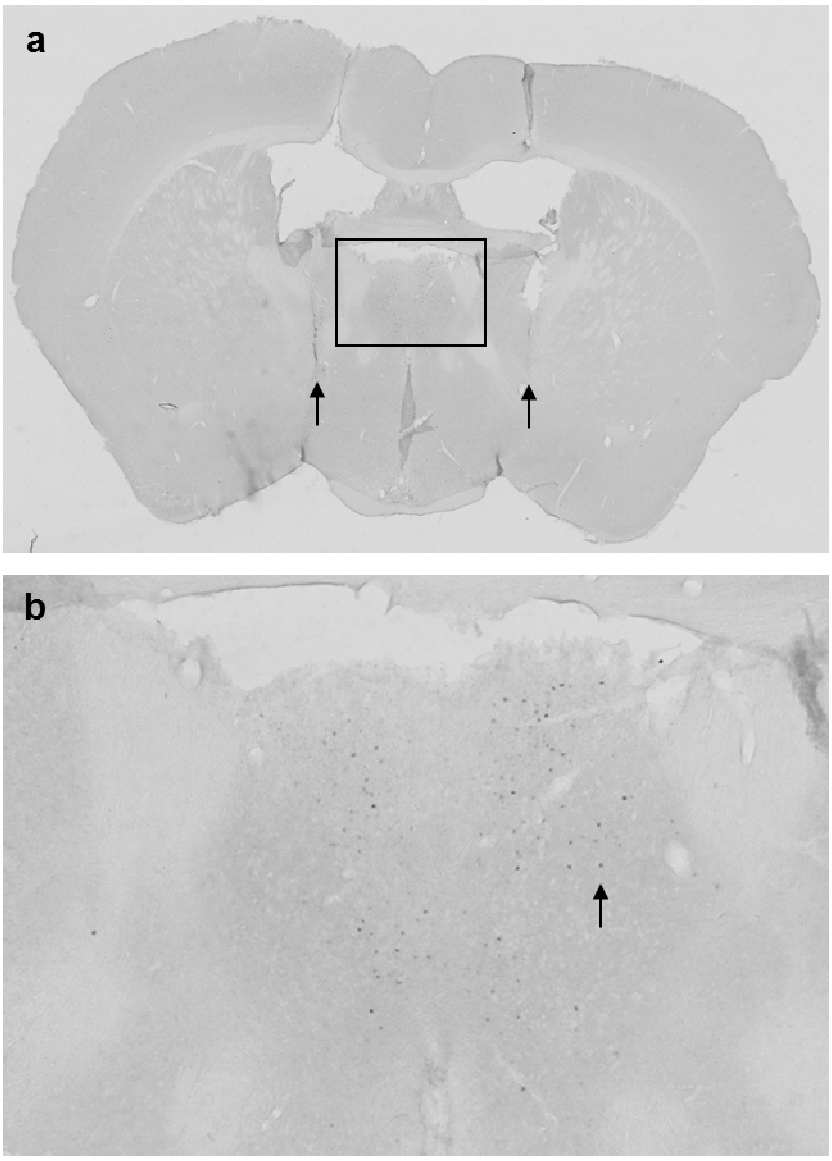

Fig. 21: DAB-nickel immunohistochemistry of *Sapap3*$^{-/-}$ brain. Slices were stained with an antibody specific for c-Fos. **a**, overview of coronal section, arrows indicate the tract of the electrodes; **b**, amplification of a small area from the image a, arrow point to the expression of c-Fos represented through the dark dotes.

- Chapter 4-

Discussion

4.1. C57BL/6 and *Sapap3*$^{-/-}$ failed to learn the discrimination task

In order to study the neurobiological mechanisms that underlie human disorders and find new and more effective treatments, researchers use rodent models. However, it is hard to mimic the conditions of a disorder, even more when it comes to a psychiatric disorder, where little is known about the causes. The best researchers can do is to find evidence that fulfil the validity criteria for animals models.

Sapap3$^{-/-}$ mice were developed and characterized for the first time by Welch et al, 2007. Indeed, mice fulfilled the three criteria, presenting OCD-like behaviours, impaired CSTC circuit and showing improvement of its symptoms when administered with fluoxetine, a SSRI effectively used in OCD patients.

Our goal was to find new evidences that would strengthen the value of *Sapap3*$^{-/-}$ mice as an OCD mouse model. Thus, we investigated a new behavioural feature that is known to be impaired in OCD patients, but as far as we know has never been assessed in *Sapap3*$^{-/-}$ mice: cognitive flexibility. For this purpose, we tested *Sapap3*$^{-/-}$ mice in a touch-screen chamber under a discrimination reversal task.

Our results were inconclusive as both C57BL/6 and *Sapap3*$^{-/-}$ mice failed to learn the discrimination task.

Indeed, little is known about the ability of *Sapap3*$^{-/-}$ mice to learn. Our studies have suggested that young animals are able to learn a task in an operant box, while older animals present difficulties to be trained in a more complex task. In this project we used animals older than 5 months, period when they start presenting the OCD-like phenotype. However, at this age, it is likely that these animals are no longer able to learn the task of interest, not only due

to cognitive decay, but mainly because they present high levels of grooming which might indirectly prevent them from learning. In other words, the lack of "learning success" could be due to the fact that *Sapap3*$^{-/-}$ mice are too focused grooming, not paying attention to the task, rather than due to a cognitive impairment. One solution for this problem would be to start training the animals before the appearance of OCD-like behaviours, when excessive grooming would not be present. However, the reversal phase of the test would have to be performed after the presentation of the symptoms in order to mimic the human condition.

More surprising than the assumable difficulties of the *Sapap3*$^{-/-}$ mice to learn this cognitive task, was the fact that wild type C57BL/6 mice also failed, after several studies have shown the contrary (Mar et al, 2013; Dickson et al, 2014; Horner et al, 2013).

The C57BL/6 mice used in this task were not naïve but had been used before in an impulsivity task, also performed in the same touchscreen chamber. It is important to point out is that animals that had the worst performance in the impulsivity task also had the worst performance in the discrimination task. Moreover, before the impulsivity task, the same mice were used in another operant task in a nose-poke box (which they learnt normally).

At the moment, we are still trying to understand the cause of this failure and a series of questions pop out. Is the problem related with the task itself? Is the food delivery not enough or should we change the reward from sucrose to milkshake (there is the claim that milkshake is more motivating to mice than sucrose)? Should we use a more intensive training schedule (usually animals are trained for 7 days a week, while we trained them for only 5)? Should we use

another task, as digging for rewards in a maze-like box (Garner et al., 2006; Bissonnette et al., 2008), to assess the cognitive flexibility? In this task, the reward is located in the place where the animal is. Thus, the animal does not need to make the choice and move to a different location in order to get the reward, which could increase the performance. Or is the problem related with mice themselves rather than the task? Were the mice too old and should we train them before the OCD-behaviour is detectable?

In conclusion, it was not possible to draw any conclusions and work is still ongoing in that sense. We are testing younger wild-type C57BL/6 and we may try other approaches. After successfully train wild-type C57BL/6 mice we will train and test *Sapap3*$^{-/-}$ in order to assess cognitive flexibility in this mouse model of OCD.

4.2. DBS in the IC has a positive effect on compulsive behaviour

DBS has shown to be effective in the reduction of compulsive grooming. Indeed, looking at the average of the percentage of grooming, it can be observed that DBS stimulated mice had a significant decrease as compared with the Sham stimulated mice.

Compulsive behaviour has been linked with a disruption of coordinated function within the basal ganglia or between striatal and forebrain structures. Indeed, studies have suggested that the motor loop, as well as the prefrontal loop might be associated with inappropriate repetition of movement. Several hypotheses have been proposed to explain how basal ganglia circuitry may modulate repetitive behaviour but one has received special attention: authors

suggested that the compulsive behaviour is due to an impairment of the direct (striato-nigral) versus indirect (striato-pallidal) pathways (Tepper et al., 2007) (fig. 22). In general, the compulsive behaviour is thought to be caused by a suppression of the indirect pathway or activation of the direct pathway (Langen et al., 2010).

Due to the localization of the internal capsule, it is not surprising that stimulation in this area would affect repetitive movements. In fact, all fibres entering and leaving the cerebral cortex from the thalamus, brainstem, and spinal cord pass through the internal capsule. With this in mind, it seems possible that stimulation in the internal capsule is able to restore the impairment in those circuitries.

No differences between male and female mice were seen in the percentage of time grooming. This suggests that compulsive behaviour is regulated by the same mechanism in both sexes.

It is also important to draw the attention for the fact that, as presented in the introduction, there is a typical order by which DBS has an effect in the clinic, with mood and anxiety reacting first and compulsions only later. However, our results show that DBS has an immediate action in compulsive behaviour. Does this mean that *Sapap3*$^{-/-}$mice do not mimic entirely OCD conditions? Is this difference due to a distinction between humans and rodents? Indeed, this is not clear and therefore, more studies are required.

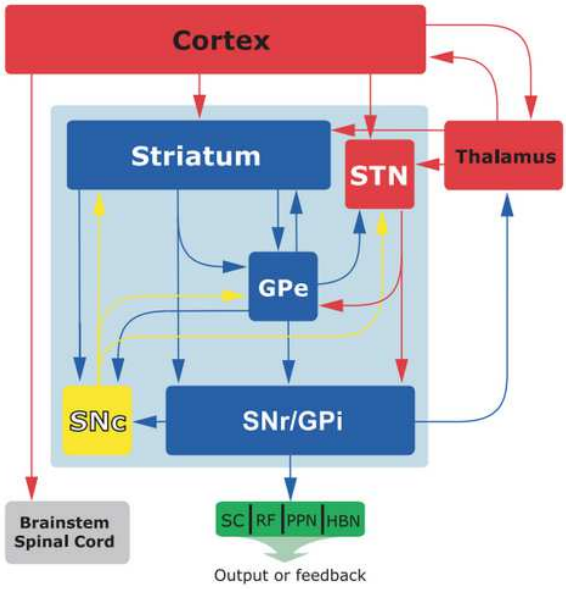

Fig. 22: Basal Ganglia Pathways involved in the control of movements. The fibres involved in the direct pathway travel from the striatum to the substantia nigra pars reticulata/ globus pallidus internal (SNpr/GPi), then to the thalamus. The ones in the indirect pathway travel from the striatum to the globus pallidus external (GPe), the subthalamic nucleus (STN) to the SNpr/PGi, and finally to the thalamus.

4.3. Is the DBS in the IC able to control unconditioned anxiety? Is this behaviour controlled by different neurobiological processes in males and females?

Results from unconditioned anxiety show that DBS had no effect in male mice. However, the results related with female mice are not that clear (so far).

In what concerns male*Sapap3*$^{-/-}$ mice, both Sham and DBS stimulated groups present an anxious pattern, spending more time in the closed arms than in the open ones. The lack of difference between the two stimulation groups suggests that DBS has no effect in unconditioned anxiety.

However, results obtained from *Sapap3*$^{-/-}$ female mice are not clear. Due to the low number of animals per group and to the high intra-group variability observed, we were not able to conclude whether DBS had an effect in unconditioned anxiety. For this reason, we will next discuss both possibilities.

Firstly, if one assumes that increasing the number of animals (to number suggested by power analysis) will reveal an anxiolytic effect of DBS, the most likely explanation is that this is due to differences in sexual hormones' levels. Thus, suggesting that DBS has an effect in sexual hormones.

As far as we know, no studies were published where the levels of sexual hormones, depending of DBS condition, were assessed. However, we had access to a recent study that shows that prolactin's levels change depending on whether DBS is on or off. Although often associated with the production of human milk, prolactin plays a wide range of other roles such as the control of maternal behaviour, energy balance and food intake, stress and trauma responses, neurogenesis, pain and anxiety. Indeed, prolactin controls these functions by regulating receptor potential thresholds, neuronal excitability and/or neurotransmission efficiency (Patil et al., 2014). Thus, our hypothesis is that DBS interferes with the levels of prolactin, which consequently interfere with levels of anxiety.

Indeed, confirming this hypothesis would be a big step, not only in understanding the way DBS acts but also because this would show us that the neurobiology of unconditioned anxiety is different in females and males. It is well accepted that sex differences influence not only the symptoms as well as the therapeutic for OCD patients. Thus, a hormonal explanation might be behind this difference.

Secondly, similar to males, females' unconditioned anxiety might not be affected through DBS in the IC (after repetition of this experiment with a bigger group). Looking at the results that we have until now, we observe that, in fact, DBS stimulated mice seem to equally explore the closed and the open arm. However, no significant difference is seen also in Sham stimulated mice. Indeed, even if they seem to spend more time/enter a higher number of times in the closed than in the open arm, this effect was not significant, as it was in the case of males. Thus, this could suggest that *Sapap3*$^{-/-}$ female mice are in general, less anxious than *Sapap3*$^{-/-}$ male mice. No evidence of this exists in literature. In fact, both cases have been shown, with some results suggesting that females are more anxious than males and other suggesting the opposite effect (Xiang et al., 2011; Belviranli et al., 2012). Whereby, no conclusion can be driven.

4.4. DBS in the IC has no effect in conditioned anxiety or in fear extinction

It is described that unconditioned and conditioned anxiety are regulated by different circuits and areas in the brain. As a matter of fact, it was suggested that the baso-lateral complex of the amygdala and the central nucleus of the amygdala are part of the neural circuitry for conditioned anxiety but not for unconditioned anxiety (Rosen, 2004).

OCD is characterized by anxiety-related behaviours; however, these symptoms are generally related to a specific situation (obsession) rather than meaningless fears.

In order to assess the effect of DBS in the conditioned anxiety we tested *Sapap3*$^{-/-}$ mice in a VCT task. Our results suggest that DBS in the IC has no effect in conditioned anxiety.

To the best of our knowledge, conditioned anxiety in a mouse model of OCD was not assessed in previous studies. Dijk et al, 2013 tested wild-type Wistar rats and reported an anxiolytic effect in conditioned anxiety when DBS was administered in the IC. However, the rodent model used by Dijk and colleagues makes comparisons to our study hard to establish.

It is important to note that our study only measured DBS' effect on *Sapap3*$^{-/-}$ male mice. Thus, it is only possible to establish a comparison between unconditioned and conditioned anxiety for *Sapap3*$^{-/-}$ males. As described above, no effect was seen in *Sapap3*$^{-/-}$ male mice in the unconditioned anxiety; however this effect is not clear for *Sapap3*$^{-/-}$ females. Thus, it would be of great interest to use *Sapap3*$^{-/-}$ female in order to see if the same pattern is kept and therefore, if both symptoms are linked.

Associated with the conditioned fear is the avoidance of certain behaviours that fail to extinguish. This characteristic suggests that OCD patients have impairment in the circuits that regulate fear extinction.

Previous studies have shown that DBS in the ventral striatum has an effect in the orbito-frontal cortex, prelimbic and infralimbic regions that modulate fear through its projections to the amygdala (McCraken et al., 2009; Sotres-Bayon et al., 2010).This raised the hypothesis that DBS might be effective in extinguish fear. Indeed, stimulation in the IC seems logical as it contains fiber bundles interconnecting cortical areas implicated in fear extinction, such as the

ventromedial prefrontal cortex, the dorsal anterior cingulate cortex and the orbito-frontal cortex, with sub-cortical areas implicated in conditioned fear, such as the amygdala (Lehman et al., 2011).

We tested this hypothesis after conditioning mice in a VCT task.

Although the results that we obtained were not conclusive, they suggest that DBS had no effect in fear extinction. Contrarily, a previous study by Rodriguez-Romaguera and colleagues found facilitation in fear extinction when DBS was applied in the dorsal part of the medial striatum, whereas DBS applied in the ventral part impaired extinction. With this in mind, the lack of an extinction process might have resulted from the fact that the electrodes were implanted in the IC, however, not always in the same region. In fact, targeting the internal capsule in mice has shown to be highly technically demanding, as the IC is a small area. In the IC, fibres connecting different areas are topologically in different positions; therefore, in order to target specific fibres, the electrodes have to be positioned in specific sectors of this fibre bundle. Thus, our results might not be conclusive due to differences in the position where the electrodes were implanted.

4.5. DBS in the IC is not able to induce chronic changes

Little is known about the underlying neural mechanism of DBS. Whether DBS acts in an acute way or is capable to induce chronic changes is not clear, however, clinical studies done not only regarding OCD but as well as in other disorders have shown that DBS-induced effects are gone as soon as

stimulation is switched off. In other words, DBS is not capable to have an effect when turned off (Denys et al., 2010; Gabriels et al., 2003).

Here we demonstrated that DBS is able to induce acute changes; as seen by the percentage of grooming assessed in the initial test. However, this effect only lasts when the stimulation is ongoing. In fact, when the same test was performed at the end, with stimulation off, we could observe that both groups behaved in a similar way. Thus, these results suggest that DBS in the IC is not able to induce chronic changes.

In order to hypothesize about the reason by which DBS acts only in an acute way and not chronic, we would need to first understand how DBS works in general. We could hypothesize that DBS would be capable to induce changes but that this effect would not be enough to induce synaptic plasticity. However, there are several indications showing that some effects may appear or develop in time. Nevertheless, effects on clinical symptoms depend on continuous stimulation, suggesting that long-term plasticity does not play a major role in the therapeutic effects.

4.6. Expression of c-Fos as a marker of neuronal activity

In order to underlie the areas that were activated through DBS we stained the brains of the *Sapap3*$^{-/-}$ mice against c-Fos, as it is a marker for neuronal activity.

Although the staining was successful and c-Fos expression could be appreciated, we were not able to always find the tract of the electrodes. Indeed, the tract of the electrodes was clearer in cresyl-violet staining than in c-Fos

staining. This is mainly due the fact that cresyl-violet binds readily to the acidic components of the neuronal cytoplasm, especially to the RNA-rich ribosomes, which are present in large numbers in neurons, besides the nuclei and nucleoli of the cells. On the other hand, in the c-Fos staining, only c-Fos was marked, thus becoming harder the visualization of different components.

As the quantification of c-Fos positive puncta of the areas surrounding the electrode was our first goal, we did not proceed with the quantification of any other areas. However, work is still ongoing and one idea to improve the visualization of the electrodes is to keep them for longer in the brain, after perfusion, in order to strengthen their tract.

- Chapter 5 -

Conclusion

The present study had two main goals: further assess the validity of *Sapap3*$^{-/-}$ mice as a multidimensional model of OCD and unveil the involved neurobiology as well as optimize treatment for OCD by testing the effects of DBS on these mice.

Sapap3$^{-/-}$mice present OCD-like behaviors such as compulsive behavior and increase levels of anxiety and we were interested if they would also show impaired cognitive flexibility. However, both C57BL/6 and *Sapap3*$^{-/-}$ mice were not able to learn the discrimination task which prevented us to draw any conclusion about cognitive flexibility in this model. Thus, this project did not allow us to find evidences that support or refute the validity of these mice as a model of cognitive defects in OCD.

Regarding the effect of DBS, our results suggest that stimulation in the IC is effective in compulsive behaviour for both male and female *Sapap3*$^{-/-}$ mice and has no effect in unconditioned anxiety, conditioned anxiety, fear extinction and memory consolidation in males. Interestingly, results concerning females are not clear whereby more work is required. Furthermore, DBS seems to act in an acute way, not being able induce chronic changes.

In fact, in clinical stimulation in the anterior IC at the border of the nucleus accumbens has shown to be effective not only in compulsion as well as in anxiety, contradicting our results. This might mean that either there is a difference between the anxiety shown by *Sapap3*$^{-/-}$mice and the clinical anxiety, or that the target cannot completely mimic the target that was successfully used in humans and therefore, not be ideal to study the neurobiology of OCD. Nevertheless, we found in all experiments a high variability in the results of

Sapap3$^{-/-}$ mice. In fact, OCD is a very heterogeneous disorder, whereby this variability could be representative of the heterogeneity seen in humans.

In conclusion, the use of mouse models to study such a complex disorder as OCD is, to say the least, challenging. Fundamental studies in OCD are scientifically demanding and extremely complex to be analysed from only one point of view. Therefore, this and more studies should be further investigated and discussed, in order to draw strong conclusions that can be taken to the clinic with the ultimate goal of helping the patients. In order to obtain that, scientists are working on finding an animal model that can be used to carefully study OCD, and then use this model to clarify the neurobiology and improve the treatment of OCD. Thus, in future work, we plan to target other relevant anatomical areas with DBS in order to determine the best brain region to be stimulated and therefore improve treatment for OCD patients.

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