

Characterization of Cortico-Striatal Myelination in the Context of Pathological Repetitive Behaviours

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ABSTRACT

Several neuropsychiatric conditions are characterized by repetitive behaviours such as simple tics or ritualized sequences of complex behaviour. Although different types of repetitive behaviours are likely emerging from different aetiologies, a wide array of both clinical and preclinical evidence suggests that these behaviours are imprinted in dysfunctions within the cortico-striatal-thalamic-cortical (CSTC) circuitry. Numerous published studies have demonstrated white matter abnormalities in various brain regions comprising mainly frontal and prefrontal cortical projections (both of which crossing the corpus callosum into striatum) in patients with psychiatric disorders with repetitive behaviours, such as Obsessive-Compulsive Disorder. The conductive properties of myelin provide a fascinating tool for neural networks to adapt to environmental and experience-derived cues, considering the fact that minor alterations in myelin thickness or axonal coverage patterns can provide significant changes in conduction speed. Thus, we believe that aberrant myelination of cortico-striatal pathways may participate to the emergence of repetitive behaviours. Conversely, we hypothesized that the promotion of myelination in these pathways could rescue abnormal behaviour.

In order to address these questions, we assessed myelin-related differences between wild-type mice and a well-characterized model for repetitive behaviours, the *Sapap3*-KO mouse. We performed immunohistochemical analysis for the quantification of oligodendroglial cells, and complemented our results with the measurement of myelin thickness and density of myelinated axons. Additionally, aiming for an understanding of the topographical organization of the different cortico-striatal circuits, and their direct implication on the diversity of existing categories of repetitive behaviours, we performed a detailed analysis on the behavioural heterogeneity displayed by the *Sapap3*-KO mouse and correlated it with two different striatal regions: dorsomedial, and ventrolateral striatum. Our working hypothesis was that compulsions are imprinted within dysfunction of associative cortical areas that project to the dorsomedial striatum, whereas the pathological signatures of tic events are anatomically related to motor cortical areas who project to the ventrolateral part of the striatum.

We observed a slight, but non-significant, tendency towards a reduced number of mature oligodendrocytes in striatal regions, and, in opposition, a noteworthy increase in myelin thickness was demonstrated in the dorsomedial striatum. Contrary to what we expected, we did not find a correlation that corroborated our hypothesis. However, a noteworthy correlation between tic-like events and the number of mature oligodendrocytes in the dorsomedial striatum was observed.

Combination of these results seem to point towards a validation of the hypothesis that myelination abnormalities can be underlying pathological repetitive behaviours, but a further analysis and characterization will be required to prove such causal correlation.

Finally, targeting a possible behavioural rescue, we administered a drug that has been shown to promote remyelination - Clemastine, and quantified behavioural changes. However, under the tested conditions, no apparent effect of this drug was noted throughout the course of the experiment.

RESUMO

Diversas patologias neuropsiquiátricas são caracterizadas por comportamentos repetitivos, que podem abranger desde simples e involuntários tiques, a sequências ritualizadas de comportamentos complexos. Apesar de se considerar que diferentes tipos de comportamentos repetitivos se encontrem associados a diferentes etiologias, um largo espectro de evidências clínicas e pré-clínicas sugerem que estes comportamentos estão correlacionados com disfunções nos circuitos cortico-estriato-tálamo-corticais. Estudos recentes têm vindo a demonstrar alterações na substância branca em diversas regiões cerebrais, com principal enfoque em projecções frontais e pré-frontais (projecções que atravessam o corpo caloso e o corpo estriado), em pacientes com perturbações psiquiátricas tais como transtorno obsessivo-compulsivo. As propriedades condutoras da mielina proporcionam uma ferramenta fascinante para as redes neuronais se modificarem perante sinalizações do microambiente e alterações que derivam de experiências adaptativas, uma vez que pequenas alterações de espessura ou dos padrões de cobertura axonal podem influenciar significativamente a velocidade de condução. Desta forma, colocamos como hipótese que uma mielinização aberrante de projecções cortico-estriatais pode participar no aparecimento e desenvolvimento de comportamentos repetitivos. Adicionalmente, propusemos testar se a promoção da mielinização destes feixes poderia atenuar as alterações comportamentais observadas.

De forma a endereçar estas questões, verificamos a existência de possíveis diferenças de mielinização entre murganhos *wild-type* e um modelo animal de comportamentos repetitivos, o murganho *Sapap3-knockout* (KO). Para tal, quantificamos células da oligodendroglia através de análise imunohistoquímica, e complementamos os nossos resultados medindo a espessura da mielina e a densidade de axónios mielinizados. Adicionalmente, com o intuito de inferir sobre a organização topográfica dos diferentes circuitos cortico-estriatais, e a sua implicação directa na diversidade de categorias comportamentais, avaliamos detalhadamente a heterogeneidade comportamental característica do modelo animal utilizado e correlacionamos com duas regiões estriatais: porções dorsomedial e ventrolateral do corpo estriado. A nossa hipótese prende-se à existência de correlação entre o surgimento de comportamentos compulsivos e disfunções entre áreas corticais associativas que projectam para a porção dorsomedial do estriado e, por outro lado, o aparecimento de tiques ter por base disfunções em áreas corticais motoras que projectam para a porção ventromedial do estriado.

Resultados preliminares indicam uma tendência, embora sem significância estatística, para um reduzido número de oligodendrócitos em regiões estriatais e, em oposição, um aumento da espessura na porção dorsomedial desta estrutura cerebral. Contrariamente ao que havíamos considerado, não foi encontrada uma correlação que corroborasse a nossa hipótese. No

entanto, uma correlação significativa foi observada entre a frequência de tiques e um reduzido número de oligodendrócitos na porção dorsomedial do corpo estriado.

A combinação dos resultados obtidos parece apontar para a validação da hipótese que alterações no processo de mielinização podem estar na base do surgimento de comportamentos repetitivos patológicos. Contudo, análise e caracterizações mais detalhadas deverão ser efectuadas para provar uma relação causal.

Por fim, com vista a uma possível atenuação de comportamento patológico, administramos um fármaco que tem vindo a ser descrito como promotor de remielinação – clemastina, e quantificamos alterações comportamentais. Contudo, nas condições testadas, nenhuma evidência do efeito deste fármaco foi denotado no decorrer desta experiência.

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“If I see anything vital around me, it is precisely that spirit of adventure, which seems indestructible and is akin to curiosity.”

Marie Curie

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ABBREVIATIONS AND SYMBOLS

\emptyset	diameter
ACC	anterior cingulate cortex
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BSA	bovine serum albumin
CC1	adenomatous polyposis coli clone CC1
CNS	central nervous system
CSTS	cortico-striatal-thalamic-cortical
DMS	dorsomedial striatum
DMSO	dimethyl sulfoxide
DTI	diffusion tensor imaging
ECM	extracellular matrix
fMRI	functional magnetic resonance imaging
GA	glutaraldehyde
GABA	gamma-aminobutyric acid
I.P.	intraperitoneally
KO	knock-out
IOFC	lateral orbitofrontal cortex
M1	motor cortical area
M2	premotor cortical area

MBP	myelin basic protein
MSN	medium-spiny neuron
NMDA	<i>N</i> -methyl-D-aspartate
OCD	Obsessive-Compulsive disorder
OFC	orbitofrontal cortex
OL	oligodendrocyte
OPC	oligodendrocyte progenitor cell
PB	phosphate buffered
PBS	phosphate buffered saline
PV	parvalbumin
ROI	region of interest
RT	room-temperature
SMA	supplementary motor area
TEM	transmission electron microscopy
VLS	ventrolateral striatum
WT	wild-type

CHAPTER 1 – INTRODUCTION

1.1 REPETITIVE BEHAVIOUR

Repetitive, ritualistic behaviour is a core feature of animal behaviour, including for humans, and can range from habitual thumb sucking to bedtime rituals as part of normal development in children. However, several neuropsychiatric disorders are characterized by a pathological expression of repetitive behaviours in the form of rumination, compulsions, tics, mannerisms, or stereotypies. Among these disorders are Autism Spectrum Disorders, Gilles de la Tourette Syndrome, and Obsessive-Compulsive Disorder (OCD) (Langen, Kas, Staal, Engeland, & Durston, 2011; Langen, Durston, Kas, Engeland, & Staal, 2011; Ridley, 1994). These symptoms can seriously impair daily functioning, with repercussions that go from impaired capacity to engage in relationships to the diminished ability to be occupationally functional, increasing stigma and diminishing the quality of life of the patients (Bokor & Anderson, 2014).

The term repetitive behaviour is broadly used, and sometimes refers to quite different classes of comportment, nevertheless linked by repetition, rigidity, and inappropriateness. Two clusters have been proposed: on the one hand, repetitive behaviours with predominantly motor features such as stereotypies or tics, and on the other hand repetitive behaviours of cognitive character such as compulsions or rituals (Turner, 1999), though it has been argued that grouping disparate behaviours into broad characteristics may oversimplify the subject. In fact, this distinction is not trivial. Different forms of repetitive behaviours are associated with different neuropsychiatric disorders and may derive from different neural mechanisms, although comorbidity exists (Worbe, 2015).

1.1.1 Cortico-striatal-thalamic-cortical pathways, the candidate circuitry of Repetitive Behaviour

The hypothesized mechanism mediating the expression of repetitive behaviour points to abnormalities in the cortico-striatal-thalamic-cortical (CSTC) pathways. Cortico-striatal loops are implied in processing limbic, cognitive and sensorimotor information, and learning, and are thought to be implicated in several neuropsychiatric conditions that exhibit movement disorders (such as repetitive behaviours). (Alexander, DeLong, & Strick, 1986)

Motor and non-motor information processing is thought to be mediated by functionally different, topographically organized CSTC circuits. The three proposed anatomo-functional subdivisions within this circuitry (sensorimotor, associative, and limbic) are therefore thought to be responsible for distinct classes of repetitive behaviour. The sensorimotor cortex projects to the dorsolateral part of the striatum (corresponding to the putamen in primates), and abnormalities in this loop might be responsible for stereotypical motor behaviour, where repetitive motor actions are continuously completed without the presence of a specific goal. (Tremblay, Worbe, Thobois, Sgambato-Faure, & Féger, 2015) Additionally, functional magnetic

resonance imaging (fMRI) studies have shown that supplementary motor area (SMA), a structure that highly projects into the ventrolateral striatum, has been described to be activated in the onset of a tic event in Tourette patients (Neuner, et al., 2014). In the light of this hypothesis, vocal and motor tics, which are a key characteristic in Gilles de la Tourette syndrome, might be due to changes in the circuits comprising both the dorso- and ventrolateral striatum (DLS and VLS) (Langen, Durston, Kas, Engeland, & Staal, 2011).

On the other hand, the associative cortex projects to the dorsomedial part of the striatum (DMS) (corresponding to part of the caudate nucleus in primates) and the limbic cortex to the more ventromedial part of the striatum (Tremblay, Worbe, Thobois, Sgambato-Faure, & Féger, 2015). These two loops are thought to be closely related to the pathological repetition of more complex sequences of actions, where the associative loop is considered to be associated with an exacerbated repetition of a goal, which can be related to its role in monitoring performance and correcting errors and may indicate a deficit in the interpretation of feedback and reward, or in the update of working memory (Groenewegen, Berendse, Cools, Voorn, & Mulder, 2009); and the limbic loop is associated with increased impulsivity and difficulty to suppress behaviour, regardless of the outcome, due to its implication in motivation as a component of behavioural control. (Langen, Durston, Kas, Engeland, & Staal, 2011)

Nonetheless, there is solid evidence that different cortico-striatal circuits are differently implied in different types of information processing, and, despite clinical and fundamental research in the field of repetitive behaviours and striatal sub-regions (Xu, Li, & Pittenger, 2016; Kalanithi, et al., 2005; Kataoka, et al., 2010), the hypothesis still remains to be causally tested whether dorsomedial striatum underlies repetitive behaviours of cognitive character and the ventro- and dorsolateral striatum repetitive behaviours of sensorimotor character.

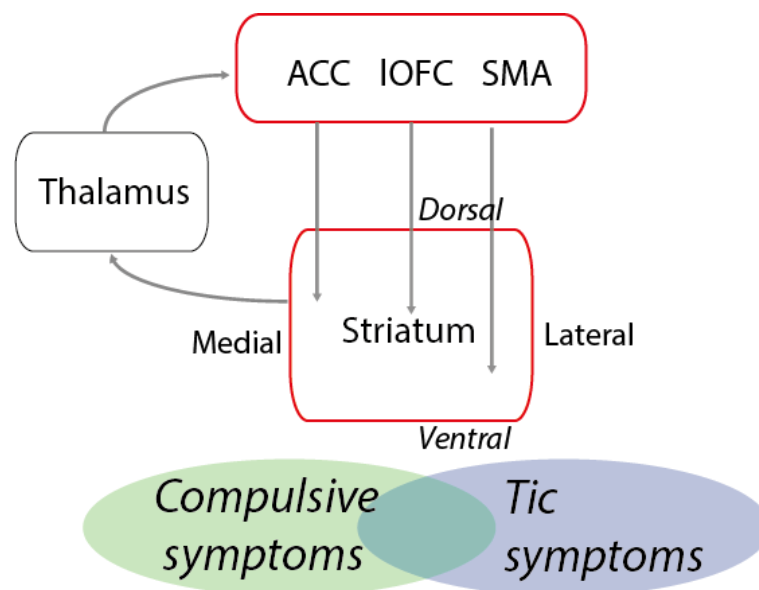


Figure 1: Hypothesized mechanisms underlying compulsive and tic symptoms.

In a simplified schematic manner, we hypothesize that compulsions are more closely related with dysfunctions within associative cortical areas, such as anterior cingulate cortex (ACC) and lateral orbitofrontal cortex (IOFC), who heavily project into the more dorsomedial part of the striatum, whereas

tics should be imprinted within more sensorimotor regions, namely supplementary motor area (SMA), that project into the lateral striatum.

1.1.2 *Sapap3*-KO mouse and the lack of inhibition

Assessing manifestations of neuropsychiatric disorders in animal models constitutes both a great challenge and a great opportunity to understand the neural mechanisms involved in the pathophysiology of these diseases. A powerful insight into repetitive behaviour can be achieved through the analysis of self-grooming in rodents (**Figure 2**), attending to the fact that it establishes a model of a complex, sequentially patterned, and repetitive behaviour. Self-grooming in rodents comprises a series of movements that form functional sequences that are crucial for hygiene maintenance, thermoregulation, and social communication, and its manifestation is known to be controlled by basal ganglia. Aberrant rodent self-grooming provides a great parallelism for symptomatic displays of several neuropsychiatric disorders (e.g. reduced grooming activity may be correlated with depression, or increased frequency and duration may be bridged with obsessive-compulsive disorder), thus numerous animal models have been proposed, which rely on distinct grooming phenotypes (Kalueff, et al., 2016).

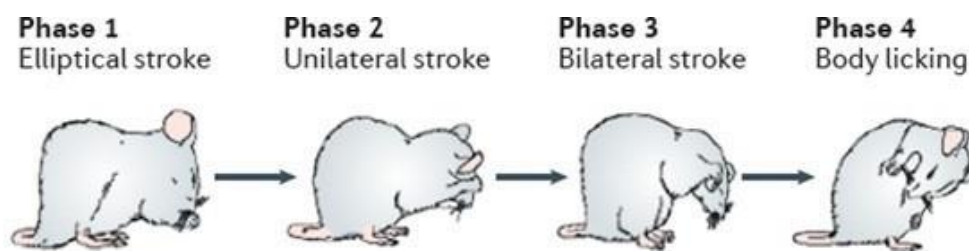


Figure 2: Rodent sequential self-grooming behaviour.

Phase 1 comprises bilateral elliptical strokes nearby the nose, corresponding to the grooming of the nose and paws. Phase 2 corresponds to face grooming and consists of alternated unilateral strokes near the eyes. Phase 3 is again characterized by bilateral strokes, but now corresponding to head grooming, and starting behind the ears. Phase 4 comprises body and tail licking. *Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Kalueff, et al., 2016), copyright (2016)*

One interesting mouse model that presents pathological repetitive behaviours, the *Sapap3*-KO mouse, has been developed and widely studied in this context, putatively presenting OCD-like behaviours (Welch, et al., 2007; Burguière, Monteiro, Feng, & Graybiel, 2013). SAPAP3 (SAP90/PDS-95-Associated Protein 3) belongs to the SAPAP family of proteins, which are postsynaptic density components of glutamatergic (excitatory) synapses, and it is the only one that is highly expressed in the striatum. Deletion of the *Sapap3* gene leads to the development of self-inflicted, bilateral, facial lesions by the age of 4-6 months (**Figure 3**). These lesions are a consequence of disproportionate and injurious levels of self-grooming throughout all day, a behaviour that has been characterized as compulsive. Additionally, *Sapap3*-KO mice present increased anxiety-like behaviour when performing open-field, dark-light emergence, and elevated zero maze tests (Welch, et al., 2007).



Figure 3: Facial lesions on *Sapap3*-KO mouse due to excessive grooming.

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Studies in this mouse model have provided powerful insights into the neurocircuitry of repetitive behaviours. Chronic electrophysiological recordings have shown a significant increase in the baseline firing rates of medium spiny neurons (MSNs) in the DMS, but not in the lateral orbitofrontal cortex (IOFC), an associative cortex that has been shown to be differentially activated in patients with OCD and that projects to the DMS (Mar, Walker, Theobald, Eagle, & Robbins, 2011; Burguière, Monteiro, Feng, & Graybiel, 2013; Burguière, Monteiro, Mallet, Feng, & Graybiel, 2015). Furthermore, no thalamostriatal synaptic alterations were observed, indicating that the impaired circuitry may be restricted to cortico-striatal pathways (Wan, et al., 2014).

Remarkably, the *Sapap3*-mutant mice revealed an abnormally low number of parvalbumin (PV)-immunostained interneurons, which correspond to the fast-spiking interneurons (FSIs) within the striatum, known for their role in feed-forward inhibition of MSNs. Moreover, optogenetic stimulation of FSIs greatly increased the inhibition of MSNs and rescued compulsive behaviour (Burguière, Monteiro, Feng, & Graybiel, 2013). Altogether, these results indicate that a deficit in the inhibitory mechanism at the cortico-striatal level may be implicated in the development of repetitive behaviours in this mouse model. These mice were capable of learning a simple behavioural task as efficiently as their non-transgenic littermates, however, they displayed the inability to adapt to conditioned paradigms, showing they are bound to maladaptive compulsive response resulting from a defective inhibition (Burguière, Monteiro, Feng, & Graybiel, 2013).

Surprisingly, on a detailed analysis and behavioural quantification in the *Sapap3*-KO mouse, our group detected, not only the well-established repeated syntactic self-grooming, but also brief and isolated myoclonic jerks (sudden and involuntary contraction of a muscle or group of muscles), which may correspond to tic-like behaviours (**Figure 4A**), indicating a more complex model than previously thought (unpublished data). Indeed, this phenotype is consistent with what can be observed in clinical practice, where patients often present comorbidity between compulsions and tic-like behaviours (Worbe, 2015). Moreover, an extremely evident behavioural aspect of this model is the increased number and duration of hind paw scratches (**Figure 4B**). In fact, this behaviour is usually the most obvious and severe phenotype of the *Sapap3*-KO, and it remains poorly described. This behaviour might probably be paralleled with the complex tics observed in some Tourette patients.

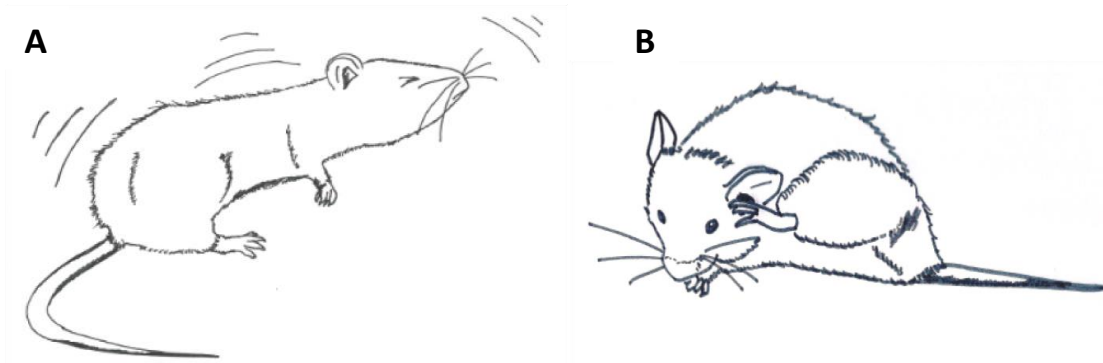


Figure 4: Additional characteristic repetitive behaviours detected in the *Sapap3-KO* mouse

Apart from the already well-described disrupted self-grooming on the *Sapap3-KO* mouse, two other characteristic behaviours have been noted: **A** – myoclonic jerk: a simple and brief tic, mostly detected in the head; **B** – hind paw scratch: a repetitive, alternated unilateral movement.

Taken together all the aforementioned behavioural characteristics, *Sapap3-KO* mouse arises as a highly complex and interesting model of repetitive behaviours. Considering the multitude and diversity of the phenotype, it leaves space to raise questions on the topographical organization of the different types of repetitive behaviours observed.

1.1.3 Potential implication of neuroplasticity

Neuroplasticity within CSTC circuitry has been highlighted as a key player in compulsive, repetitive behaviour (Koob & Moal, 2005). An interesting approach that reiterates this thought was provided by the development of an optogenetic mouse model of obsessive-compulsive disorder. This model consists in the optogenetic stimulation of the OFC-DMS pathway in awake, behaving wild-type mice. Notably, whereas acute stimulation revealed no significant alterations on mice behaviour, chronic stimulation (15 minutes a day for 5 days) led to the development of abnormal levels of self-grooming, which persisted for 2 weeks after stimulation cessation (Ahmari, et al., 2013).

This optogenetically-introduced long-term change in the associative cortico-striatal pathway opens the window for new questions, such as the molecular signatures that underlie adult plasticity and their involvement in the development of abnormal behaviour.

Until a recent paradigm shift, neuroplasticity mechanisms were ultimately attributed to neuronal changes in neuron morphology, synaptic mechanisms and neurogenesis (Kulak & Sobaniec, 2004; Fuchs & Flügge, 2014). The fundamentals that describe these mechanisms are based on Hebbian plasticity (Hebb, 1949) and were elegantly made famous by Löwel with the sentence “neurons that fire together wire together” (Löwel & Singer, 1992). This principle determines that excitatory presynaptic neurons that fire milliseconds before the postsynaptic neuron fires will strengthen the connectivity between them and cause long-term potentiation, whereas if the presynaptic firing occurs milliseconds after the postsynaptic, the connectivity is weakened and cause long-term depression (Power & Schlaggar, 2016; Cohen, Quarta, Bravi,

Granato, & Minciacchi, 2017). In a broader level, morphological changes in dendrites have been reported as a stress-induced response, with observable regression of their length in the hippocampus (Watanabe, Gould, & McEwen, 1992) and prefrontal cortex (Cook & Wellman, 2004), and enhancement in the amygdala (Vyas, Mitra, Rao, & Chattarji, 2002). The length of the dendritic branches regulates the number of axons that are able to connect and therefore the number of synapses (Fuchs & Flügge, 2014). However, in the light of recent development in brain imaging, it was possible to detect fractional anisotropy in white matter during learning, which broke ground to understand the possible role for activity-dependent myelination in both motor and cognitive modifications resulting from functional experience (Fields, 2008).

One particularly interesting study in OCD patients measured white matter abnormalities using a diffusion tensor imaging (DTI) technique. DTI is a MRI technique that provides measurement for fractional anisotropy, mean and radial diffusivity, and provides information about white matter microstructure. Disperse white matter irregularities were found in OCD patients, with specific relevance to abnormalities in the internal capsule, which were positively correlated with the percentage of errors that were committed during a cognitive task (Magioncalda, Martino, Ely, Inglese, & Stern, 2016). Internal capsule in primates does not have a specific correspondent structure in rodents, but comprises cortico-striatal projections (Steiner & Tseng, 2010), and might therefore constitute an important parallel to what has been described in the *Sapap3*-KO mouse model for obsessive-compulsive disorder, in which optogenetic stimulation of the IOFC-DMS permitted behavioural rescue (Burguière, Monteiro, Feng, & Graybiel, 2013).

Combination of the abovementioned premises led us to question a potential role for myelin-related abnormalities in the development of cortico-striatal connectivity impairment, and its causal relationship with the emergence of pathological repetitive behaviours.

1.2 MYELINATION: THE HOW'S AND WHY'S

Myelination of neurons increases electrical conduction by working as an insulator, reducing transverse capacitance and increasing transverse resistance across the axonal membrane. Furthermore, conduction velocity is also potentiated by clustering voltage-gated sodium channels in gaps (Nodes of Ranvier) between myelin sheaths, which translates in a saltatory conduction of action potentials (Nave & Werner, 2014). Bearing in mind these aforementioned properties, it is not surprising that much attention has been attributed to myelination.

1.2.1 Oligodendroglial cell lineage

In the CNS, myelin sheaths are formed by one type of multipolar glial cells, the oligodendrocytes, which wrap around the axons by spreading their membrane and growing both radially and longitudinally. Oligodendrocytes are part of the oligodendroglial lineage, and derive from the differentiation of oligodendrocyte progenitor cells (OPCs). During embryonic development, undifferentiated progenitor cells migrate from ventral and dorsal domains. After birth, these cells start their differentiating process and go through different stages until the final differentiation into mature, myelinating oligodendrocytes (**Figure 5**) (Nishiyama, Komitova, Suzuki, & Zhu, 2009; Bradl & Lassmann, 2010). OPCs extend and multiply their ramified membrane until they reach their mature state. These protrusions are crucial for cell-cell communication with the axonal portion of the surrounding neurons, and will be determinant for their subsequent ensheathment.

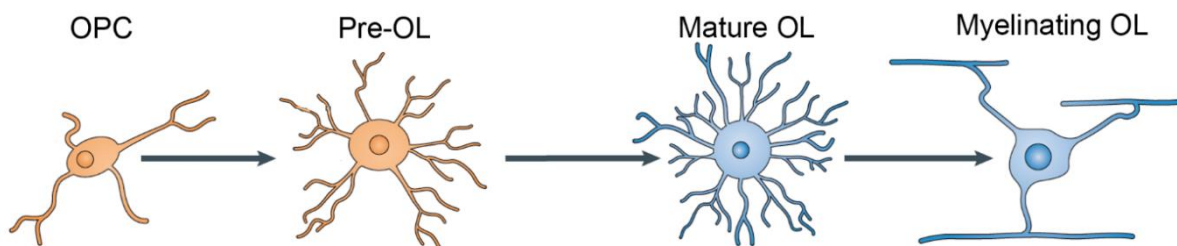


Figure 5: Representation of the oligodendroglial lineage progression during differentiation

Oligodendrocyte progenitor cells (OPCs) expand and multiply their protrusions until reaching a star-shaped immature oligodendrocyte (Pre-OL). Further expansion and arborization of this protrusions leads to the full maturation of these cells (Mature OL), allowing the contact with the surrounding axons until reaching their myelinating state (Myelinating OL).

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The biological cues that drive the OPCs into a differentiation process remains not fully understood. Several mechanical, electrical, and biochemical factors are believed to work together in providing the appropriate environment for these precursor cells to enter into a maturing state. OPCs are known to be mechanosensitive cells, who respond to variances in

the rigidity of the ECM. Their interaction with proteins of the cytoskeleton are thought to be essential for the extension and ramification of the protrusions (Lourenço, et al., 2016; Jagielska, Norman, Whyte, & Vliet, 2012; Domingues, Cruz, Chan, & Relvas, 2017). Other environmental cues from the extracellular milieu are held responsible for this process, as such the Notch signalling pathway, which is thought to be determinant in the timing of the differentiation (Shimizu, Osanai, & Ikenaka, 2017).

1.2.2 Dynamics of axon-OPC communication

Interestingly, from a biochemical point of view, OPCs have been shown to express receptors for glutamatergic (AMPA and NMDA receptors), GABAergic, and acetylcholinergic neurotransmitters (Barres, Koroshetz, Swartz, Chun, & Corey, 1990; Wyllie, Mathie, Symonds, & Cull-Candy, 1991; Bergles & Richardson, 2015). Complementary to this observation, axon-OPC synapses have been described both in grey and white matter, in all studied CNS regions (Zonouzi, et al., 2015; Bergles & Richardson, 2015; Chittajallu, Aguirre, & Gallo, 2004; Jabs, et al., 2005). These findings intuitively lead to the idea of an activity-dependent activation of a differentiation process in the OPCs, however, these synaptic inputs remain poorly understood. One curious aspect of the aforesaid findings is the fact that the activation of either glutamatergic or GABAergic synapses induces depolarization on the OPCs (Bergles, Roberts, Somogyi, & Jahr, 2000; Tanaka, et al., 2009), which presents as counter-intuitive, as one would expect a GABAergic synapse to induce hyperpolarization. This fact is related to the high intracellular concentration of Cl⁻ in the OPCs, and the GABAergic promotion of Cl⁻ efflux.

Glutamatergic signaling through AMPA receptors enhances oligodendrocyte survival, but the impact of this cell-cell communication has yet to be better elucidated and proven significant for the myelination process (Kougioumtzidou, et al., 2017). Nevertheless, recent studies have been gathering evidence that point to the idea that GABAergic axon-OPC communication may indeed have some impact on maintaining activity-dependent plasticity (Tanaka Y. , et al., 2009), and that the blockade of this synaptic transmission increases the proliferation of OPCs, but decreases the number of mature oligodendrocytes (Zonouzi, et al., 2015; Hamilton, et al., 2017).

The relevance of the concept of activity-dependent OPC differentiation is highly related to the idea of myelination as a form of adult plasticity. If proven correct, neuronal activity may be essential in potentiating and modulating *de novo* myelination and/or myelin replacement.

1.2.3 Axonal ensheathment

The most recently proposed model of myelin formation indicates that oligodendrocytes spread their membrane around the axon, wrapping it, and expanding their projections both in a radial and longitudinal manner. This growth takes place concomitantly and results in a greater number of wraps at the site of attachment, decreasing gradually towards the nodes (**Figure 6**) (Chang,

Redmond, & Chan, 2016). Afterwards, these lipid layers compact, extruding most of the cytoplasm and extracellular space, achieving, in this way, the insulating properties. Oligodendrocytes are capable of myelinating several axons simultaneously in response to certain attractive or inhibitory environmental cues (Chang, Redmond, & Chan, 2016; Fields, 2015). This characteristic particularity of oligodendrocytes might additionally contribute to their role in neuroplasticity.

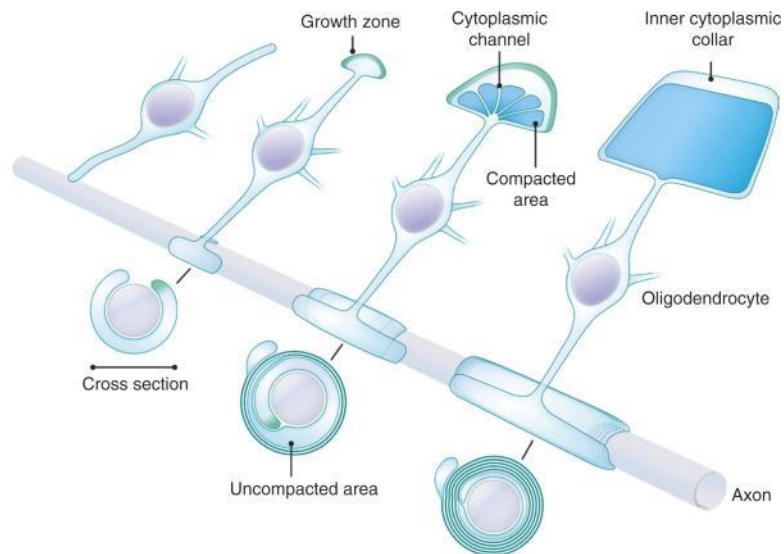


Figure 6: Current model of myelination in the CNS.

On the left, a newly differentiated oligodendrocyte contacts the unmyelinated axon by extending one of its protrusions. Afterwards, it begins to wrap its membrane around the axon by spreading this lipidic bilayer in a radial and longitudinal manner. Finally, after approximately three layers of myelin membrane, intracellular compaction starts from the outermost layer towards the inner layers. *Adapted by permission from Macmillan Publishers Ltd: Nature Neuroscience (Chang, Redmond, & Chan, 2016), copyright (2016)*

In the light of the hypothesis of an activity-dependent myelination as a form of neuroplasticity, it is interesting to understand how adult axonal remodelling may be determinant in fine-tuning conductive properties. Several factors such as sheaths' thickness, length and axonal coverage patterns may constitute the adaptive changes that emerge as a response to learning and environmental fluctuations (**Figure 7**) (Bujalka & Emery, 2016; Tomassy, et al., 2014).

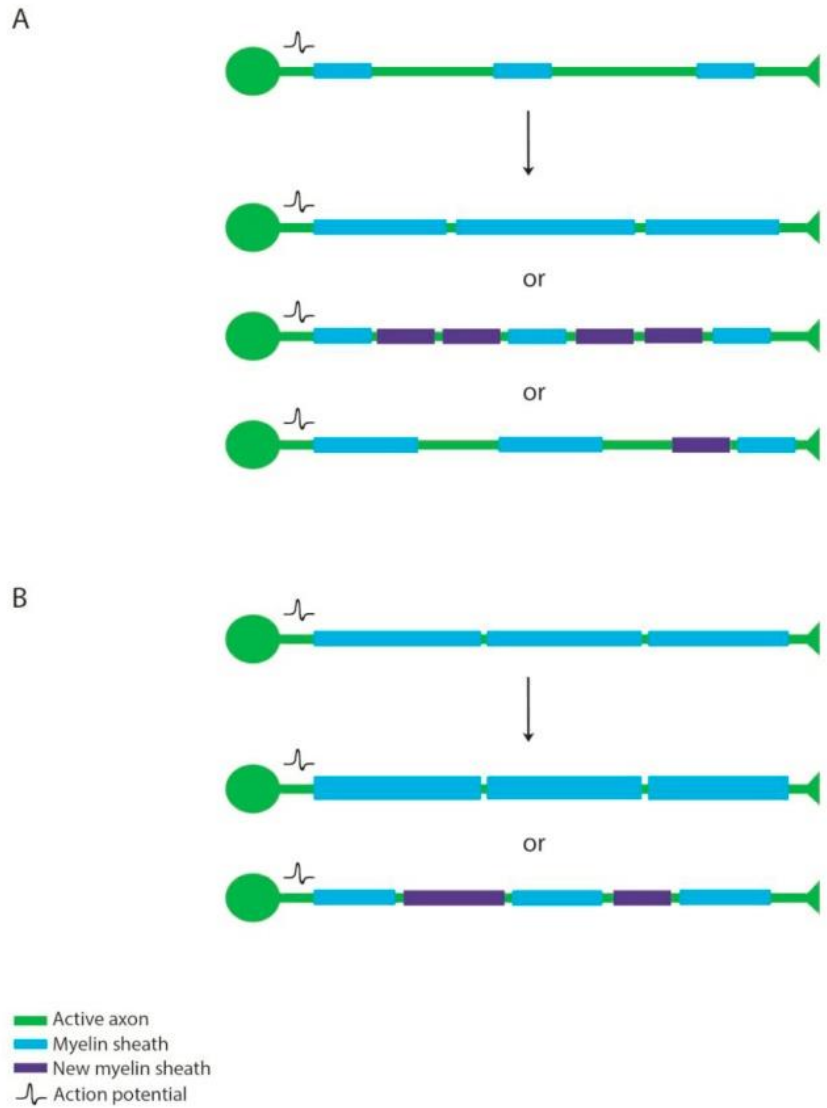


Figure 5: Models of adaptive myelination in partially and fully myelinated axons.

In partially myelinated axons (A), myelin remodeling may occur by the longitudinal extension of the already existing internodes or by the addition of *de novo* myelin sheaths with different coverage patterns. In already fully myelinated axons (B), internodes may increase their thickness or demyelination may occur, with subsequent, differentially patterned, remyelination.

(Baraban, et al., 2016) *This is an open access article under the terms of the Creative Commons Attribution License, copyright (2016)*

1.3 PATHOLOGICAL EXPRESSIONS OF WHITE MATTER ABNORMALITIES

Parallel to findings that adult myelination in humans can be triggered during learning, impaired oligodendroglial and myelin integrity have been detected in patients with psychiatric disorders, including depression or schizophrenia by *in vivo* neuroimaging as well as in *postmortem* studies (Roussos & Haroutunian, 2014; Edgar & Sibille, 2012). This dual approach leaves the thought that “myelin is not only a prerequisite, but also a consequence of normal activity” (Nave & Werner, 2014).

Several animal studies have been demonstrating an important role of myelination in different types of learning and other behaviours, thus reinforcing the idea of its involvement in neuroplasticity mechanisms.

1.3.1 Myelination in motor skill learning

Particular attention has been attributed to study the role of myelination in motor skill learning. In the first study of this kind, adult rats were assigned to three different experimental conditions, namely skilled reach for food pellets (SR), unskilled reach for food pellets (UR), and caged controls (CC). In all three groups, white matter differences were assessed by diffusion MRI fractional anisotropy, and significant difference in these values was observed between several white matter areas contralateral to the trained paw between the SR group and both control groups. Additionally, quantitative immunohistochemistry of myelin basic protein (MBP), a myelin marker, demonstrated a substantial increase in white matter subjacent to motor cortical area (M1) contralateral to the trained paw only in the SR group (Sampaio-Baptista, et al., 2013). Another interesting and detailed approach came from *Gibson et al., 2014*, where mice that were optogenetically stimulated in the premotor cortex (M2) have shown proliferative response of OPCs in ipsilateral premotor area and subcortical white matter projections into the corpus callosum. OPC differentiation was assessed by immunohistochemistry, showing increased expression of CC1 (marker for mature oligodendrocytes), and histone modifications that translate the epigenetic changes required for progression into differentiated states. Transmission electron microscopy (TEM) analysis demonstrated increased myelin thickness in layer VI of M2 axons and subcortical fibers entering corpus callosum. Complementing this finding, MBP expression levels also increased in these optogenetically stimulated mice in comparison to their wild-type littermates. Remarkably, chronically stimulated mice have shown enhanced speed in limb swing only in the stimulated limb when performing a CatWalk task, correlating better motor performance with increased myelin thickness (Gibson, et al., 2014). On the other hand, blocking differentiation of novel oligodendrocytes in adult mice prevented the animal from learning new motor tasks such as mastering a complex wheel (McKenzie, et al., 2014). Altogether, these results show that active myelination is required for adult motor skill learning.

1.3.2 Social isolation

Two different studies addressed the question on whether social isolation would impact myelination, one in juvenile and one in adult mice. The first study determined a critical period for oligodendrocyte maturation (P21 to P35), during which socially isolated mice developed impairment in neuregulin 1-ErbB3 signaling pathways in the oligodendrocytes of the medial prefrontal cortex. This results in morphological differences in these oligodendroglial cells (simpler, shorter processes, less branching and fewer internodes), whereas their density remains unaltered. This, in turn, translates into reduced MBP expression and myelin thickness, which culminated in compromised social interactions and working memory that were not reversible by the reintroduction into a social environment (Makinodan, Rosen, Ito, & Corfas, 2012). Conversely, eight weeks of social isolation in adult mice led to changes in chromatin organization and in ultrastructural changes in oligodendrocyte, resulting in myelin thinning. When put back into a social environment, these manifestations were rescued, along with the social behaviour of the mice with their littermates (Liu, et al., 2012).

1.4 A PHARMACOLOGICAL APPROACH

As myelination has been resurfaced as a potential neuroplasticity mechanism involved in learning and behaviour, several studies aimed for a therapeutic approach by the enhancement of adult *de novo* myelination. A pharmacological therapy that has been in the limelight is the treatment of pathological behaviours with clemastine (Li, He, Fan, & Sun, 2015; Liu, et al., 2016).

Clemastine is a Food and Drug Administration-approved drug that has been shown to promote remyelination. This small anti-muscarinic compound can easily pass the blood-brain barrier and promote OPC differentiation in the CNS (Li, He, Fan, & Sun, 2015) (Liu, et al., 2016). Mice that were submitted to cuprizone administration (cuprizone mouse model of demyelination) for six weeks developed schizophrenia-like behaviours. When treated with clemastine for three weeks after cuprizone withdrawal, they showed recovery of MBP intensity and an increase in mature oligodendrocytes, which promoted behavioural rescue and improvement of spatial working memory (Li, He, Fan, & Sun, 2015). Another approach that pronounces the pharmaceutical potential of clemastine was recently provided by Liu et al. These authors orally administrated this compound to two to eight week old, socially isolated mice, and demonstrated its ability to rescue social behaviour by increasing the levels of repressive histone methylation in oligodendroglial cells, a crucial step for OPC differentiation into oligodendrocytes (Liu, et al., 2016).

CHAPTER 2 – AIM OF THE STUDY

Considering recent findings on the role of myelination in learning, behaviour, and adult plasticity, we hypothesized that a deficit in myelination of cortico-striatal circuits may be underlying the emergence of repetitive behaviours in the *Sapap3*-KO mouse, as well as a topographical organization of these circuits being the substrate for the heterogeneity of these behaviours. Thus, we defined as aims of this work:

- Assess myelin-related differences between *Sapap3*-KO and *Sapap3*-WT mice in three regions of interest: lateral orbitofrontal cortex, dorsomedial and ventrolateral striatum;
- Test causal correlation between compulsive-like behaviours and myelin deficits in dorsomedial striatum, and tic-like behaviours with deficits in ventrolateral striatum;
- Behavioural rescue by a pharmacological promotion of remyelination.

CHAPTER 3 – METHODOLOGY

3.1 ANIMALS

Animals used for immunohistochemistry and electron microscopy analysis were six- to eleven-month old male *Sapap3*-KO and non-transgenic littermates (*Sapap3*-WT), C57BL/6J background (Welch, et al., 2007). Animals were kept at the animal facilities of the “Institut du Cerveau et de la Moelle Épineuse”, housed in Techniplast ventilated polycarbonate cages under positive pressure with hard-wood bedding and provided with food and fresh water, *ad libidum*. All animals were housed in environmentally controlled cages with 40 air changes per hour. The temperature was maintained at 21–23 °C and the relative humidity at 55 ± 10% with a 12-h light/dark cycle. Each study animal was assigned a unique number and identified by tattoos in the paws. All experiments were approved by the French ministry of research under the agreement number (APAFIS) #1418-2015120217347265.

3.2 BEHAVIOURAL ANALYSIS

All animals were filmed for 24 hours in the automated behavioural boxes developed by the team, with food and water *ad libidum*, without being submitted to the performance of any behavioural task.

Videos were analyzed with Kinovea (version 0.8.15), an open-source software adapted to the study of movement and motor performance. 15 minute videos were selected for each animal between 8 and 9 a.m. when the mouse was awake, after an overnight period of adaptation to the new cage.

During the selected period, three types of behaviour were considered for quantification: myoclonic jerks, grooming events (with discrimination of the four different phases), and hind paw scratches. Frequency and duration of these behaviours were the parameters used for analysis.

3.3 IMMUNOHISTOCHEMISTRY

Animals were divided in two different groups (*Sapap3*-KO and *Sapap3*-WT, N=7 per group). Animals from both groups were deeply anesthetized with Euthasol®, and transcardial perfused with 2% PFA (Sigma-Aldrich). The brain of each animal was collected and post-fixed overnight in 2% PFA. Samples were then rinsed in 1X PBS (Euromedex).

With the purpose of cryo-protecting the tissue for the upcoming freezing procedure, samples were immersed in a 15% sucrose solution for 24 hours, and subsequently immersed in 30% sucrose for 48 hours, until the brains sink in the solution as sign it is completely impregnated.

Samples were embedded in Tissue-Tek O.C.T. compound (Thermo Scientific) for 30 minutes, in order to fully impregnate the tissue and provide a stabilizing matrix, and frozen in dry ice. Brains were subsequently cut in a Microm HM 560 cryostat, in 12µm coronal slices, on a glass slide, and stored in -80°C.

Slices were defrost at RT and rehydrated in 1X PBS.

With the purpose of increasing the intensity of the immunostaining, the slides were immersed in 10mM sodium citrate buffer ($C_6H_5Na_3O_7 \cdot 2H_2O$) and put in water-bath at 85°C for 30 minutes. The acidic environment provided by the citrate buffer disrupts the protein cross-linking caused by the PFA and enables a more efficient penetration of the antibodies. When taken off the water-bath, the samples were cooled-down to RT and rinsed in 1X PBS.

In order to decrease the non-specific binding of the antibodies, thus staining with less background noise, samples were saturated in 4% BSA/0.1% Triton X-100 for 1 hour. Triton X-100 (VWR Chemicals) is a detergent that lyses the lipids in the membranes, and therefore increases membrane permeability for the antibodies.

The antibodies anti-Olig2 rabbit (Millipore, #AB9610) and anti-CC1 mouse IgG2b (Calbiochem, #OP80) were diluted in 4% BSA/0.1% Triton X-100 with a concentration of 1/1000 and 1/100 respectively, and incubated overnight at 4°C.

Samples were rinsed with 1X PBS and incubated with the secondary antibodies anti-Rabbit 488 Alexa and anti-Mouse IgG 568 Alexa (Invitrogen, #A21206 and #A21144 respectively) in a 1/2000 dilution, for 1 hour at RT, in dark. The slides were rinsed in dark, under agitation.

The slides were mounted with Mowiol® as a mounting medium, and left overnight to dry.

Samples were imaged in a Zeiss Axio Scan.Z1, in fluorescence, objective Plan-Apochromat x20, Hamamatsu camera.

3.4 ELECTRON MICROSCOPY

Animals (N=3 per group) were deeply anesthetized with Euthasol® and transcardial perfused with 5% glutaraldehyde (GA) (Electron Microscopy Sciences)/5mM $CaCl_2$. The brain of each animal was collected and post-fixed overnight in 5% GA. Samples were then rinsed in 0.12M phosphate buffered solution (PB) (pH=7.4).

Brains were cut in 200µm slices in the vibratome Leica VT1200 S, and the slices that comprised the target areas were selected. In order to be specific about our regions of interest, a 1mm Ø biopsy punch was used to extract them. The following protocol was performed only in the collected punches.

Attending to the fact that myelin is a lipidic structure, another post-fixation step with 4% osmium tetroxide (Electron Microscopy Sciences) was added. After 1hour of incubation, samples were carefully rinsed.

As a first contrasting step, samples were immersed in 5% uranyl acetate (Electron Microscopy Sciences), a heavy metal who interacts with proteins and lipids. After 1hour of incubation, samples were carefully rinsed.

Afterwards, samples were dehydrated in increasing concentrations of ethanol and finally with 100% acetone. To prepare the samples for the embedding with epoxy resin, they were first let overnight, at 4°C, immersed in a solution of 50:50 acetone and epoxy resin.

Samples were transferred into embedding molds and immersed in resin (Embed 812 Kit, Electron Microscopy Sciences). After 2hours the epoxy resin was replaced and left to polymerize at 60°C for 48hours. Samples were then cut in an ultramicrotome Leica EM UC7 into 70nm slices and places in a TEM grid.

Finally, as a complementary contrasting agent, lead citrate (ChromaLys) was used under CO₂ free conditions.

Samples were imaged in the Transmission Electron Microscope HITACHI 120kV HT7700, camera AMT XR41-B. Magnification used for quantification of g-ratios was x26.0k, and x11.0k for the quantification of the density of myelinated axons.

3.5 IMAGING ANALYSIS

All images were analyzed with Fiji from ImageJ2 (Schindelin, et al., 2012; Schindelin, Rueden, Hiner, & Eliceiri, 2015).

3.5.1 Immunohistochemistry – Quantification of mature oligodendrocytes

For the quantification of the density of oligodendrocytes, we first chose the slices that matched the Bregma levels of our regions of interest: lateral orbitofrontal cortex – Bregma +2.46; dorsomedial striatum – Bregma +0.50; ventrolateral striatum – Bregma +0.14. For each animal, four slices per area were chosen, only on the left hemisphere. Lateralization effects have been previously assessed, and no different values were found between the two hemispheres (data not shown).

We drew a square on the specific area of interest in each sample by using the ROI tool on Fiji, and counted all cells that were positively stained for Olig2 and CC1 (Olig2+/CC1+ cells). Olig2 is a nuclear marker for all cells of the oligodendroglial lineage, and CC1 is a cytosolic marker specific for mature oligodendrocytes. Combination of these two markers allowed us to be confident on the quantification of mature oligodendrocytes. To calculate the density, we divided the total number of cells by the area of the drawn ROI.

3.5.2 Electron Microscopy – G-ratio measurement

To assess myelin thickness, we analyzed the TEM images with x26.0k magnification that were previously collected from two regions of interest: dorsomedial and ventrolateral striatum.

In order to be specific on the quantification of local striatal cells, myelinated axons within the fibers that cross the striatum (corresponding to internal capsule) were not considered. To obtain a satisfactory level of confidence in our results, we quantified ~150 myelinated axons per region, per animal.

The tool “Polygon selections” in Fiji was used to draw both the inner axonal area and the outer (myelinated) area of each axon. To calculate the g-ratio, we divided the inner area by the outer.

3.5.3 Electron Microscopy – Quantification of myelinated axons

For the quantification of the number of myelinated axons, we analyzed the TEM images with x11.0k magnification that were previously collected from two regions of interest: dorsomedial and ventrolateral striatum.

In order to be specific on the quantification of local striatal cells, myelinated axons within the fibers that cross the striatum (corresponding to internal capsule) were not considered.

For each animal, we analyzed ~20 images per region and counted the number of myelinated axons in each image.

3.6 PHARMACOLOGICAL TREATMENT

3.6.1 Administration protocol

The animals used were seven- to fourteen-month old female *Sapap3*, C57BL/6J background, housed in the same conditions as previously described.

Sapap3-KO females were divided in two groups (N=6 per group): clemastine-treated (CT) and the control, vehicle-treated (VT) groups.

Clemastine (Sigma-Aldrich) was dissolved in NaCl 0.3% DMSO (Sigma-Aldrich) and administrated daily to the CT group via I.P. injection at the concentration of 10mg/kg body weight (Apolloni, Fabbrizio, Parisi, Amadio, & Volonté, 2014) for 12 days. VT group was treated with equivalent volumes of NaCl 0.3% DMSO. Animals were weighted and health monitored daily.

3.6.2 Behavioural analysis

All animals were filmed for 24 hours with food and water *ad libitum* at different time points: day 0, day 6, day 12, and day 7 after treatment cessation.

Videos were analyzed with Kinovea (version 0.8.15). 15 minute videos were selected for each animal between 1 and 2 a.m. when the mouse was awake, after a six hour period of adaptation to the new cage. Analyzed parameters were the same as previously described.

3.7 STATISTICAL ANALYSIS

All data was visually inspected prior to statistical analysis, which was then performed in the programming environment R (a language and environment for statistic computing, R Development Core Team - R Foundation for Statistical Computing, Vienna, Austria, 2008). Due to small sample size, behavioural parameters (number of hind paw scratches, number of myoclonic jerks, and number of grooming events), as well as the number of myelinated axons in the two genotypes were assessed using the function “wilcox test” to perform non-parametric Mann Whitney U tests. Severity of individual behavioural readouts and the number of Olig2+/CC1+ cells in each of the three brain regions were correlated using the function “cor test”, the non-parametric Spearman correlation. Even though a small sample was used to measure the g-ratios, considering the apparent normal distribution, a Student’s T test was performed.

All p-values > 0.05 were considered non-significant.

CHAPTER 4 – RESULTS

4.1 BEHAVIOURAL ANALYSIS

Aiming for a descriptive analysis on the behaviour of the *Sapap3*-KO mouse, we analyzed the categories that provide the most obvious phenotype. The first notable difference is on the average weight of the *Sapap3*-KO (p-value=0.00053) (**Figure 8A**), who are visibly smaller and lighter than their non-transgenic littermates (*Sapap3*-WT).

The subsequent analysis, targeting the validation of what has been described on the model, was to count the number of compulsive-like events (grooming events) on both groups. Results indicate a significant increase in the average frequency of grooming behaviour in the *Sapap3*-KO mice (p-value=0.0333) (**Figure 8B**), which goes in line with what has previously been stated (Welch, et al., 2007). Remarkably, the most significant displays of abnormal behaviour come from the phenotypes that have not yet been described in the literature. Both the average frequency of myoclonic jerks (tic-like events), and the hind paw scratches present a notorious increase in the *Sapap3*-KO mice (p-value=0.00695; p-value=0.00183, respectively) (**Figure 8C-D**). Altogether, these results seem to reinforce our working hypothesis of a complex model, who presents repetitive behaviours of mutually motor and cognitive character.

4.2 DENSITY OF MATURE OLIGODENDROCYTES

The first approach on assessing possible myelin-related deficits in the *Sapap3*-KO mouse relies on the evaluation of the cells that are actively myelinating the axons. Different values regarding the density of mature oligodendrocytes in our regions of interest (lateral orbitofrontal cortex, dorsomedial striatum, and ventrolateral striatum) (**Figure 9A-C**) may constitute the initial evidence of this matter. For that purpose, we double-immunostained our samples (N=7 per group) for Olig2: nuclear marker of all the cells that are part of oligodendroglial lineage, and CC1: specific cytosolic marker for mature oligodendrocytes, and counted all Olig2+/CC1+ cells (**Figure 9D-I**).

Our results indicate a slight, non-significant increase in the number of oligodendrocytes per μm^2 in the IOFC (p-value=0.2285) of the *Sapap3*-KO mouse (**Figure 9J**). However, this tendency seems to be inverted both in the DMS (p-value = 0.3024) and VLS (p-value = 0.1332) (**Figure 9K-L**) where the number of oligodendrocytes counted by μm^2 was reduced in the *Sapap3*-KO, in comparison with their non-transgenic littermates.

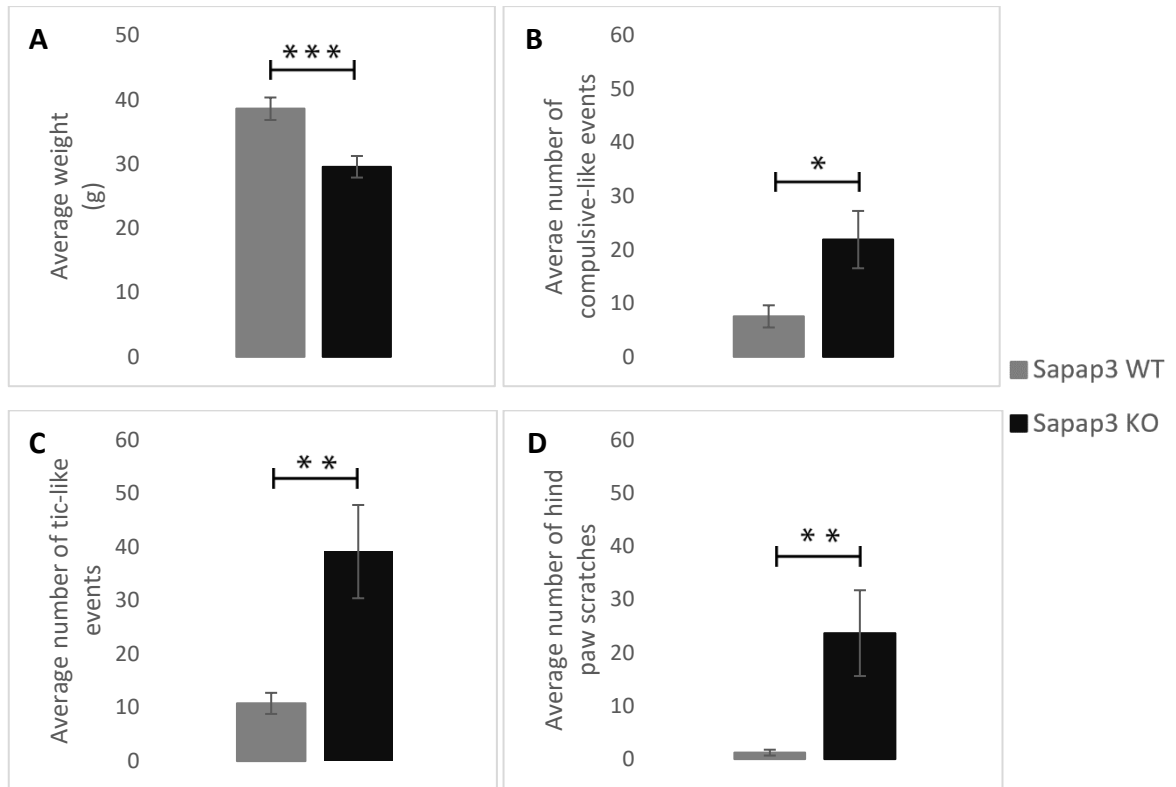


Figure 6: Quantitative behavioural comparison between the Sapap3-KO and the Sapap3-WT mice

A – Comparison between the average weights of the two groups of animals. *Sapap3*-KO animals are notoriously smaller and lighter than the *Sapap3*-WT group; B – Average number of compulsive-like events in the two groups validating the characteristic excessive grooming seen in the mutant mice; C – Average number of tic-like events (myoclonic jerks) and D – Average number of hind paw scratches, demonstrating the significant increase on the frequency of these pathological repetitive behaviour on the *Sapap3*-KO mouse. Bars represent average value and error bars show standard error. Mean and standard error were calculated from video analysis of 18 animals (N=9 per group). Represents * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

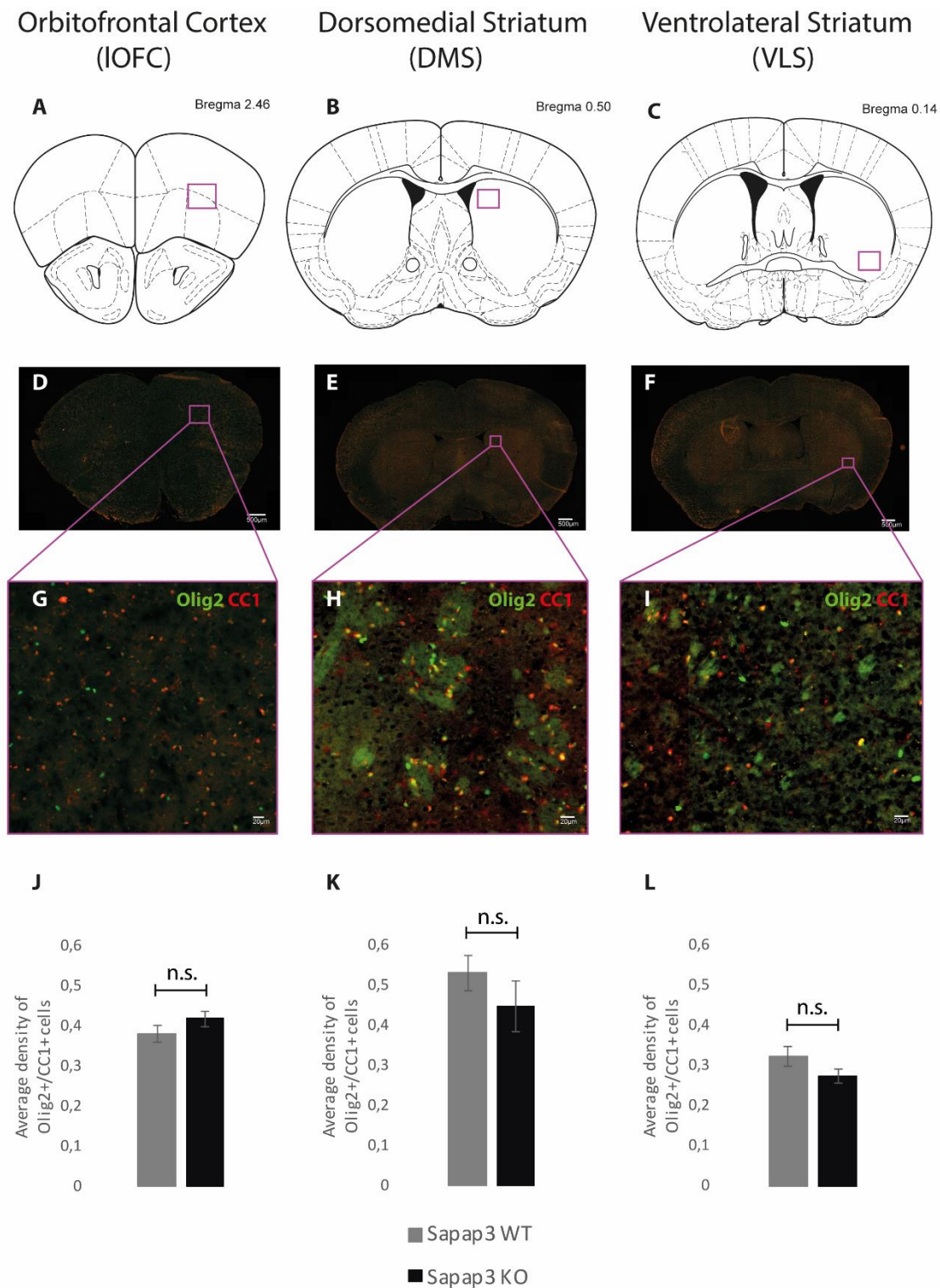


Figure 7: Quantification of the density of mature oligodendrocytes

Figures A, B, and C represent a diagram of the Bregma levels we aimed in order to incorporate the defined ROIs (IOFC, DMS, and VLS, respectively). Figures D, E, and F correspond to the fluorescence imaging of the entire brain slice that match the Bregma levels we targeted, and a representation of where we outlined the abovementioned ROIs. Figures G, H, and I correspond to a zoom-in of the ROIs. Olig2+ cells are stained in green and CC1+ cells are stained in red. Figures J, K, L represent the quantification of the number of Olig2+/CC1+ positive cells per μm^2 in the regions abovementioned. A slight decrease in the average

density of Olig2+/CC1+ cells in DMS (K) and VLS (L) was noticed in the *Sapap3*-KO group. In OFC (J), this tendency was inverted, with a slight increase in the average density of mature oligodendrocytes in the *Sapap3*-KO group. Bars represent average value and error bars show standard error. Mean and standard error were calculated from image analysis of 14 animals (N=7 per group), 4 images per animal. Represents n.s. = non-significant.

4.3 FUNCTIONAL ANALYSIS

In order to verify our hypothesis that compulsive-like behaviours are correlated with dysfunctions within associative circuits and their projections in the DMS, and tic-like behaviours within SMA-VLS circuitry, we tested a possible correlation between the behavioural quantifications and the density of Olig2+/CC1+ cells in these areas.

Contrary to what we expected, our results indicate that both the correlation between the compulsive-like events with the density of oligodendrocytes in the DMS, and the tic-like events with the density of oligodendrocytes in the VLS are not significant (p-value=0.3811, p-value=0.8986, respectively) (**Figure 10A-B**).

However, a surprising and noteworthy negative correlation was found between the frequency of myoclonic jerks and the density of Olig2+/CC1+ cells in the DMS (p-value=0.0231) (**Figure 10C**). This result demonstrates a clear indication that an increased number of tic-like events relates to a decreased number of mature oligodendrocytes in this brain region. Additionally, a tendency towards an also negative correlation between the number of hind paw scratches and the density of oligodendrocytes was also denoted in the DMS (p-value=0.0585) (**Figure 10D**).

All other tested correlations were non-significant (data not shown), indicating that there seems to be no apparent implication of myelin-related deficits in VLS nor IOFC. Additionally, myelination deficits that could be underlying compulsive-like behaviours do not appear to correlate with neither of the hypothesized areas.

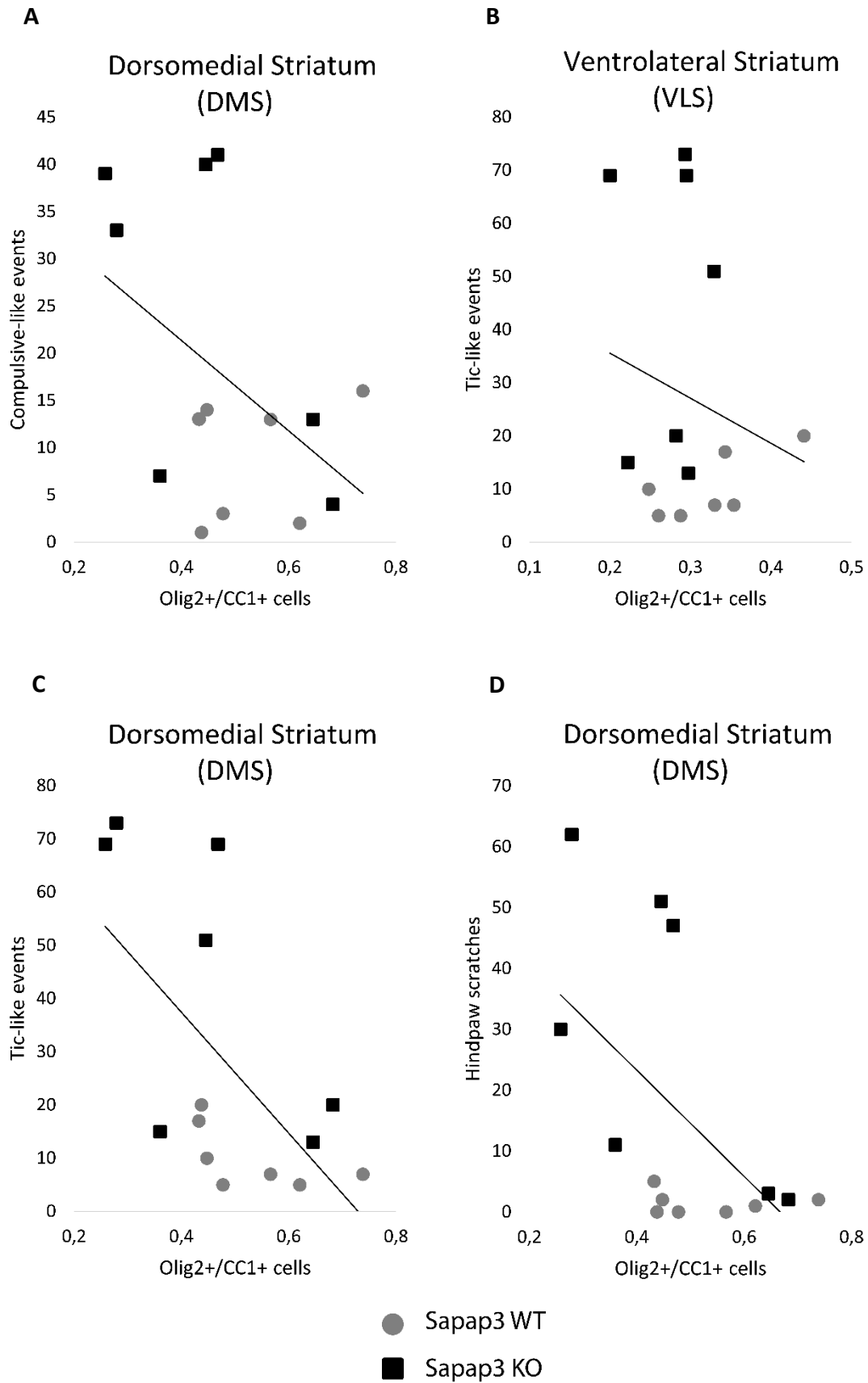


Figure 8: Correlation between behavioural quantifications and the number of oligodendrocytes in DMS and VLS
We looked for a correlation between the frequency of compulsive-like events (grooming events) with a decreased number of oligodendrocytes in the DMS (A) and a correlation between the frequency of tic-like

behaviours (myoclonic jerks) with a decreased number of oligodendrocytes in the VLS (B). However, contrary to our hypothesis, these correlations were not verified. On the other hand, we detected a negative correlation between the number of tic-like events with the average density of mature oligodendrocytes (p -value=0.0231) (C), and a tendency towards an also negative correlation between the frequency of hind paw scratches and the average density of oligodendrocytes (p -value=0.0585) (D), both in DMS.

4.4 MYELIN THICKNESS

Aiming for a more detailed characterization of possible myelin-related deficits in the *Sapap3*-KO mouse, we quantified myelin thickness by performing g-ratios (ratio between the diameter of the axon *per se* and the outer diameter of the myelinated axon) on electron microscopy images (N=3 per group) on our designated ROIs: DSM and VLS (**Figure 11A-D**).

Bearing in mind the first indication of a reduced number of oligodendrocytes in striatal regions, we would expect its translation into a reduction of myelin thickness. However, a decrease in the g-ratio (increased myelin thickness) was observed in DMS of the *Sapap3*-KO group (p -value=0.053) (**Figure 11E**). Considering the diversity of cells that can be found in striatal regions, we looked for a possible correlation between the g-ratio and the respective axonal diameter. This test would enable us to detect if there was a tendency that could be attributed to one specific type of striatal cells. However, no correlation was seen (data not shown), meaning that this increase in myelin thickness is consistent across the different striatal cells.

A difference in myelin thickness is a strong indication of myelination deficits on our mouse model, and thus, might be implicated on the connectivity deficits that have been shown to exist in the IOFC-DMS pathway.

In VLS, no difference in the g-ratios was detected (p -value=0.7616) (**Figure 11F**).

4.5 NUMBER OF MYELINATED AXONS

Our final approach on the characterization of myelination on the *Sapap3*-KO mouse was to seek for a reduced density of myelinated axons on the obtained TEM images, as a possible consequence of a reduced number of oligodendrocytes. No apparent difference in the average number of myelinated axons was detected in DMS (p -value=0.7000) (**Figure 12A**). However, our results demonstrate a slight, yet non-significant, tendency to an increase in the number of myelinated axons in VLS (p -value=0.2000) (**Figure 12B**).

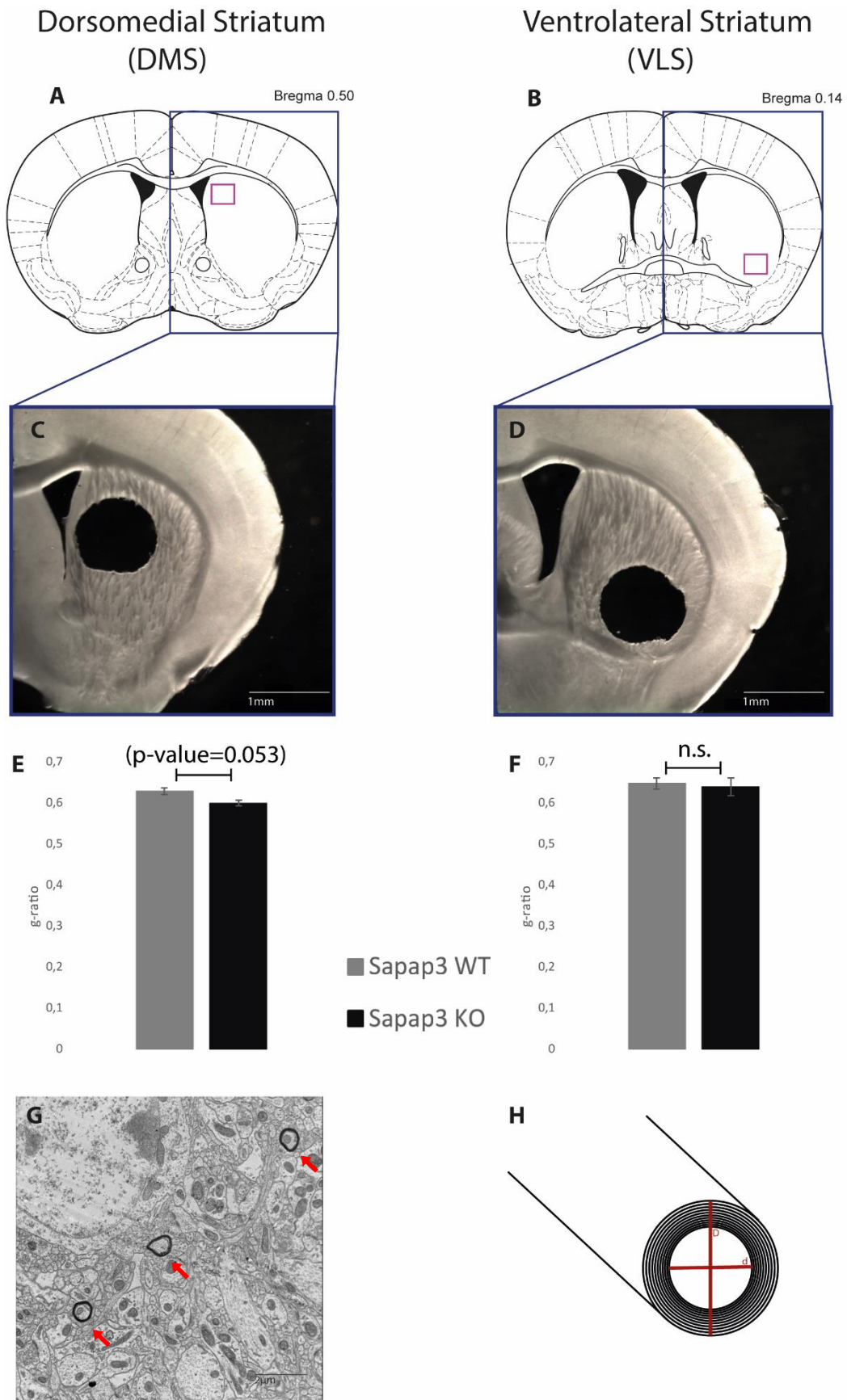


Figure 9: Quantification of myelin thickness by g-ratio measurement

Figures A and B represent a diagram of the Bregma levels we aimed in order to incorporate the defined ROIs (DMS and VLS, respectively). Figures C and D correspond to the slices that match the target regions

(DMS and VLS, respectively) under the stereo microscope, with the regions where we removed the ROIs for analysis. Figures E and F represent a quantification of myelin thickness by the measurement of the g-ratio (N=3 per group). In the DMS (E) a significant increase in myelin thickness can be observed, while no difference is seen in VLS (F). Figure G corresponds to a TEM image in the DMS, with three visible myelinated axons. Figure H schematically demonstrates how a g-ratio measurement is performed, by dividing the inner axonal diameter by the outer diameter of the myelinated axon. Bars represent average value and error bars show standard error. Mean and standard error were calculated from image analysis of 6 animals (N=3 per group), 150 g-ratios measured per animal. Represents n.s. = non-significant.

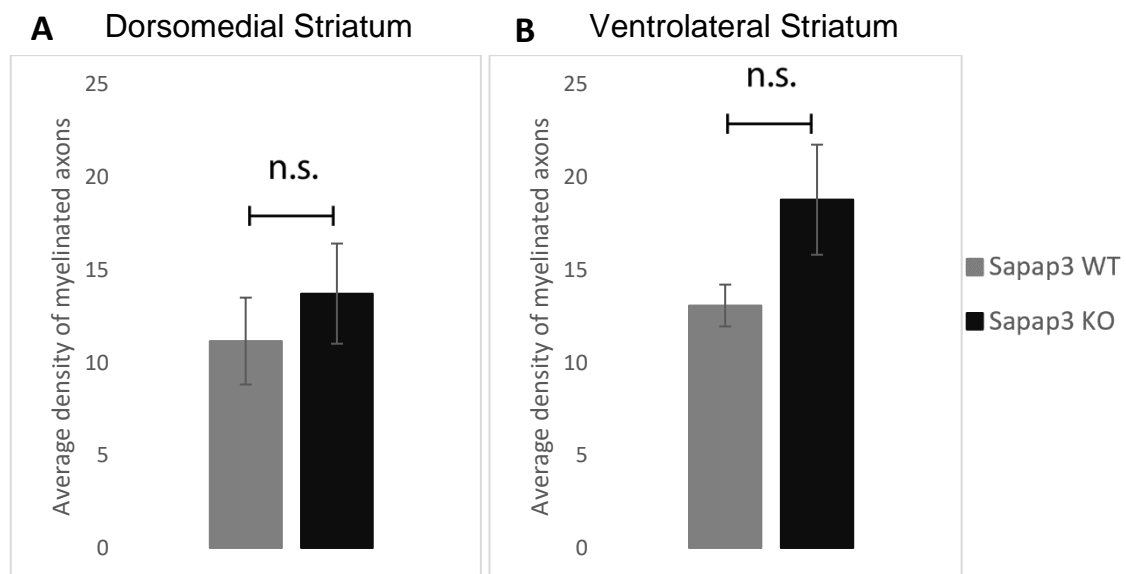


Figure 10: Quantification of the average density of myelinated axons

A tendency towards an increased number of myelinated axons can be seen in the DMS (A) and, more prominently, in VLS (B). Bars represent average value and error bars show standard error. Mean and standard error were calculated from image analysis of 6 animals (N=3 per group), 20 images per animal. Represents n.s. = non-significant.

4.6 PHARMACOLOGICAL TREATMENT

Motivated by the putatively reduced number of oligodendrocytes observed, we aimed for a behavioural rescue by the promotion of oligodendrocyte maturation. For that purpose, we used Clemastine, a drug that has been pointed out by its pro-remyelinating properties by inducing OPC differentiation. Animals were divided in two groups: clemastine-treated (CT) and vehicle treated (VT) (N=6 per group). Our administration protocol consisted on daily I.P. injections of 10mg/kg body weight for 12 days. To address behavioural changes, we quantified weight, compulsive-like and tic-like behaviours, and hind paw scratches on day 0 and on day 19 (seven days after treatment cessation).

Results demonstrate a slight, non-significant, weight loss on both groups (**Figure 13A**). An alleviation of behaviour was observed equally on both groups that was probably due to the adaptation of being manipulated (**Figure 13B-D**). No significant differences were observed between groups.

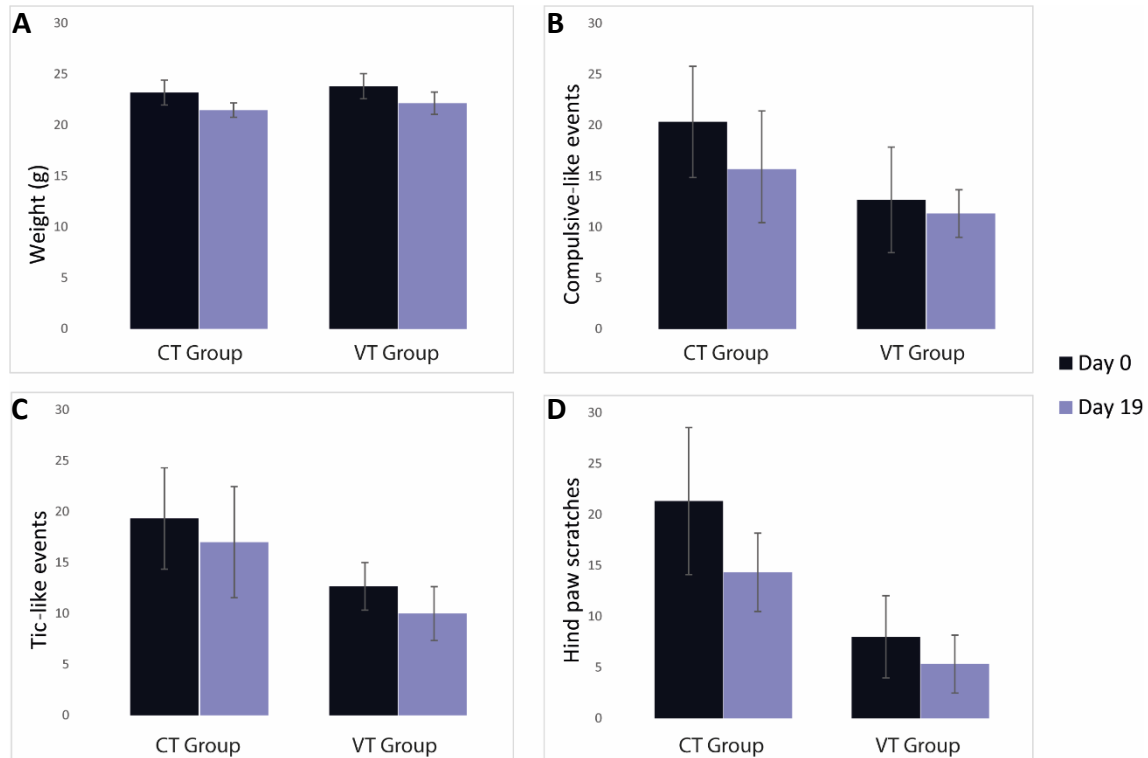


Figure 11: Quantitative behavioural comparison between Day 0 and Day 19 on Clemastine-treated and Vehicle-treated groups

A- Comparison between average weights on the first day of treatment and seven days after treatment cessation on both clemastine- and vehicle-treated groups. B- Comparison between average number of compulsive-like events on the first day of treatment and seven days after treatment cessation on both clemastine- and vehicle-treated groups. C- Comparison between average number of tic-like events on the first day of treatment and seven days after treatment cessation on both clemastine- and vehicle-treated groups. D- Comparison between average number of hind paw scratches on the first day of treatment and seven days after treatment cessation on both clemastine- and vehicle-treated groups. No statistically significant decrease in abnormal behaviours between CT and VT groups was noted.

CHAPTER 5 – DISCUSSION

Cortico-striatal dysfunctions have been widely studied for their involvement on the expression of pathological repetitive behaviours. Nonetheless, a satisfactory characterization of the nature of these dysfunctions has yet to be shown. Several recent studies have been highlighting the role of impaired myelination in neuropsychiatric disorders, as well as its relevance in learning and brain plasticity (Edgar & Sibille, 2012; Gibson, et al., 2014; McKenzie, et al., 2014). Thus, the primary aim of the present work was to assess whether aberrant myelination could be detected in the cortico-striatal circuits of the *Sapap3*-KO mouse, a well characterized model of repetitive behaviours (Welch, et al., 2007; Burguière, Monteiro, Feng, & Graybiel, 2013). Altogether, our results strongly support this hypothesis, paving the way for a future detailed characterization of the subjacent mechanisms.

The first indication of an impaired myelination comes from the quantification of the number of cells that actively myelinate the axons, the oligodendrocytes. We observed a decreased number of these cells in striatal regions on the *Sapap3*-KO group of animals when compared with their non-transgenic littermates. In fact, this decrease was not retrieved as statistically significant, however, several factors may be contributing to this lack of significance. In the first place, after detailed observation on the pathological behavioural displays of the *Sapap3*-KO group of animals, we were able to notice a great heterogeneity within the group. Some animals did not present an obvious phenotype, and behaved very similarly to the *Sapap3*-WT animals. This heterogeneity, combined with a small number of animals (N=7) might be masquerading the actual significance of the decreased number of oligodendrocytes in the *knock-out* mice. Future analysis with an increased number of animals will be performed in order to address this issue. Additionally, one other important factor resides on the anatomical characteristics of the striatum. This region contains a considerable number of fibers that cross it (corresponding to the internal capsule), which, on an immunohistochemical analysis, we could not properly differentiate from the specific striatal structure. Considering the highly number of oligodendrocytes present on these fibers (white-matter projections), our results are accompanied with a substantial amount of noise. To address this issue, we consider for our future analysis a higher magnification imaging. Nevertheless, assuming the observed tendency as real, a decreased number of oligodendrocytes in striatal regions is consistent with what we expected on our mouse model. The *Sapap3*-KO mouse model arises from the deletion of the SAPAP3 protein, which is a postsynaptic scaffolding protein present on excitatory (glutamatergic) synapses. Recent literature on axon-OPC communication has shown that glutamatergic synaptic input into OPCs enhances oligodendrocyte survival (Kougioumtzidou, et al., 2017). Thus, in a mouse model with impaired glutamatergic synapses, a causal correlation with a lower survival rate of oligodendrocytes may be hypothesized. To address this question, we believe that the first step would be to detect the presence of the SAPAP3 protein in the cells of the oligodendroglial lineage in wild-type animals, as it would provide evidence of a direct consequence. Additionally, it has been described that the *Sapap3*-KO mouse presents a deficient number of PV-positive interneurons (Burguière, Monteiro, Feng, & Graybiel, 2013), a GABAergic type of cells that modulate the activity of the MSNs. Considering the role of GABAergic input into OPCs, putatively implicated in their differentiation into mature oligodendrocytes (Arellano,

et al., 2016; Hamilton, et al., 2017), it is possible to question whether a deficient GABAergic synaptic transmission into OPCs may be contributing to this matter.

Curiously, we detected a slight increase in the density of oligodendrocytes in the IOFC on the *Sapap3*-KO group. This result was highly unexpected, considering the fact that no abnormal activity was previously detected on this region (Burguière, Monteiro, Mallet, Feng, & Graybiel, 2015). A further characterization of myelination in this area is currently being addressed, as its validation would be denoted as a possible compensatory mechanism.

Taken the result of a decreased number of oligodendrocytes, we intuitively hypothesized to see a reduction of either myelin thickness, or on the number of myelinated axons. However, our results clearly indicate an increase in myelin thickness in the DMS (with no difference in the VLS), and a tendency towards a greater number of myelinated axons in the VLS (with no significant difference in DMS). Even though highly counter-intuitive, these results sturdily fit with what has been described so far concerning our mouse model. Considering that the MSNs constitute the vast majority of striatal neurons (Kreitzer, 2009), the significance of this study should be attributed to the measurement of these cells. In addition, according to what has been described, an abnormal increase in the baseline firing rates of MSNs is present in the DMS of the *Sapap3*-KO mouse (Burguière, Monteiro, Feng, & Graybiel, 2013). The combination of these two premises strongly suggests that the augmented myelin thickness on MSNs underlies its hyperactivity. Likewise, the same reasoning can be done for the observation of an increased number of myelinated axons in the VLS. It has been previously demonstrated that a hyperactivity in cortical motor-lateral striatal regions are implicated in the emergence of tics (Neuner, et al., 2014; Rapanelli, Frick, Bito, & Pittenger, 2017). Thus, an increased number of myelinated axons on these regions may additionally be underlying its abnormally high activity. The reason why myelin abnormalities are expressed differently between dorsomedial and ventrolateral striatum should be further addressed, since it may shed light on the interplay between several factors that could be implicated on the regulation of myelination under pathological conditions.

The second aim of the present study arises from the hypothesis that different types of repetitive behaviours are imprinted within dysfunctions of topographically distinct cortico-striatal projections. Compulsive behaviours, as a complex type of repetitive behaviour, is associated to impairment in associative cortical projections into the more dorsomedial part of the striatum, whereas tics, as a motor type of repetitive behaviour, is associated with dysfunctions within motor cortical projections into the more lateral part of the striatum (Langen, Durston, Kas, Engeland, & Staal, 2011; Langen, Kas, Staal, Engeland, & Durston, 2011). Considering that the *Sapap3*-KO mouse displays both types of repetitive behaviours, we proposed to assess if they were compartmentally organized within the described areas. For that purpose, we quantified the number of grooming events (as a parallelism with compulsive behaviours), the number of myoclonic jerks (as a parallelism with tic behaviours), and a third category, the number of hind paw scratches, that may be categorized as an in-between category like the complex tics that can be seen in clinical practice. We proceeded to test all possible correlations between the abovementioned behaviours, and the number of oligodendrocytes previously counted on the IOFC, DMS, and VLS. Surprisingly, the only correlations that presented some significance were the tic-like behaviours within DMS, and hind-paw scratches, also within DMS, both indicating

that an increase on the frequency of these events was linked to a decreased number of mature oligodendrocytes in this region. Being so contrary to our expectations, these results raise several questions, as well as point towards the need of an optimization of the methodology used for this analysis. In the first place, we believe that the lack of correlation of compulsive-like behaviours with the number of oligodendrocytes in all the studied regions may be influenced by the shortened analysis on the behavioural quantifications. Indeed, only 15 minutes may be insufficient for this specific type of repetitive behaviours, considering that its display is not as striking evident as the tics and hind paw scratches. Attending to the need of a better characterization of behaviour, we are currently addressing this issue by developing an automated quantification that resolves the difficulties of a manual analysis. The second question that arises is the reason why no correlation was found within the VLS. This region constitutes the main target for SMA projections, a region that has been shown to be implicated in Tourette patients (Neuner, et al., 2014), and has not been yet fully characterized. The apparent lack of implication of myelination on this area in the emergence of tic-like behaviours may be an indication that only motor and sensorimotor projections are implicated in this phenotype. To test this hypothesis, more dorsolateral areas should be analyzed. The third question that arises is related to the reason why we detect correlation between the frequency of tic-like behaviours to a decrease in the number of oligodendrocytes in the DMS. Being a region that mainly receives projections from associative cortical areas, it is not intuitive to understand its implication on typically motor types of behaviour. This result may be in fact an indication that the different categories of repetitive behaviour may not be topographically organized in our mouse model. To further test this hypothesis, selective depletion of the SAPAP3 protein in the different specific striatal regions could constitute a stronger approach to this matter. Finally, one cannot exclude that the reduced number of animals may not be sufficient to significantly detect correlations.

Finally, motivated by the reduced number of oligodendrocytes observed, we tested a pharmacological approach on the promotion of OPC differentiation, and its expected subsequent alleviation of symptoms. For that purpose, we administered a drug that has been shown to enhance *de novo* myelination, clemastine (Li, He, Fan, & Sun, 2015; Liu, et al., 2016), and quantified behavioural changes. Our results display a slight symptomatic alleviation throughout the course of this experiment. However, this behavioural rescue was transversal to both Clemastine and Vehicle (control) groups. We believe that this behavioural change was related to habituation of the animals to a daily manipulation, rather than any pharmacological effect. Nevertheless, further characterization will be addressed, with additional time points, as well as immunohistochemical analysis on the brain tissue after treatment cessation. Nonetheless, considering the results obtained through electron microscopy analysis, where we could observe an increase in myelin thickness and in the number of myelinated axons, promotion of oligodendrocyte differentiation may not constitute the ideal methodology. We believe that an interesting approach would be a precise modulation of an activity-dependent myelination through optogenetic stimulation, as we could address to unveil the interplay between striatal microcircuitry.

Albeit further characterization and understanding of the specific mechanisms are still required, combination of these results point towards a validation of our hypothesis on the implication of myelin deficits on the emergence of repetitive behaviours.

CHAPTER 7 – CONCLUSION

Pathological repetitive behaviours constitute a core feature of several neuropsychiatric disorders, and can be manifested by different degrees of complexity, ranging from simple motor tics to ritualized sequences of abnormal behaviour. Considering the significant impact that these behaviours have in the quality of life of patients, and the poor understanding of the mechanistic that underlies them, we aimed for a better characterization of the circuitry involved on their emergence. In the light of recent discoveries concerning the participation of myelination in adaptive learning and neuropsychiatric disorders, we hypothesized a causal role for aberrant myelination to the development of repetitive behaviours.

For this purpose, we assessed myelin-related differences in a mouse model of pathological repetitive behaviours, the *Sapap3*-KO mouse, and its non-transgenic littermates.

After analysis of our results, we could indeed detect a deficient regulation of myelination in cortico-striatal pathways, translated by a reduced number of oligodendrocytes, and an increase in either myelin thickness or number of myelinated axons.

This preliminary findings open the path for future characterization of more specific mechanisms that might be responsible for regulation of myelin formation under pathological conditions.

CHAPTER 8 – BIBLIOGRAPHY

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