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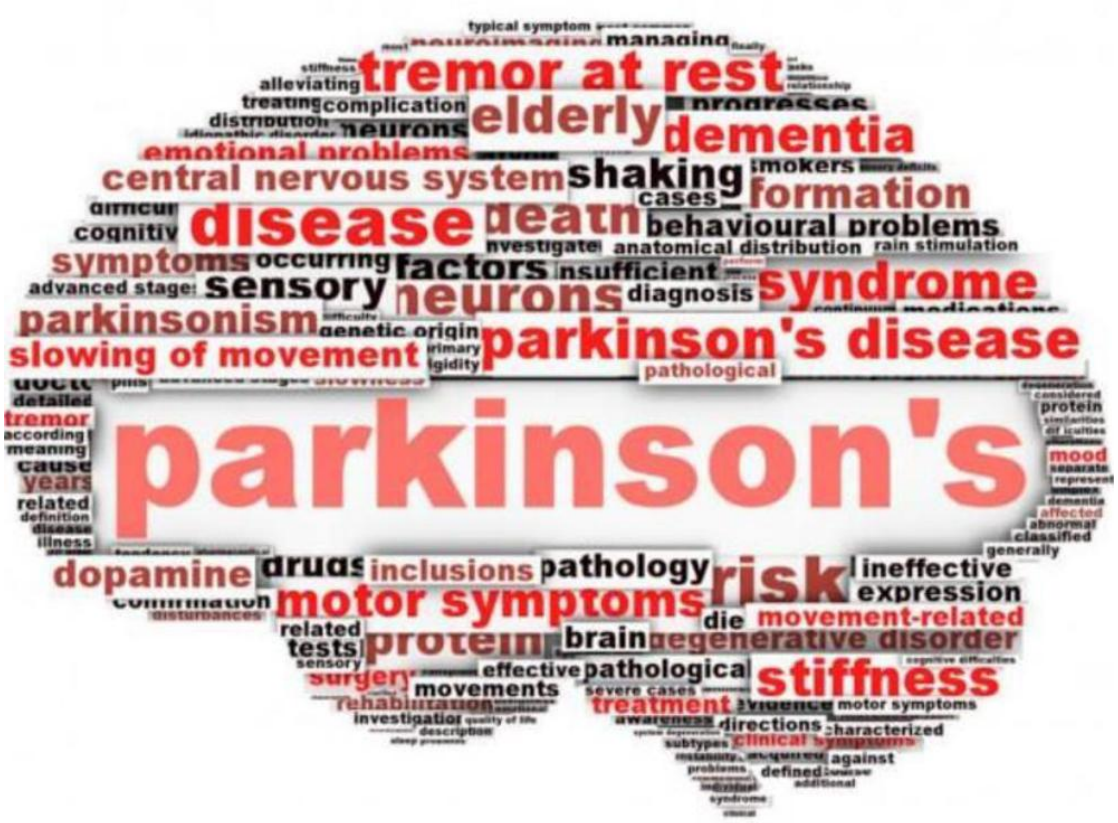
Investigating the role of alpha-synuclein in dopamine related behaviors

MSc Thesis



In the context of MSc Program: Molecular Biomedicine

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Abstract

Parkinson's Disease (PD), in spite of being known as a motor-impairing neurodegenerative disease, has numerous non motor symptoms. Parkinson's disease Psychosis (PDP) is the most challenging non-motor symptom with an estimated 50% incidence, affecting both the prognosis and the progression of the disease and dramatically reducing patients' quality of life. PDP is commonly perceived as a complication of dopamine therapy used to treat motor symptoms. However, some evidence indicates that PDP may precede motor symptoms and manifest in the absence of PD medications. In addition, there are no safe therapeutic options and no specific animal models available to study this condition.

Herein, we used in-house bred humanized alpha-synuclein BAC (AS BAC) transgenic rats created by Nuber et al (2013). We initially evaluated locomotor activity in an open field, prepulse inhibition, striatal, cortical and olfactory dopamine levels with HPLC and electrochemical detection and markers of dopaminergic activity with Western immunoblotting. Locomotor activity was assessed following pharmacological manipulations with haloperidol, a typical antipsychotic, clozapine, an atypical antipsychotic, pimavanserin, an atypical antipsychotic approved specifically for PDP, D-amphetamine, a psychostimulant or SCH 23390, a D1 receptor antagonist, and ropinirole, a D2 receptor agonist.

We show that compared to their wild type littermates, AS BAC rats exhibit increased striatal dopamine levels and locomotor hyperactivity from the early age of 3 mo and a prepulse inhibition deficit at 12 mo. Their hyperactive phenotype is reversed following the administration of haloperidol, clozapine, SCH-23390. In addition, this phenotype is exacerbated following the administration of ropinirole and d-amphetamine. The animals had a broad response spectrum regarding pimavanserin administration and no conclusive results could be drawn. Western blot data demonstrated similar levels between WT and AS BAC rats in two proteins regulating dopamine levels in the synapse, Dopamine Transporter (DAT) and Vescicular Monoamine Transporter (VMAT). Immunohistochemical analysis did not reveal differences in the levels of D1 receptors in the striatum between the two groups.

These data support a connection between aberrant human alpha-synuclein expression and a psychosis-like phenotype in AS BAC rats. Fascinatingly, this in vivo data may have analogies to clinical PD, where recent findings suggest that a premotor hyperdopaminergic state may occur.

Περίληψη

Η Νόσος του Πάρκινσον παρόλο που είναι ευρέως γνωστή ως μία ασθένεια που επηρεάζει την κινητικότητα του ασθενούς, διαθέτει πολλά μη κινητικά συμπτώματα. Η Ψύχωση στη Νόσο του Πάρκινσον (PDP) είναι ίσως το πιο απαιτητικό μη κινητικό σύμπτωμα με περίπου 50% συχνότητα, επηρεάζει την πρόγνωση και την εξέλιξη της νόσου και μειώνει δραματικά την ποιότητα ζωής των ασθενών. Η Ψύχωση στη Νόσο Πάρκινσον συχνά θεωρείται μία επιπλοκή της θεραπείας υποκατάστασης ντοπαμίνης που χρησιμοποιείται για να αμβλύνει τα κινητικά συμπτώματα. Παρόλα αυτά υπάρχουν ενδείξεις πως η Ψύχωση στη νόσο του Πάρκινσον μπορεί να προηγείται των κινητικών συμπτωμάτων και να εκδηλώνεται απουσία θεραπείας υποκατάστασης ντοπαμίνης. Επιπλέον δεν υπάρχουν ασφαλείς θεραπευτικές επιλογές και μέρος του προβλήματος είναι πως δεν υπάρχουν εξειδικευμένα ζωικά μοντέλα για να μελετήσουμε την νόσο.

Γι αυτή τη μελέτη χρησιμοποιήσαμε διαγονιδιακούς επίμυες που είχαν ενσωματωμένο στο γονιδίωμα τους το ανθρώπινο γονίδιο για την α-συνουκλεΐνη (AS BAC) οι οποίοι είχαν δημιουργηθεί από τους Nuber et al (2013). Αρχικά εκτιμήσαμε την κινητικότητα τους στη δοκιμασία ανοιχτού πεδίου, την προπαλμική αναστολή, τα επίπεδα ντοπαμίνης σε ραβδωτό σώμα, προμετωπιαίο φλοιό και οσφρητικούς λοβούς με Υγρή Χρωματογραφία Υψηλής Απόδοδης (HPLC) με ηλεκτροχημικό ανιχνευτή και δείκτες ντοπαμινεργικής δραστιριότητας με τύπωμα κατά Western. Η οριζόντια κινητικότητα εκτιμήθηκε μετά από τους ακόλουθους φαρμακολογικούς χειρισμούς: Αλοπεριδόλη - τυπικό αντιψυχωσικό, Κλοζαπίνη – άτυπο αντιψυχωσικό, Πιμαβανσερίνη – Άτυπο αντιψυχωσικό εγκεκριμένο για την PDP, Δεξτροαμφεταμίνη – Διεγερτικό του Κεντρικού Νευρικού Συστήματος, SCH 23390 – Ανταγωνιστής D1 ντοπαμινεργικών υποδοχέων, Ροπινιρόλη – Αγωνιστής D2 Ντοπαμινεργικών υποδοχέων.

Αποδεικνύουμε ότι εν συγκρίσει με τα ζώα αγρίου τύπου, οι διαγονιδιακοί επίμυες εμφανίζουν αυξημένα επίπεδα ντοπαμίνης στο ραβδωτό σώμα, υπερκινητικότητα από την ηλικία των τριών μηνών, και έλλειμα προπαλμικής αναστολής από τους 12 μήνες. Ο υπερκινητικός φαινότυπος τους αναστρέφεται με τη χορήγηση αλοπεριδόλης, κλοζαπίνης και SCH-23390. Επιπλέον αυτός ο υπερκινητικός φαινότυπος εντείνεται με τη χορήγηση ροπινιρόλης και δεξτροαμφεταμίνης. Αναφορικά με την πιμαβανσερίνη τα ζώα εμφάνισαν ευρύ φάσμα ανταπόκρισης και δεν ήταν δυνατό να εξαχθεί κάποιο οριστικό συμπέρασμα. Τα δεδομένα από το τύπωμα κατά Western δείχνουν παρόμοια επίπεδα συγκεντρώσεων δύο πρωτεϊνών που διαδραματίζουν σημαντικό ρόλο στην συγκέντρωση της ντοπαμίνης

στη σύναψη, του Μεταφορέα της Ντοπαμίνης (DAT) και του Κυστιδιακού Μεταφορέα Μονοαμινών (VMAT). Η Ανοσοϊστοχημική ανάλυση δεν υπέδειξε κάποια διαφορά στα επίπεδα του ντοπαμινεργικού υποδοχέα D1 στο ραβδωτό σώμα ανάμεσα στις δύο ομάδες ζώων.

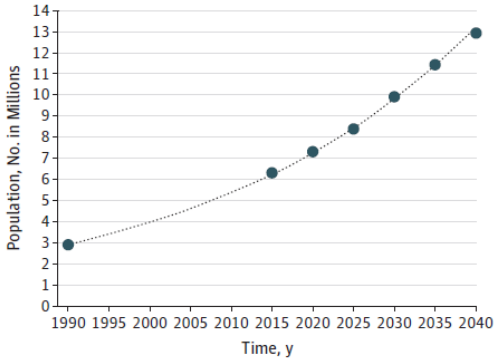
Αυτά τα δεδομένα υποδεικνύουν μία συσχέτιση μεταξύ της υπερέκφρασης της α-συνουκλεΐνης και ενός φαινοτύπου που προσομοιάζει ψύχωση στους διαγονιδιακούς επίμυες. Εξαιρετικά ενδιαφέρον είναι το γεγονός πως αυτή η κατάσταση μπορεί να έχει αναλογίες με τη νόσο του Πάρκινσον κατά την οποία μπορεί να εκδηλωθεί με μία υπερντοπαμινεργική περίοδο όπως υποστηρίζουν πρόσφατα δεδομένα.

Contents

1. Parkinson's Disease

The primary causes of disability nowadays are neurological diseases and among them Parkinson's disease (PD) is the fastest growing, to the point where it is now being called a pandemic.[1]

Figure. Estimated and Projected Number of Individuals With Parkinson Disease, 1990-2040



Sources: Global Burden of Disease Study (1990 and 2015) and projections based on published[2] and public[3] sources.

PD is one of the most common neurodegenerative diseases second only to Alzheimer's disease and it is the most common movement disorder.[2] It was first identified and described as 'shaking palsy' by James Parkinson in 1817. It is an age related condition with a significant prevalence in the general population. It affects about 1% of people over 60 years of age and this percentage rises to 3% for people over the age of 80. [3]

Clinically, PD is characterized by motor symptoms including static tremor, rigidity, muscle stiffness, bradykinesia and postural instability. In spite of being known as a motor-impairing disease it has numerous debilitating non motor symptoms, such as neuropsychiatric manifestations (depression, anxiety and psychosis), anosmia (olfaction deficit) and constipation- many of which often precede the appearance of motor symptoms. [4]

The most characteristic pathological feature of PD is the degeneration of dopaminergic neurons in the substantia nigra pars compacta which project their axons to the putamen, thereby compromising the dopaminergic nigrostriatal pathway. In post mortem patient samples, the major histopathological findings in the cell somata, are the 'Lewy bodies' (Fig. 1), formations that comprise of accumulations of aggregated proteins such as α-synuclein, ubiquitin, and occasionally Tau protein, crowded organelles and lipid membranes.[5]

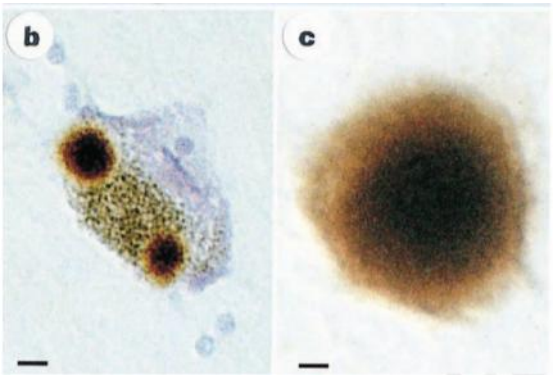


Figure 1. Substantia Nigra, Intracellular (b) and extracellular (c) α-synuclein-positive Lewy bodies in Parkinson's disease patient from the MRC Brain Bank, immunostained for α-synuclein[44]

Nowadays we know PD is a multifactorial disease. Genetic background is an important parameter since about 5-10% of PD cases are genetically inherited. More than 90 risk loci have been identified and more than 20 genes have been linked with monogenic forms of PD. Some of these include SNCA (alpha-synuclein), the first one to be associated to PD, LRRK2 and Parkin.[6]

Environmental toxic factors such as a narcotic drug, MPTP, or pesticides used in the past (i.e. paraquat, rotenone) have been shown to be linked to PD. Animal models were developed using these substances in order to study PD and assess their ability to inhibit mitochondrial function and damage the dopaminergic neurons in the substantia nigra *pars compacta*-mimicking motor and some of non motor (gastrointestinal dysfunction) symptoms.[7]

Biochemically, whether an inherited or a sporadic mutation is the reason, the cause of the cellular degeneration is associated with protein dysfunction. First of all, α-synuclein can lose its tertiary form due to a point mutation, overexpression or defective clearance and obtain an abnormal structure forming dimers, oligomers and fibrils which aggregate causing cellular dysfunction. Defective clearance is caused by lysosomal or proteasomal dysfunction. Mutations in the Glucocerebrosidase (GBA) gene, that encodes the enzyme that catalyses the conversion of glucocerebroside into glucose and ceramide, cause accumulation of the substrate and defective lysosomal clearance of α-synuclein.[8]

Another important factor that is implicated in PD is mitochondrial dysfunction. First of all, the toxic factors mentioned above, paraquat and rotenone, are inhibitors of the Complex I in the mitochondrial energy production chain leading to an increase of Reactive Oxygen Species (ROS) and oxidative stress. Furthermore, mutations in PINK1 and Parkin, two proteins critical for mitochondrial function have been shown to have a causative effect in the appearance of PD. The reason why dopaminergic neurons of the substantia nigra are more vulnerable in oxidative stress lies in their morphology and function. DA neurons have very long unmyelinated axons projecting to the striatum and formulating a massive amount of synapses thus requiring a great deal of energy.[9] However, the total mitochondrial mass is low compared to other neurons in the midbrain, thus any loss is critical.[10]

The pharmacological approach used nowadays in clinical practice is mostly symptomatic, substituting the lack of dopamine and alleviating motor symptoms. Patient quality of life is improved significantly, however, the progression of the disease is not decelerated. To that cause, dopamine analogues (Levodopa), Dopamine Receptor agonists (Pramipexole,

Ropinirole), MAO inhibitors (Rasagiline), COMT inhibitors (Entecapone, Tolcapone), etc., are currently being used.

2. Alpha-Synuclein

The Synuclein family of proteins consists of three isoforms, α, β and γ synuclein. They share a highly conserved N-terminus region with a unique sequence that contains seven 11 amino acid residue repeats that form a helix that can interact with membranes. This structure is not found outside of the synuclein family, and is only found in vertebrates. The C-terminus varies significantly depending on the isoform and consists of mainly hydrophilic residues susceptible to phosphorylation which implies a possible role in the regulation of synucleins' function.[11]

The α and β synuclein isoforms are expressed in red blood cells and in the majority of the nervous cells where they often colocalize in the presynaptic boutons- evidence that indicates their possible role in neurotransmission. The γ isoform in contrary is mostly expressed in glial cells and in specific neuronal populations[11] and has often been found in ovarian and breast cancers.[12]

α-synuclein is a small protein comprised of 140 amino acids with a molecular weight of 14 kD, smaller than the 40 kD cut-off of nuclear pores and was originally detected in the nuclear envelope, where it got its' name.[12]

It is the main component of Lewy bodies, the pathological hallmark of PD and it is found in the brains not only of mutation carriers but of patients with sporadic disease as well. Upon aggregation, it inhibits the actin microtubule function, thus impairing intracellular transportation such as vesicle movement in synaptic function and other mechanisms of the neuronal cells.[8]

α-synuclein plays a major role not only in the motor symptoms in PD but also in numerous non motor symptoms. It can be found aggregated in nerve cells of the intestines, the olfactory bulb and the dorsal motor nucleus up to 20 years before the appearance of motor symptoms and thus it can be held accountable for the symptoms of constipation, hyposmia and REM sleep disorder that appear. Furthermore, these symptoms are not relieved by the administration of dopamine replacement therapy, indicating that they do not occur due to the loss of dopaminergic neurons in the substantia nigra.[12]

3. Dopamine

Dopamine is one of the main neurotransmitters of the nervous system. It is implicated in motor control, motivation and reward pathways regulating a plethora of behaviours.[13] In PD, motor symptoms occur after the loss of about 60% of the dopaminergic neurons projecting from the substantia nigra *pars compacta* to the striatum.[14]

With respect to psychiatric conditions, dopamine imbalance plays a crucial role in schizophrenia and Attention Deficit Hyperactivity Disorder (ADHD). The central dogma for schizophrenia so far suggests that there is a dopamine augmentation in the mesolimbic system, in the neurons projecting from the midbrain into the limbic regions. However, recent findings suggest that there is a significant dopamine imbalance in the nigrostriatal pathway. Dopaminergic neurons projecting from the substantia nigra *pars compacta* play a pivotal role in initiating motor response and locomotion but also in perceiving rewards and reacting to novelty.[15,16]

ADHD is a spectrum of hyperactivity, attention deficit, anxiety and sleep disorders. ADHD is characterized by dysfunction of the reward system.[15] The ventromedial prefrontal cortex, orbitofrontal cortex and ventral striatum are the main brain regions involved in the anticipation and receipt of reward.[15] The prevailing hypothesis suggests that there is an imbalance (decrease) of dopamine and noradrenaline in these areas. Therefore, patients do not get enough rewarding stimuli from the tasks they perform, and normally reinforced behaviours are extinguished. [17]

Dopamine binds mainly to D1 and D2 receptors (D1DR and D2DR, respectively). D1 receptors are G protein-coupled receptors (GPCR) that are found mostly in the striatum, olfactory bulb and substantia nigra *pars reticulata.* They regulate the function of adenylate cyclase, Ca^{2+} ion channels and therefore, intracellular calcium concentration and neurotransmitter exocytosis. D2 receptors on the contrary are autoreceptors found mainly in the striatum, ventral tegmental area and cerebral cortex. They inhibit the action of adenylate cyclase, and consequently neurotransmitter release.[18] In addition, D2 receptors inhibit tyrosine hydroxylase, the enzyme responsible for dopamine synthesis. Postsynaptically, they initiate cascades which lead into the expression of dopamine- associated behaviours. Dopaminergic

D1 and D2 receptor types interact in an interesting way since D1 receptor antagonists block D2 agonist action.[19]

The regulation of dopamine synthesis, metabolism and release in the brain is mostly regulated by three enzymes, Tyrosine Hydroxylase (TH), Monoamine Oxidase (MAO) and Catechol-O-methyl-Transderase (COMT), and the two trasnporters, Dopamine Transporter (DAT) and Vescicular Monoamine Transporter (VMAT) (Fig. 2). TH is the key enzyme that catalyses the conversion of the amino acid tyrosine into L-DOPA, the precursor molecule of dopamine. MAO, along with COMT and other enzymes catalyze the metabolism of dopamine into DOPAC, HVA and 3-MT, the three main dopamine metabolites in the brain.[20] DAT is the key regulatory element regarding the reuptake of dopamine in the presynaptic cell, while VMAT activation is responsible for the fusion of dopamine-containing vesicles with the cell membrane and the release of dopamine in the synaptic cleft.[16]

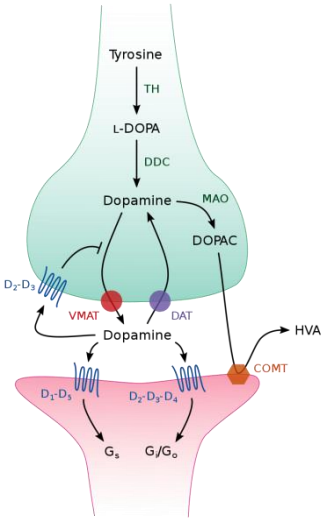


Figure 2 : Dopamine processing in a synapse.

4. Parkinson's Disease Psychosis

PD is commonly known as a motor-impairing disease, however non motor symptoms play an equally important role in the quality of the patient's life and often precede the appearance of motor symptoms. Some of these symptoms include depression, anxiety, sleep dysregulation, anosmia and constipation.[2,21] Psychosis in PD, also referred to as Parkinson's Disease Psychosis (PDP) is one of the most prominent and debilitating non motor conditions that manifests in PD patients.[22]

PDP consists of a combination of visual and auditory hallucinations and delusions. Visual hallucinations are the most common type and affect approximately one third of patients while auditory ones appear in 20% of cases and finally delusions, most commonly of spousal infidelity take the third place.[22,23] It greatly affects the patients' quality of life, is a major reason for hospitalization, reduces life expectancy and causes severe stress both to patients and caregivers.[22]

PDP could be characterized as the most challenging non motor symptom for several reasons. Usually it is not diagnosed at an early stage as patients maintain insight of their condition and do not inform their physician and later on it is undertreated by physicians who focus more on dealing with the extrapyramidal symptoms. Finally, the pharmacological treatment of PD could be triggering the exacerbation of psychotic phenomena. The basic theory of how psychosis develops is based on excessive dopamine neurotransmission. Therefore, the drugs used in PD to relieve motor symptoms such as dopamine analogues or dopamine receptor agonists can induce psychosis.[24]

Furthermore, the pathophysiology behind PDP has not yet been elucidated. Even though the excess of dopaminergic stimulation could be an explanation, minor hallucinating phenomena have been reported before the diagnosis of PD and the initiation of dopamine replacement treatment.[25] Therefore, psychosis precedes the appearance of motor symptoms and this suggests it should be treated as an independent non benign condition that is worsened by dopamine medications and its symptoms may worsen over time. [22]

In addition, it has been observed that the hallucinations in PDP are mostly visual, resembling those provoked by psychoactive substances such as LSD and mescaline and differentiate from the primarily auditory hallucinations in schizophrenia. This supports the current theory that a serotonin imbalance may be the leading cause of PDP.[24]

The prevailing hypothesis suggests that Lewy bodies accumulate in the prefrontal cortex and cause a degeneration of postsynaptic cells that carry serotonergic receptors. This causes the remaining cells to increase the number of 5-HT_{2A} receptors. Simultaneously due to neuronal loss in raphe nuclei, serotonin turnover increases which combined to the augmented post synaptic receptors result in an overexcitation of the prefrontal cortex leading to the visual hallucinations mentioned above. This consequently increases the excitation of the Ventral Tegmental Area (VTA) which projects into the ventral striatum causing the auditory hallucinations.[4]

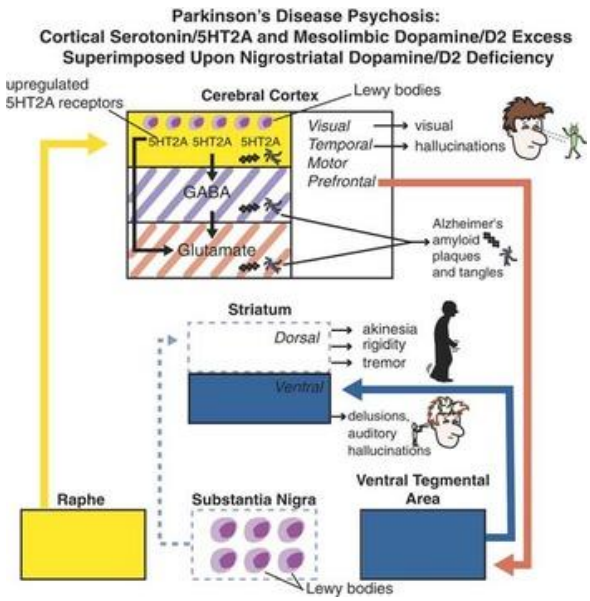


Figure 3: The cortical and mesolimbic pathways implicated in PDP

However, because the mechanisms behind this disease are not known, it is not a surprise that there is no safe and effective treatment for PDP. The role of serotonin in psychosis is defined by the response of patients to atypical antipsychotics that do not have high affinity to dopamine receptors but rather bind to serotonin receptors. These types of antipsychotics are currently being used, since they do not interfere with the therapy for motor symptoms. Usually clozapine is currently the go-to option, which supports the serotonin imbalance theory.[26] However, it has some serious side effects such as agranulocytosis, a potentially fatal condition where white blood cell counts drop drastically and thus requires close monitoring and regular blood tests. Quetiapine is also being used but as shown by Ferreira et al. 2013, it is probably ineffective.

Since 2016, a new drug, pimavanserin, has been approved by the FDA to treat hallucinations and delusions linked to PD.[22] Pimavanserin (*N*-(4-Fluorophenylmethyl)-*N*-(1-methylpiperidin-4-yl)-*N*_-(4-(2-methylpropyloxy)phenylmethyl) Carbamide (2*R*,3*R*)-Dihydroxybutanedioate) is a $5HT_{2A}$ inverse agonist. Pharmacologically, it binds to the $5HT_{2A}$ serotonergic receptor and decreases its constitutive signaling activity.

5. Animal models of Psychosis

In order to evaluate psychosis in animal models, cross-species comparability is required in the tests performed, so that the results observed in the animal models can be generalized in the human context. Usual markers are drug (d-amphetamine)- induced head twitches, hyperactivity, and prepulse inhibition (PPI).[27]

Some animal models used to study psychosis include:

- DISC-1 (Disrupted in Schizophrenia) mutant mice, which have a dysfunction in a gene linked to susceptibility for this disease.
- COMT transgenic mice, which, due to a single nucleotide polymorphism, have a non functional enzyme that normally metabolizes dopamine. This leads to the accumulation of the neurotransmitter and a psychosis-like phenotype.
- Drug-induced psychotic models, following the administration of d-amphetamine, or 5-$HT_{2A/2C}$ agonist, 1-[2,5-dimethoxy-4-iodophenyl]-2 aminopropane (DOI).[27]

Prepulse Inhibition

Prepulse Inhibition (PPI) is the physiological phenomenon in which an organism's response to a certain startle stimulus (pulse) is lowered if a weaker startle stimulus (prepulse) precedes the pulse 30-300 ms earlier (Fig.4). It is a mechanism developed so that organisms do not unnecessarily respond to repetitive stimuli. It applies in humans and rodents and therefore can be useful in translational research. A deficit in PPI is observed in patients with schizophrenia as well as animal models of the disease. Pharmacologically, PPI can be disrupted by dopaminergic drugs, such as apomorphine, a dopamine receptor agonist, and this disruption is reversed with the administration of atypical antipsychotics such as Clozapine.[28]

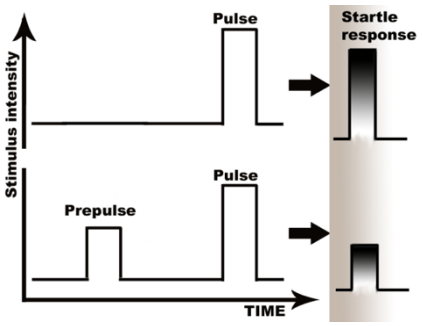


Figure 4: Prepulse Inhibition: Response is attenuated in the presence of a preceding stimulus.

6. Pharmacology

Haloperidol

Haloperidol is a typical antipsychotic. It manifests its antipsychotic effects by antagonizing several receptors and reducing mostly the positive symptoms of schizophrenia (auditory and visual hallucinations, delusions). It blocks several receptors such as DRD1 and DRD2, glutamate receptor NMDA-2B, serotonergic receptor 5-$HT2_A$ and various other dopaminergic, muscarinic and adrenergic receptors and does not have a fully elucidated pharmacological action. However, it has a higher affinity for DRD2 and for that reason it cannot be used to treat PDP since it interferes with the dopamine substitution therapy.

Clozapine

Clozapine is an atypical (second-generation) antipsychotic. It has a strong affinity for serotonergic receptors 5-$HT_{2A/2C}$ and a strong binding capacity for D2 dopaminergic receptor. It is the drug currently used for the treatment of PDP however it is not selective since it partially antagonized D2 receptors and has some severe side-effects. The most severe and possibly fatal one is agranulocytosis (1% incidence) a condition in which white blood cells (usually neutrophils) of patients are depleted and their immune system fails.[26]

D-Amphetamine

D-amphetamine is the dextrorotorary enantiomer of amphetamine. It is a CNS stimulant whose mechanism of action has not yet been fully elucidated but we know that it results in increased dopamine in the brain. It binds to VMAT (Vesicular Monoamine Transporter) and increases vesicular release, but also inhibits presynaptic reuptake by inhibiting DAT (Dopamine Transporter).[15]

Psychostimulants such as amphetamine are used in the treatment of ADHD to restore the dopamine deficit and provide the patients with the same satisfactory feeling the endogenous neurotransmitter would cause.[29]

DAT knockout rats are used to model of ADHD. They do not express DAT receptor which is responsible for the presynaptic reuptake of dopamine and regulation of cellular stores. These rats have increased extracellular but decreased tissue DA concentration. They also exhibit pronounced hyperactivity which is reversed upon administration of psychostimulants such as amphetamine.[16]

Pimavanserin

Pimavanserin (ACP-103) is a $5HT_{2A}$ inverse agonist, meaning it can bind to this serotoninergic receptor and attenuate the basal constitutive signaling of the receptor. Therefore, not only does it block the activation of the receptor by its ligand, serotonin, but it also inhibits the endogenous baseline activity the receptor has without any kind of activation. At the same time, it does not interfere with dopaminergic therapy and clinical trials have shown it does not worsen motor symptoms.[30]

The existing theory regarding PDP suggests that serotonin imbalance in the prefrontal cortex impacts the appearance of PDP, and thus, pimavanserin has been approved as a novel antipsychotic agent that does not affect the dopamine replacement therapy of PD since it has zero affinity for dopamine receptors.

Experiments in vitro suggest a strong binding capacity of pimavanserin to $5HT_{2A}$ receptors. Experiments in vivo, in several animal models have provided ambiguous results. Some experiments indicate a promising reversal of psychosis in animal models[31] , while in other studies the $5\text{-}HT_{2A/2C}$ agonist, 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) induced head twitches (a marker for psychotic behavior) in rats and mice have been significantly reduced only by the administration of 3mg/kg of pimavanserin ($p<0.05$) and not

any other, higher or lower, dose. It has been proven to be more efficacious in reversing prepulse inhibition caused by DOI administration in a broader range of doses (0.1 to 10mg/kg) ($p<0.05$). [21]

Phase II clinical trials have shown only a trend towards improvement of psychotic incidences regarding visual hallucinations in PD patients, but overall a significant improvement in the decrease of psychotic events. Recently it was reported that pimavanserin has also failed to reach a statistically significant result in treating Alzheimer's disease Psychosis[32] and positive symptoms of schizophrenia. [22]

SCH-23390

SCH-23390 is a selective D1 and D5 dopamine receptor antagonist in vitro, however, in vivo the doses required to block D5 receptors are 10 times higher than the ones necessary to block D1. Therefore, practically we do not consider it to be a drug, since it is not used in clinical practice but rather a pharmacological tool used to inhibit selectively D1 Dopamine receptors.[33]

Ropinirole

Ropinirole is a non-ergot derived dopamine receptor agonist used in clinical practice to treat early Parkinson's Disease. It has a high affinity for D2 receptors but it can also bind to D3 and D4 receptors.

7. AS-BAC rats

As mentioned above, α-synuclein has been implicated in the pathogenesis of PD, and therefore, there have been various attempts to generate animal models with alterations in the SNCA gene in order to study this disease. These attempts include overexpression of the mutations A53T and A30P which are known to cause a dominantly inherited form of PD.[34] These animals possess phenotypes that model some of the non motor symptoms of PD such as olfactory deficit, depression and colonic dysfunction. However, the effect of SNCA mutations on the nigrostriatal pathway does not necessarily translate to neurodegeneration. These differences might be a matter of integration ratio or of promoter-restricted expression[35]. Furthermore in rodents the amino acid 53 is usually a threonine, indicating that the A53T mutation is pathogenic only in the human context.[11] As an attempt to overcome the limitations of the lack of promoter region, a new model was generated by Nuber et al

(2013). The human wild type SNCA gene including the intronic sequences as well as a 35-kb upstream region containing the human promoter regulatory elements as well as a 45-kb downstream flanking region were inserted in the genome of Sprague-Dawley rats (AS BAC rats).

As reported by the team that generated them, they have certain biochemical and phenotypical traits that render them a satisfactory model to study the motor as well as the non-motor symptoms of PD. Initially, the augmentation of both the insoluble full length a-synuclein and the c-terminus truncated form leads to the degeneration of dopaminergic neurons in the substantia nigra pars compacta. This results in the demonstration of several non motor symptoms such as an olfactory deficit, impaired novelty seeking and locomotion decline, which are consistent with an animal model of PD.

The present study was performed in these transgenic AS BAC rats. Our previous findings suggest that these animals indeed demonstrate global brain α-synuclein aggregation, an olfactory deficit starting at 3 mo, and reduced locomotor activity during the dark phase as suggested by Nuber et al., as well as other behavioral alterations. Interestingly, these animals exhibit hyperactivity in a novel environment and this finding is in line with the findings of Yamakado et al. (2012)[36] who inserted the same construct in mice and observed an increase of locomotor activity and decreased anxiety-like behavior but also with earlier mouse models of α-synuclein overexpression.[37] The hyperactivity exhibited by AS BAC rats can be explained by increased striatal dopamine levels and mild nigral neurodegeneration that we have also assessed. These unusual findings gave us the impetus to assess the potential of these transgenic rats to serve as a novel model of PDP.

8. Aim of the study

Based on previous findings in the lab that demonstrate hyperactivity and striatal hyperdopaminergia in AS BAC rats, we sought out to test if these behaviors are associated with a “psychotic” phenotype. If this is the case, hu-AS BAC rats would manifest non-motor symptoms in parallel with underlying dopaminergic neurodegeneration, thus validating the hu-AS BAC rat as a model to study this phenomenon. Furthermore, we wanted to assess how the overexpression of human α-synuclein results in increased dopamine and how this affects the dopaminergic system. To do so, we proceeded with the determination of neurotransmitter levels in the striatum and the prefrontal cortex, implicated in PD and psychosis pathogenesis. Furthermore, we assessed the hyperactive phenotype of AS BAC rats following pharmacological manipulations in order to explore the role of dopamine neurotransmission regulators and the potential reversal of this phenotype with antispychotics. Furthermore, we attempted to correlate the results of the locomotor assays with biochemical analysis of the striatum through Western Blot and immunohistochemical analysis. Ultimately, we aimed to explore the potential of AS BAC rats as a novel animal model to study PDP.

9. Methods

For all experiments, we used male Sprague-Dawley rats. The animals were 10-14 weeks old and their weight was 350-550 grams. The animals were housed in pairs in the BRFAA animal facility in the following conditions: Temperature 25± 2^{o}C, humidity 55± 10% and 12-hour light-dark circles, with light-period starting at 7 a.m. Animals had access to food and water *ad libitum.* All experiments were approved by the local ethics committee.

9.1 Neurochemical profiling

3 mo AS BAC rats were decapitated and their brains were immediately separated into different regions of interest, snap frozen in liquid nitrogen, and kept on dry ice until storage at -80 ^{o}C until the day of analysis.

Tissues from the striatum, and prefrontal cortex of 3 mo and olfactory bulb of 3 and 6mo Sprague-Dawley male BAC and WT rats (n=5 per group) were homogenized by ultrasound sonication in an ice cold buffer (volume (ml) = 5 * tissue weight (mg)) containing TAE (Trizma Base 2M, EDTA 0.05M, and glacial acetic acid, pH=6.2) and 0.1% (M?) perchloric acid. The homogenized samples were left in the dark, at 0 ^{o}C for 20 minutes and then centrifuged at 13,000 rpm for 30 minutes at 4 ^{o}C. The supernatant was then collected and stored at -80 ^{o}C for HPLC analysis.

The monoamine neurotransmitters noradrenaline (NA), dopamine (DA) and its metabolites: 3,4-Dihydroxyphenylacetic acid (DOPAC), Homovanillinic Acid (HVA) and 3-Methoxytyramine (3-MT), serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5HIAA) were measured with High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED). The column used was YMC Triart C18 100 x 2mm, 3µm particle size, the mobile phase consisted of an acetonitrile, 50 mM phosphate buffer (1:9 dilution), pH 3.0, containing 300 mg/l 5-octylsulfate sodium salt as the ion-pair reagent.

All samples were diluted 1:4 in TAE buffer before being injected in the electrochemical detector. It should be mentioned that for the correct quantification of the samples every day a new standard curve was calculated by using new standard samples in concentrations of 0.08, 0.04 and 0.02 M. t-test analysis was performed to compare the two groups.

9.2 Behavioral analysis: Locomotor activity following dopaminergic system in/activation

We used the open field test in order to assess the animals' locomotor activity and/or anxiety-like behavior. Each animal was placed in a transparent Plexiglas arena (40 x 40 x 35 cm) with an overhead and side camera and their locomotor activity was recorded for 60 minutes with specialized video-tracking software (Ethovision 9.0 XT, Noldus). Distance travelled (cm) and rearings were analyzed as indices of horizontal and vertical activity, respectively and time spent in the center (s) as an index of anxiety. All animals were habituated to the procedure room for 30 minutes before testing.

Figure 5. Open field arena with an overhead camera using Noldus Ethovision visual tracking software

Vehicle (saline) or one of the following drugs were administered by injection, placed back in their homecage and 10 minutes later placed in the open field arena for the following drugs: haloperidol, clozapine, pimavanserin and SCH-23390. . For D-amphetamine and ropinirole, a 20 min habituation session in the arena preceded the injection and 60 min test session. Drugs were injected either subcutaneously (SC) or intraperitoneally (IP) (See Table 1). Two-way ANOVA analysis was performed to study the effect of the drugs administered in the different animal genotypes.

Drug	Dose (mg/kg/ml)	Administration
Haloperidol	0.05[38]	SC
D-Amphetamine	2.5[24]	IP
Clozapine	2.5[26]	SC
Pimavanserin	0.3 and 1 [26]	SC
SCH-23390	0.1[39]	IP
Ropinirole	2.5[40]	SC

Table 1. Dose and route of administration of the administered drugs

9.3 Biochemical analysis

Tissues from the striatum and the prefrontal cortex of 3 mo male Sprague-Dawley rats were obtained after decapitation of the animals. The tissues were immediately separated into different regions of interest, snap frozen in liquid nitrogen, and kept on dry ice until storage at -80 ^{o}C until the day of analysis.

In order to extract the cytosolic and membrane soluble proteins from the collected tissues, we first homogenized the tissues using 600 ul of STET buffer (50 mM Tris-base pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Triton-X-100) and a glass Teflon homogenizer. The lysate was then ultracentrifuged at 48.000 G for 1 hour at 4 ^{o}C. The supernatant was transferred to a clean tube (Triton-X soluble fraction).

The pellet was washed two times with 1X PBS and centrifuged at 13,000 rpm at 4^{o}C. The lysate was sonicated at 30% amplitude for 3-4 seconds in RIPA buffer + 2% SDS (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate) and then centrifuged at 48,000 G for 1 hour at 4^{o}C. The supernatant was transferred to a clean tube (SDS soluble fraction). Protein concentration was determined using the Bradford assay (BIORAD).

The protein samples were mixed with 4X sample buffer (20% β-mercaptoethanol) and ran on 12% (?) acrylamide gels. Following this, the proteins were transferred onto a nitrocellulose membrane and blocked using 5% milk in 1X TBST. The blots were probed with antibodies against: DAT (1:1000), VMAT (1:1000), and BDNF (1:1000), γ-Tubulin (mouse, 1:2000) and GAPDH (mouse, 1:5000). Blots were probed with horseradish peroxidase-conjugated secondary antibodies (HRP; mouse and rabbit) and then incubated with Enhanced Chemilluminescence (ECL) solution for 3 minutes. Finally, the blots were developed on Super RX film (Fuji film). The resulting films were analyzed and quantified using ImageJ. γ-Tubulin and GAPDH were used as loading controls with which all values were normalized. t-test analysis was performed to compare the two groups.

9.4 Immunohistochemistry

Animal brains from 3-4 mo male hu AS BAC rats were fixated after transcardial perfusion. The animals were anesthetized with isoflurane and a catheter connected to a pump was inserted in the left cardiac ventricle. 30 ml of PBS were used to clear the blood and then 30ml of 4% paraformaldehyde (PFA in PBS) were used in order to fix the brain. The brains were then removed and immersed in 4% PFA overnight, then in 15% and 30% sucrose solution in PBS overnight. Finally the brains were snap frozen in -55 ^{o}C using isopentane on dry ice and stored at -80^{o}C until analysis.

The perfused brains were cryosectioned through the coronal plane in 30 μm increments. We collected sections from the striatum and substantia nigra. The sections were collected free floating in anti-freeze buffer (NaH_2PO_4 ; Na_2HPO_4 ;Ethylene glycol; Glycerol; H_2O) and kept in storage at -20^{o}C until analysis.

For immunohistochemichal analysis, we selected striatal sections of 9 mo WT and AS BAC rats (n=). The sections were washed with PBS, followed by antigen retrieval with 10 mM citrate buffer at 80 ^{o}C for 20 minutes then placed on ice for an additional 20 minutes. The sections were then blocked using 5% normal goat serum (NGS) and 0.1% Triton-X for 1 hour at room temperature. Subsequently, the sections were incubated for 48 hours at 4^{o}C with the following antibodies: DRD1 (1:500; Proteintech), TH (1:2000; Millipore),. Finally, the sections were incubated for 60 minutes, room temperature, in the dark with the following secondary antibodies: rabbit red (1:2000), mouse green (1:2000), TOPRO (1:2000).

Three images from each of the stained sections were obtained using confocal microscopy (Leica SP5 mark II with conventional photon-multiplier tube, at 23°C using the Leica Advanced Fluorescence v2.7 acquisition software (Leica Microsystems, Wetzlar, Germany, n=2 sections per animal, 4 animals per group.

For the quantification of the DRD1 signal ImageJ was used in order to calculate the mean intensity in the red channel. The mean of six measurements per animal was drawn and a t-test analysis between the two groups provided the final results.

10.Results

10.1 Neurochemical profiling

In the striatum, we observed a statistically significant increase of DA and 5-HT levels in AS BAC rats (n=4, p=0.0013; n=4, p=0.0069), respectively, and a decrease in NA levels (n=4, p=0.001).

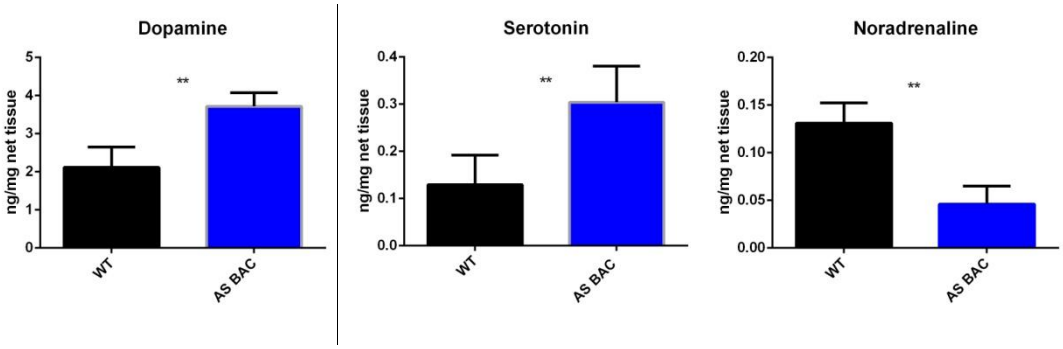


Figure 6 : AS BAC rats demonstrate increased DA (p=0.0013) and 5HT (p=0.0069) levels and decreased NA (p=0.001) levels compared with WT littermates (n=4 per group). [*(p≤0.05), **(p≤0.01)]

We believe the increases in dopamine and serotonin concentrations are not related to defective metabolism but rather to increased production since their metabolites appear to be significantly increased. DOPAC (n=4, p= 0.032), 3-MT (n=4, p= 0.0006), and 5HIAA (n=4 p= 0.033).

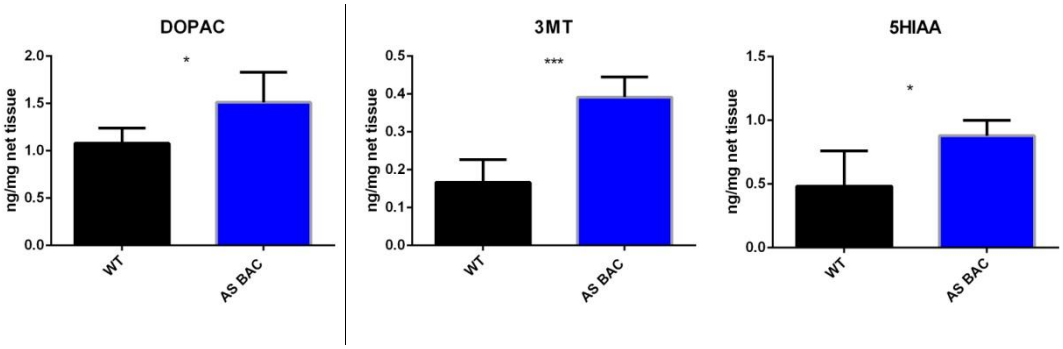


Figure 7 : DOPAC (p=0.032), 3MT (p=0.0006) and 5HIAA (p=0.033) concentration levels appear to be increased in AS BAC rats, which indicates there is no degradation deficiency. (n=4 per group) [*(p≤0.05), **(p≤0.01), ***(p≤0.001)]

No statistically significant differences in the neurotransmitter levels of cortical tissues between the two groups were observed.

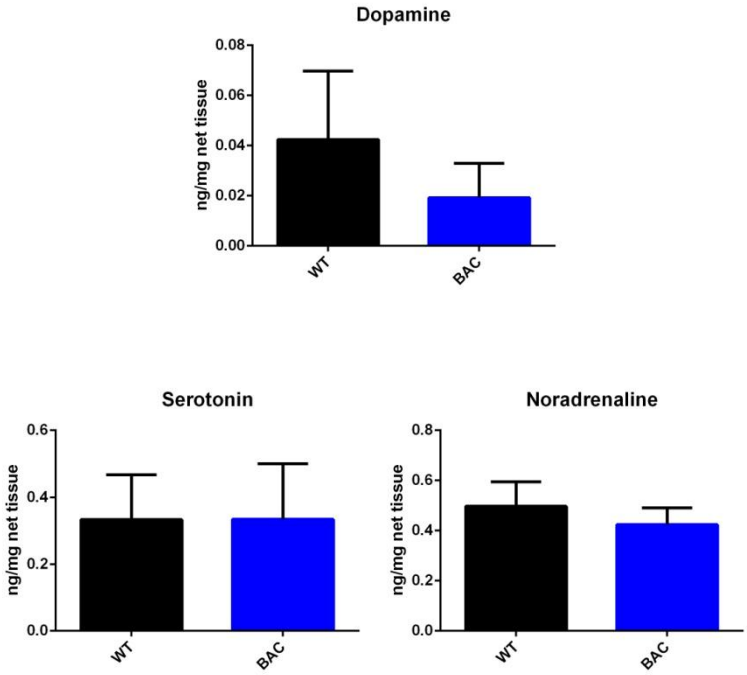
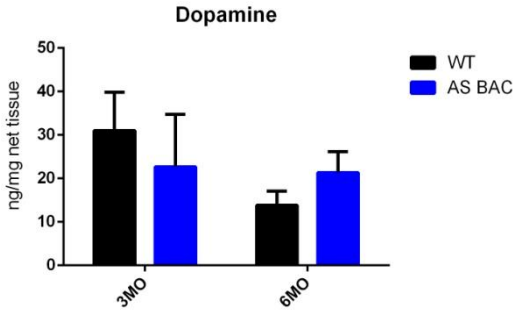


Figure 8 : Comparison between neurotransmitter levels in cortical samples (n=4 per group), no statistical differences between AS BAC and WT rats were detected.

In the olfactory bulb, we observed an age-dependent trend for decreased dopamine concentration, although the difference is not statistically significant (n=4, p=0.0557) and increased dopamine metabolism (DOPAC levels), however, no difference was observed concerning serotonin and noradrenaline levels.



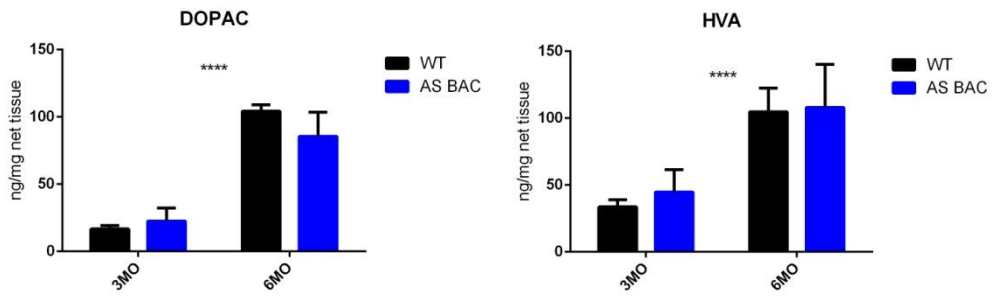


Figure 9: Comparison between neurotransmitter metabolite levels of the olfactory bulb at 3 and 6mo rats. In this tissue there is no statistically significant difference in dopamine, or its' metabolites between the two groups in each timepoint. (n=4 per group)

10.2 Behavioral analysis: Locomotor activity following dopaminergic system in/activation

Saline

To begin with, we verified the previous findings of our lab. Upon saline administration, we monitored their activity for 60 minutes. We concluded that indeed the hyperactivity of AS BAC rats is evident during the 60 minute trial. (n=6 p=0.0002)

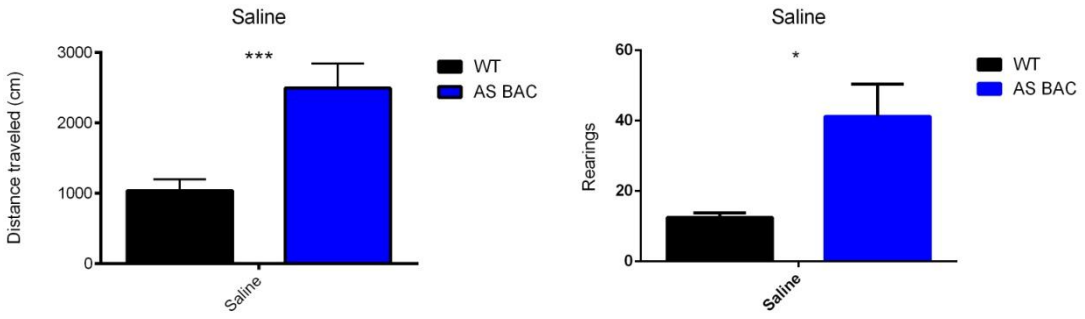


Figure 10: Horizontal (p=0.0002) and vertical (p=0.046) hyperactivity of AS BAC rats (n=6 per group) [*(p≤0.05), ***(p≤0.001)]

Haloperidol: D2 receptor antagonism

We assessed the locomotor activity of AS BAC versus WT rats upon administration of haloperidol. We demonstrated that the pronounced hyperactivity AS BAC rats demonstrate is reversed with the subcutaneous administration of Haloperidol (0.05mg/kg) at a

subthreshold dose that does not affect WT rats. The activity levels of the transgenic animals reach the levels of the WT group both with respect to horizontal and vertical activity.

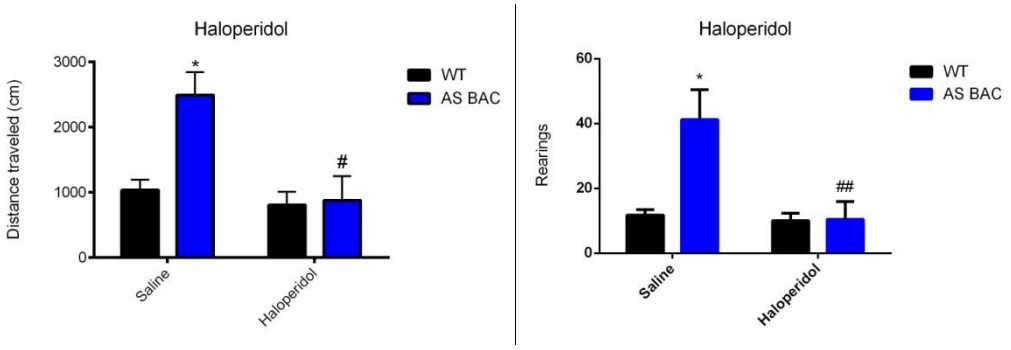


Figure 11: Horizontal (p=0.0123) and vertical (p=0.0116) hyperactivity reversed in AS BAC rats with Haloperidol (n=5 per group) [Genotype effect F(1,18)=5.306, p=0.0334 ; Drug effect F(1,18)=7.734, p=0.0123][Genotype effect *(p≤0.05), Drug effect #(p≤0.05), ##(p≤0.01)]

D-Amphetamine: CNS stimulation, DAT/VMAT antagonism

In order to assess whether the hyperactivity exhibited by AS BAC rats is a phenomenon caused by DAT receptor dysfunction, we proceeded with intraperitoneal administration of D-amphetamine (2.5mg/kg) in AS BAC rats and their WT littermates and monitored their locomotor response. In addition to exhibiting a significantly elevated baseline locomotor activity, AS BAC rats also exhibited a significantly higher locomotor response to D-amphetamine as compared to WT rats. This result suggests that this phenotype is not a consequence of dopamine reuptake dysfunction.

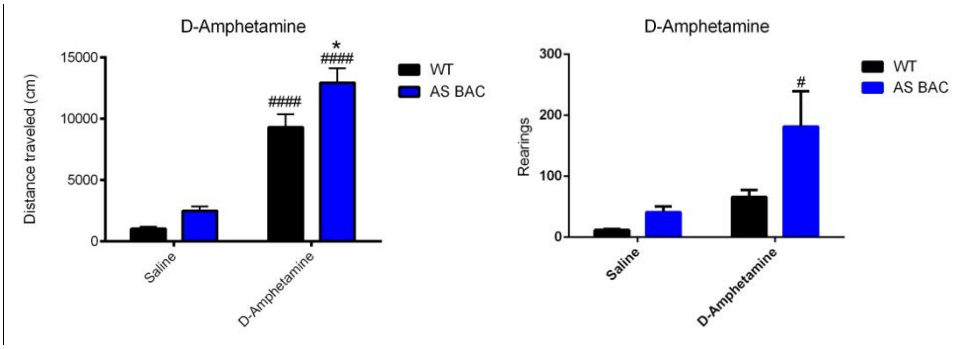


Figure 12 : Horizontal (p<0.0001) hyperactivity was increased in both AS BAC and WT rats, but vertical (p=0.0117) hyperactivity was increased only in AS BAC rats following D-Amphetamine administration. (n=5 per group) [Genotype effect F(1,20)=7.522, p=0.0125 ; Drug effect F(1,20)=101.2, p=0.0001] [Genotype effect*(p≤0.05), Drug effect #(p≤0.05), ####(p≤0.0001)]

Clozapine : 5-HT$_{2A}$ / D2 Antagonism

Following the subcutaneous administration of Clozapine (2.5mg/kg), we observed a statistically significant reduction of hyperactivity in AS BAC rats. The levels of the horizontal and vertical activity reached those of the WT animals. The low dose used did not affect the locomotor activity of WT animals.

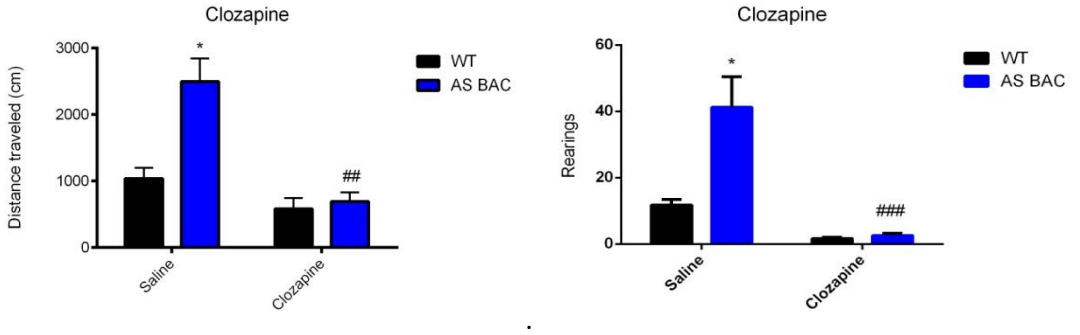


Figure 13 : Horizontal (p=0.0021) and vertical (p= 0.0009) hyperactivity reversed with Clozapine (n=5 per group) [Genotype effect F(1,18)=6.602, p=0.0214 ; Drug effect F(1,18)=13.78, p=0.0021] [Genotype effect*(p≤0.05), Drug effect ##(p≤0.01), ###(p≤0.001)]

These results indicate that antipsychotics are effective in reversing this hyperactive phenotype.

Pimavanserin

Upon subcutaneous administration of Pimavanserin (0.3 mg/kg)[26], we did not see any statistically significant differences between the two animal groups. We repeated these studies with a higher dose, (1mg/kg)[31] that the literature suggested was effective in animal models of D-amphetamine-induced psychosis. However, at both doses the variance amongst the subjects is evident and therefore, pimavanserin does not appear to modulate in a defined way the locomotor response of neither AS BAC nor WT rats.

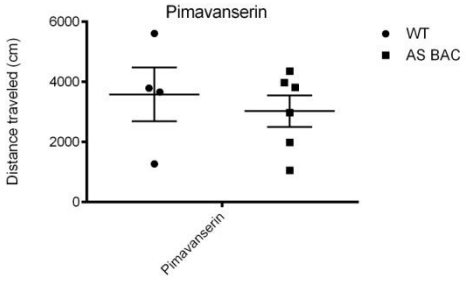


Figure 14: Variance of locomotor responses to pimavanserin treatment

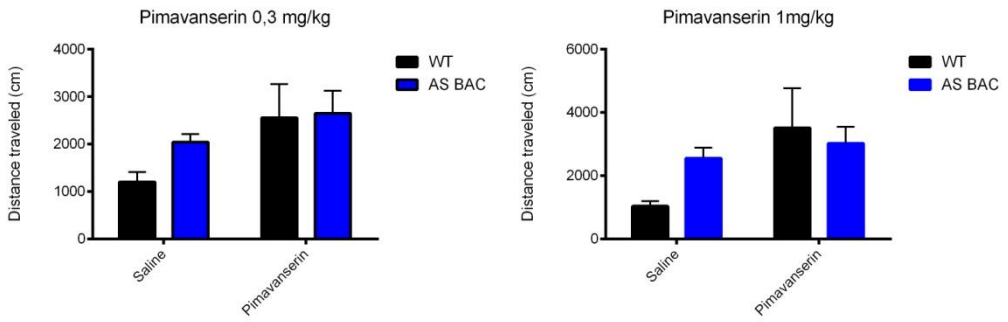


Figure 15: No conclusive results could be drawn after the administration of Pimavanserin at 0.3mg/kg and 1mg/kg

SCH-23390

We then proceeded with the intraperitoneal administration of the pharmacological compound SCH-23390 (0.1mg/kg), a selective D1 receptor antagonist and we observed a statistically significant reduction of AS BAC rats' locomotor activity while their WT littermates remained unaffected at the chosen dose (0.01mg/kg).

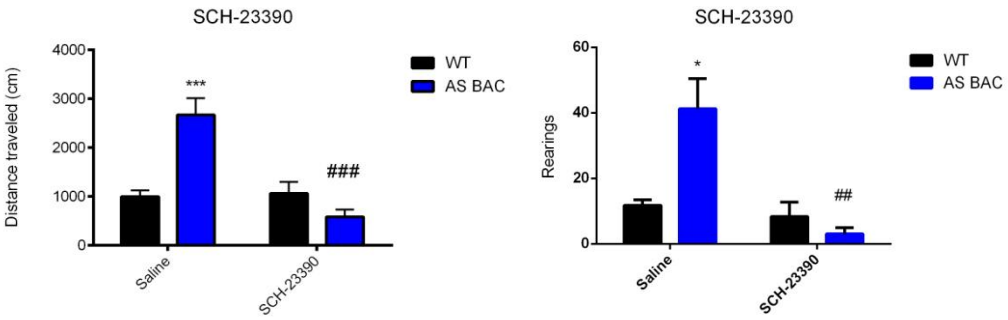


Figure 16 : Horizontal (p=0.0007)and vertical (p=0.0067)hyperactivity of AS BAC rats reversed with SCH-23390 (n=5 per group) [Genotype effect F(1,20)=5.572, p=0.0285 ; Drug effect F(1,20)=16.01, p=0.0007] [Genotype effect*(p≤0.05), ***(p≤0.001), Drug effect ##(p≤0.01), ###(p≤0.001)]

Ropinirole

Upon subcutaneous administration of Ropinirole (2.5mg/kg), both groups appeared to demonstrate increased horizontal and vertical activity, although there was no statistically significant difference treatment effect. Worthy of note is the observation that while the horizontal activity of both groups was increased under the effect of the drug, vertical activity was decreased.

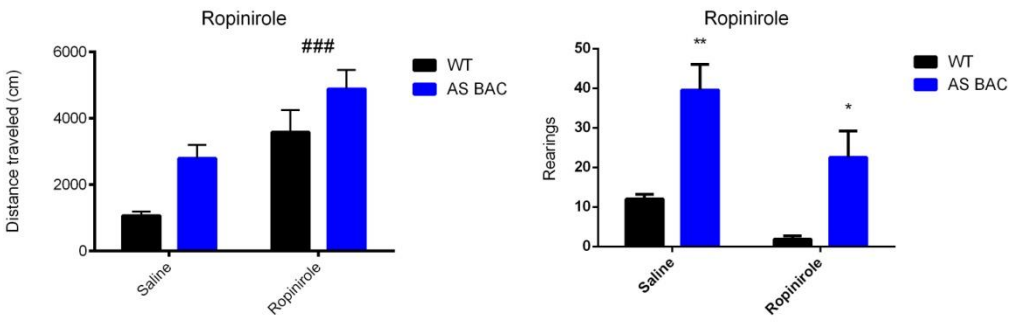


Figure 17: Horizontal (p=0.0002) activity increased and vertical (p=0.087) activity decreased with Ropinirole (n=5 per group) [Genotype effect F(1,19)=8.770, p=0.008 ; Drug effect F(1,19)=20.34, p=0.0002] [Genotype effect*(p≤0.05), ** (p≤0.01), Drug effect ### (p≤0.001)]

10.3 Biochemical analysis

Following the behavioral analysis we wanted to identify potential changes in dopaminergic signaling through biochemical analysis of proteins involved. Our data suggests that there is a non-significant trend for increased DAT expression in AS BAC rats (p=0.3222) and no change in VMAT expression in the striatum of WT and AS BAC rats. This finding agrees with the common reaction of the two groups to D-amphetamine administration and indicates that the hyperactivity observed is not related to ADHD.

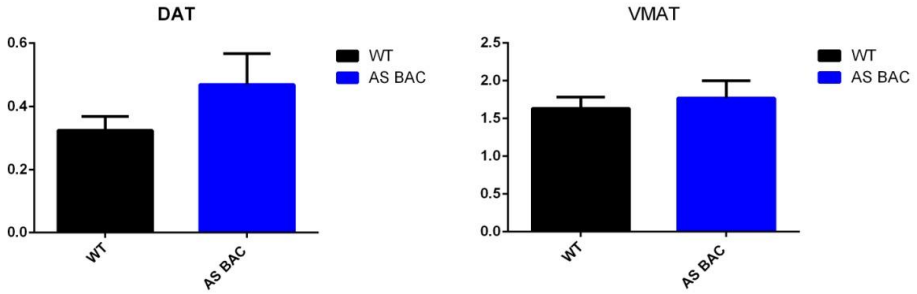


Figure 18 : Normalized WB quantification for DAT and VMAT (n=4 per group)

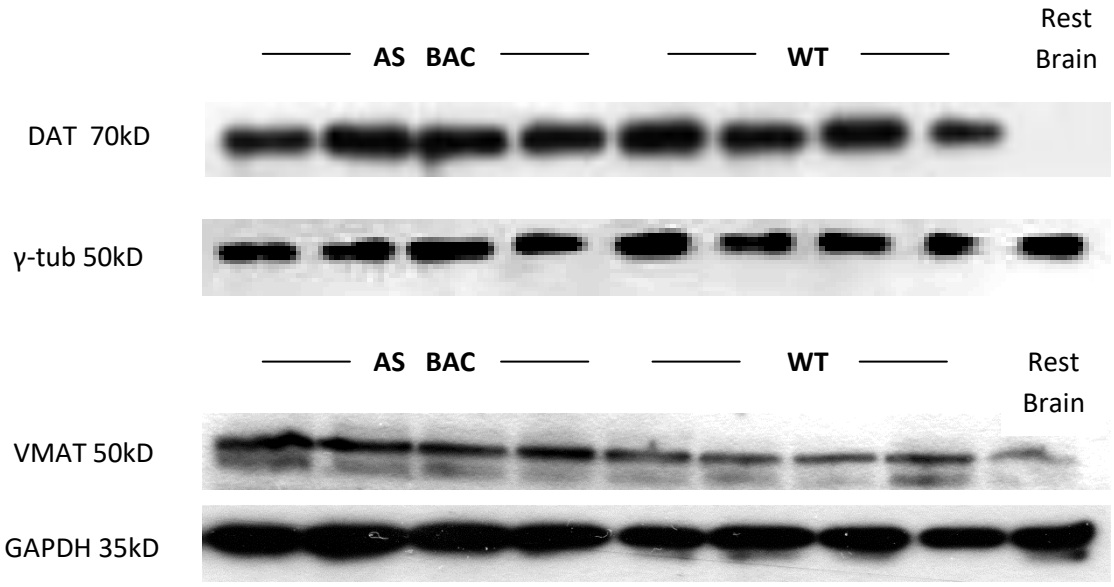


Figure 19 : Western immunoblotting analysis of striatum tissue samples of WT and AS BAC rats (n=4 per group). 20ng of protein were loaded in each well. The remaining tissues after the dissection of the areas of interest were homogenized and used as a positive control (rest brain). γ-tubulin and GAPDH were used as a loading control for the quantification respectively. (n=4 per group)

Since BDNF pathway signaling is associated with plasticity and neuronal survival, we examined the expression of mature BDNF in the striatum of 3 mo rats. Even though there appears to be a trend towards an increase of BDNF levels in AS BAC rats, the result is not statistically significant and we will have to increase our sample size with further experimentation in order to obtain conclusive results. (n=4, p=0.14)

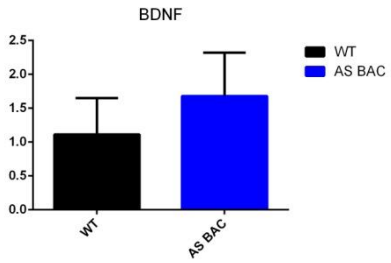


Figure 20 : Normalized WB quantification for BDNF (n=4 per group)

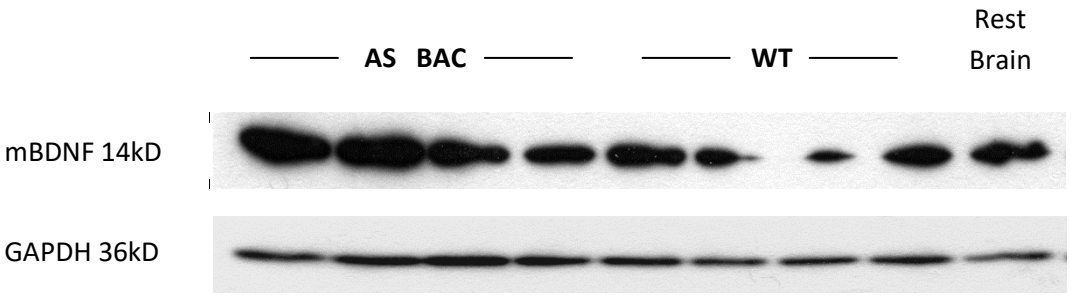


Figure 21 : Western immunoblotting analysis of striatum tissue samples of WT and AS BAC rats. For this analysis 60ng of protein were loaded in each well since with less amount of protein the mBDNF bands were undetectable. (n=4 per group)

10.4 Immunohistocemistry

Because AS BAC rats responded to the subthreshold dose of a selective D1 receptor antagonist, we proceeded with the staining of D1 receptors in the striatum. We hypothesized that the excess dopamine in the cleft would lead to an internalization of the post synaptic D1 receptors. Pharmacologically this would result in decreased receptor availability on the cell surface to occupy the D1R antagonist at very low doses and would leave the WT animals unaffected. However, there was only a trend for decreased D1R expression (p=0.19) between AS BAC and WT rats groups. (n=4 per group)

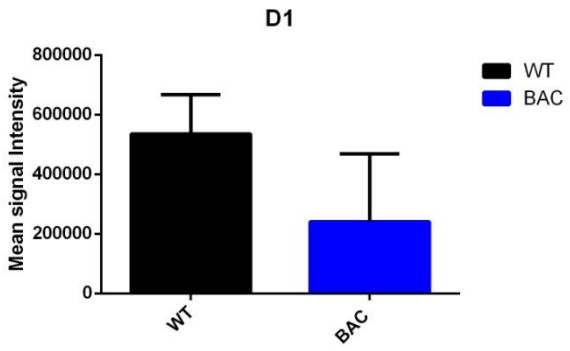


Figure 22: Immunohistochemical analysis of D1 dopaminergic receptors in the Striatum of WT and AS BAC rats. No statistically significant difference is observed between the two groups (p= 0.0812) (n=4 per group)

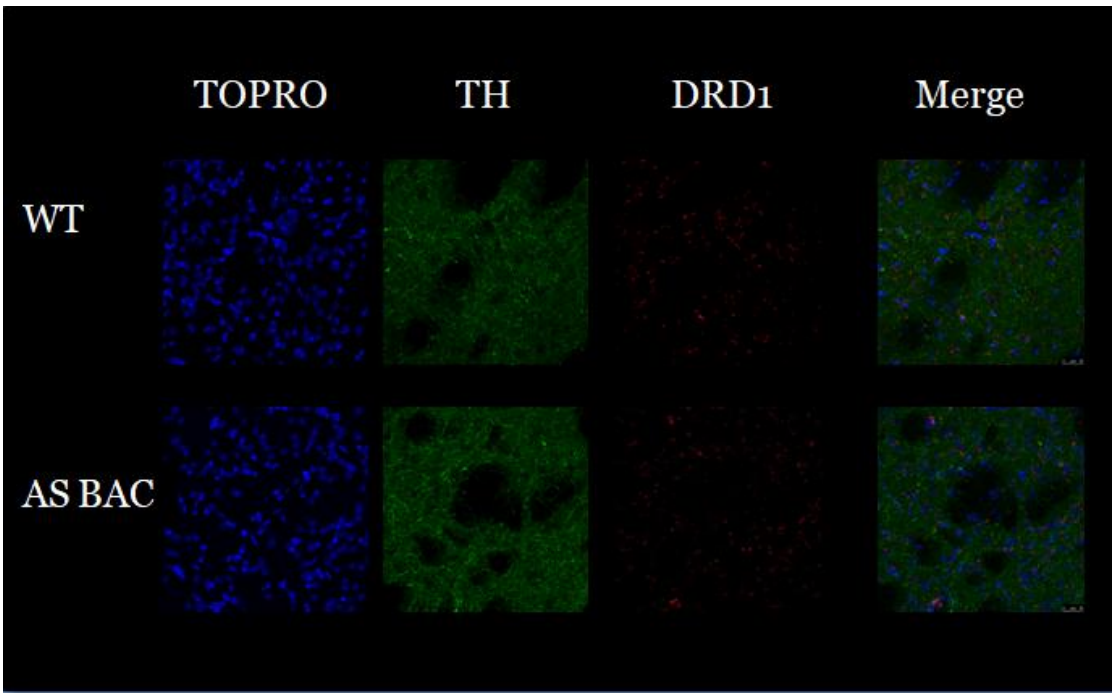


Figure 22 : Representative images from ipsilateral striatal sections of WT and AS BAC rats (n=4 per group). Cell nuclei are colored blue (TOPRO), the dopaminergic neurons (TH+) green and DRD1 receptors red.

11. Discussion

PDP is one of the most challenging non-motor symptoms of Parkinson's disease. It affects more than 30 % of PD patients, decreases their quality of life, increases caregivers' stress and overall is a marker of bad prognosis.[23] Present treatments available such as clozapine and quetiapine are not specific and have major side effects.[26] Part of the problem is that there is no specific animal model available to study this disease.

Our interest in studying of psychosis-like behavior in AS BAC rats was based on findings of hyperactivity and striatal hyperdopaminergia that persists with age. Similar behavioral hyperactivity has been observed in AS BAC mice up to 24 mo.[36] Furthermore, evaluation of these animals' phenotype showed a prepulse inhibition deficit at the age of 12 months. The observed disruption of prepulse inhibition in AS BAC rats is consistent with a psychosis-like phenotype elicited by excess α-synuclein. Notably, dopamine hyperactivation, induced by pharmacological interventions or genetic manipulation of DAT, causes PPI deficits similar to those observed in psychiatric disorders, such as schizophrenia and ADHD.[41,42,28] Therefore, we have indications of a hyperactive phenotype with PPI deficit and increased dopamine levels, so we attempted to evaluate whether this animal model could be used as a novel animal model for PDP.

Psychosis in animal models is typically measured with amphetamine-induced head twitches, hyperactivity and prepulse inhibition. We focused on the latter two tests as head twitches were not useful to us since we were examining spontaneous rather than drug-induced behavior in a genetic model.

First of all, we validated our previous findings by measuring dopamine levels in the striatum but also in the cortex and olfactory bulb of 3 mo rats and we demonstrated that indeed the striatal dopamine content was higher in AS BAC rats compared to WT rats. We also demonstrated increased striatal serotonin, a finding which supports the contemporary hypothesis of psychosis being a serotonin-dopamine imbalance rather than a sole increase of dopamine. Furthermore, we demonstrated that this increase was not due to defective metabolism and clearance of these neurotransmitters since their metabolites levels were also higher in AS BAC rats.

The literature suggests a model for ADHD which also exhibits prominent hyperactivity, DAT-KO rats. These animals, similarly to humans with ADHD, manifest a decrease of their

hyperactivity upon D-Amphetamine administration.[16] In order to address this question we administered D-amphetamine and observed that both WT and AS BAC rats increased their levels of activity, suggesting that the phenotype is not a DAT mediated dysfunction. D-amphetamine binds to DAT as well as VMAT, increasing dopamine in the synaptic cleft. Biochemical data from Western Blot show that indeed there is no difference between the amount of DAT or VMAT between the two groups supporting the behavioral results which suggest that this pathway is not implicated in the demonstrated behaviours and that AS BAC rats do not possess features of ADHD.

Furthermore, it has been suggested that DAT dysregulation causes a decrease in the transcription of the BDNF mRNA and protein levels[16], however, our results did not indicate such a decrease, and therefore, support the locomotor data that do not demonstrate a difference in D-amphetamine effects on the two animal groups.

Next, we wanted to demonstrate that the hyperactivity induced by α-synuclein overexpression could be linked to a psychosis-like phenotype and thus should be reversed by administration of antipsychotic drugs. This was indeed the case with both typical (haloperidol) and atypical (clozapine) medications showing reversal of the hyperactivity, since AS BAC horizontal and vertical activity levels decreased to the levels of their WT littermates.

This was not the case when we administered pimavanserin since the results were inconclusive with variance between the values obtained. However, this does not go against our hypothesis since pimavanserin is quite an ambiguous drug. It has been approved by the FDA for PDP despite the fact it has only been proven to be significantly different than the placebo in only one out of 4 clinical trials and has failed to show anything but a trend for improvement in patients with Alzheimer's Disease Psychosis or Schizophrenia.[22]

In order to examine potential differences in dopaminergic receptor function, we administered a selective D1 antagonist (SCH-23390) and a selective D2 agonist (ropinirole). AS BAC rats responded to the SCH-23390 administration with decreased locomotion while WT animals remained unaffected. Our results are broadly consistent with those of Unger et al. (2006), who observed hyperactivity in mutant A53T AS transgenic mice and linked it to Dopamine D1R-dependent hyperactivation. Both AS BAC and WT groups had similar responses to ropinirole however while the horizontal activity of both groups was increased the vertical activity was decreased.

What we assumed was that the excess dopamine present in the synapses of AS BAC rats, observed by the early age of 3 mo would possibly affect the levels of various receptorsin the striatum. We speculated that the high concentrations of dopamine in the synaptic cleft would cause the post synaptic dopamine receptors to be internalized, so that the post synaptic cell would not be overexcitated by continuous stimulation.

Since the transgenic animals responded differently to selective D1 antagonism we decided to delve deeper and measure D1R expression. We observed a non significant decrease in the levels of D1 receptors. Taking into consideration that, 1) D1 receptor antagonists can block the action of D2 receptor agonists[19], 2) we did not observe a reduction of D1 receptors that would justify the difference in the locomotor phenotype upon SCH-23390 administration and 3) haloperidol, a D2 antagonist had a similar effect, we could speculate the involvement of D2 receptors in this phenomenon. To address this question, our future goals include studying the D2 receptor expression pattern. Furthermore we should take into consideration that the observed responses could not be a receptor-dependent effect, since there are effects in the locomotion phenotype both with selective D1 and D2 antagonists, but rather a consequence of the overproduction of dopamine. Therefore, we plan to measure extracellular dopamine levels with in vivo microdialysis to verify whether the increased stored DA in the striatum is associated with increased release as well; indirect evidence for this is provided by the increased levels of DA metabolites, which suggest that there is increased turnover of DA. Furthermore, in order to validate that this phenotype is indeed attributed to the overexpression of human α-synuclein in the nigrostriatal and mesolimbic dopaminergic pathways, we plan to downregulate this protein in the related areas with the use of miRNA and monitor the locomotor effect of the manipulation.

Our data on AS BAC rats demonstrate that their hypersensitivity to the locomotor stimulating effects of d-amphetamine suggests that the hyperactivity displayed by these rats is inconsistent with that of Attention Deficit Hyperactivity Disorder (as manifested in e.g. DAT KO rats[16], but rather, that these rats exhibit behaviors associated with psychosis. Furthermore, the ability of clozapine, used to treat PDP and haloperidol to reduce the hyperactive phenotype, is also consistent with a psychosis-like profile. We hypothesize that this is related to increased striatal dopamine levels, which lead to increased excitation of the mesolimbic pathway.

There is clinical evidence showing that even in the premotor phase of PD such a hyperdopaminergic state may occur, independent of dopaminomimetic drugs, and be

responsible for psychotic-like behavior.[25] Most strikingly, subjects with a genetic risk for PD harboring the 22q11.2 deletion show elevated striatal 11C-DTBZ binding, indicating a hyperdopaminergic condition prior to dopaminergic neurodegeneration. Such subjects also frequently manifest psychosis, which may be linked to such a hyperdopaminergic state. [43]

In conclusion, our results indicate that AS BAC transgenic rats provide a valuable translational model to examine the biochemical and neurochemical basis of the psychotic-like behavior observed. Our data support a connection between aberrant human alpha-synuclein expression and a psychosis-like phenotype in AS BAC rats. Fascinatingly this in vivo data may have analogies to PD, where recent findings suggest that a premotor hyperdopaminergic state may occur.[43]

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