5α-reductase isoenzymes mediate stress-exacerbated Tourette-like responses in animal models

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Abstract

Tourette syndrome is a neurodevelopmental disorder characterized by purposeless, uncontrollable muscle movements known as tics. These tics are extremely sensitive to environmental factors, especially psychosocial stress. Stress has been demonstrated to increase neurosteroids in animal models, but the relationship of these neurosteroids to Tourette syndrome is unknown. The neurosteroid allopregnanolone is a key regulator of the stress cascade but has also been demonstrated to influence dopamine-mediated behaviors in animal models. Clinical results have shown that inhibiting the synthesis of allopregnanolone and other 3α, 5α steroids with the 5α-reductase inhibitor finasteride reduces tics in adult male patients with Tourette syndrome; however, the mechanism of action is largely unidentified.

In this dissertation, the mechanism by which stress exacerbates Tourette syndrome symptoms and finasteride attenuates these behaviors was examined. We found that in various animal models of Tourette syndrome, stress exacerbated tic-like behaviors and deficits in prepulse inhibition (PPI), an operational measure of sensorimotor gating aimed at filtering salient information from the environment; this process is also disrupted in Tourette syndrome patients. These stress-induced tic-like behaviors and PPI deficits were ablated by finasteride treatment, which indicated a role for 3α, 5α steroids. We found that one of these steroids, allopregnanolone exacerbated tic-like behaviors and induced PPI deficits in our animal models. In addition, we determined that allopregnanolone is mediating these effects through several possible receptors; specifically we found evidence suggesting that the pregnane xenobiotic receptor and the purinergic P2X4 receptor are involved in these processes. Finally, we demonstrated that the isoenzymes 5α-reductase type 1 and type 2 exert different effects in regulating Tourette syndrome-like symptoms, and specifically that 5α-reductase type 1 may be the more beneficial and safe target for inhibition over 5α-reductase type 2.

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1. Clinical features of Tourette syndrome

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Introduction

Gilles de la Tourette syndrome, better known as Tourette syndrome (TS) or simply Tourette's, was first described in 1825 by Jean-Marc Itard [1] and further defined by Georges Edouard Albert Brutus Gilles de la Tourette in 1885 [2, 3]. However, for almost the next century, TS was not viewed as a neuropsychiatric disorder but an issue of "weak self-control and lack of will-power" [4]. Opinions changed following reports of successful treatment with neuroleptics and further clinical descriptions published in the 1980's that highlighted the complexity of TS beyond the physical manifestations and identified the high prevalence of comorbid disorders, such as ADHD and OCD [5-7]. The results of these clinical studies and others conducted over the following decades will be further discussed in the upcoming sections.

Clinical characteristics

Tourette syndrome (TS) is a male-predominant, neurodevelopmental disorder defined by repetitive, semi-involuntary motor (multiple) and phonic (at least one) tics for more than one year (DSM) that effects approximately 0.5-1% of the pediatric population [8]. Motor tics consist of sudden, involuntary, nonrhythmic movements that most commonly occur in the head, neck, face and mouth muscles but can also be observed in the truck and limbs [9]. Phonic tics are the result

of rapid air movements through the upper respiratory tract, which can produce grunts, throat clearing or other nonsense phonic sounds, as well as, complete words or phrases [10]. These tics can be further classified as simple tics, involving one muscle group, or complex tics, which involve multiple muscle groups. Simple tics are the first to manifest in the disorder and include eye blinks, eye rolling, head jerking, and grunting or throat clearing. Alternatively, complex tics are more coordinated and akin to goal-directed movements [9]. For example, echophenomena (vocal or motor reverberations) and coprophenomena (obscene or offensive vocal or motor behaviors) are both examples of complex tics [11, 12]. As demonstrated by the examples given above, tics are indistinguishable from purposeful movements; however, they are set-apart due to their repetitive nature and misplacement in context and time.

TS symptoms generally manifest in patients around the age of 6-7 years, but diagnostic criteria require that symptoms become apparent before the age of 18. The first tics are generally simple motor tics followed by the manifestation of phonic tics [13]. Symptom severity can range from very mild cases that do not require pharmaceutical intervention and can be handled with behavioral therapy alone, to more severe cases that dramatically reduce the well-being and future outcomes for the patient. For most patients, the complexity and frequency of tic manifestations generally increase up to puberty (11-12 years of age), followed by a gradual remission as the patient ages [14]. However, 30-40% of TS patients retain their symptoms through adulthood and some studies suggest that those patients that claim a remission of symptoms continue to display tics without being aware of these behaviors [15, 16]. Tics not only fluctuate temporally but also due to environmental contexts. In particular, high mental and physical stress have been reported to increase tic severity and frequency [17].

Comorbid disorders are present in the vast majority of TS patients. The most common of these include OCD and ADHD, which further negatively impact the quality of life of these patients [13, 18, 19]. Despite the challenges posed by the large number of comorbid disorders, the core behavioral features of TS include tics, impulsivity, aggressive behaviors (self-injurious behaviors and coprophenomena) and compulsive/repetitive behaviors [13, 20-22].

Premonitory sensory phenomena

Patients have reported that tics are preceded by a premonitory urge, an intense feeling of generalized or focal discomfort that is relieved by the execution of the tic [23]. This is a hallmark characteristic of TS, and the discomfort and tension associated with the premonitory urge has been described by some to be the most problematic aspect of TS [24]. While most TS patients can suppress the execution of tics for short amounts of time, the buildup of tension associated with the premonitory urge results in an increased sense of distress and a greater urge to tic [23]. Often these urges manifest either as localized tension or itch that is generally limited to the area the tic will manifest or as a generalized feeling of unease. Despite being a core feature of TS, and according to patients, the very root of the disorder, premonitory urges are not a diagnostic criteria for TS [25].

It has been reported that premonitory urges increase in intensity up to the execution of the tic but quickly diminish, within 10 seconds, following tic execution [23, 26, 27]. Due to the discomfort associated with the premonitory urge, these phenomena are reported to be the main negative reinforcement of tics [28, 29]. Therefore, uncoupling of tics and premonitory urges may provide a useful therapeutic avenue. Tic suppression has been shown to lead to an increase in the intensity of the urge but decreases the correlation between tics and the intensity of the urge [26]. However, other conflicting studies have indicated no relationship between the intensity of the urge

and tic suppression [30, 31]. Interestingly, an increased rate of premonitory urges in patients with co-morbid OCD has been reported [32-34] and a positive correlation between the rate of premonitory urges and obsessive-compulsive symptoms has been described as well [35]. Anxiety has also been shown to correlate positively with premonitory urge reports [36]. Furthermore, the premonitory urges have a negative effect on daily life as demonstrated by a negative correlation between premonitory urges and the Global Assessment of Functioning (GAF) scale [35]. These findings further suggest that the premonitory urge is a central component of TS and therapies targeting this phenomena could assist in alleviating the problems associated with common co-morbid disorders and enhance the quality of life for these patients. Further discussion on therapeutic options will be addressed in later sections.

In addition to the premonitory urge, patients also exhibit other sensory alterations. The best defined of these is the reported deficit in sensorimotor gating as measured by prepulse inhibition of the startle reflex [37, 38]. This paradigm measures the capability of the brain to process the salience of external stimuli through measuring the natural ability of a prepulse to attenuate the startle reflex elicited from a harsh stimuli, such as sound. The deficit observed in TS patients points to alterations in informational processing, which may also contribute to the premonitory urge and resulting tic [39]; however, there have been no correlations identified between premonitory urges or tics and PPI deficits.

Other sensory phenomena are also altered in TS patients. Belluscio et al [40] reported that 80% of TS patients described enhanced sensitivity to external stimuli (such as sound, light, smell and touch) versus 35% of control subjects which the authors determined was due to altered central processing since the detection threshold for olfactory and tactile stimuli was similar in TS patients and controls. TS patients also seem to have greater interoceptive awareness. Eddy et al. [41]

described that TS patients report a heighted awareness of their internal state in comparison to controls, which has also been correlated to the number of tics [42]. Furthermore, those patients with greater internal awareness perceive more urges for the same number of tics [43]. Given the description of these premonitory urges as feelings of discomfort, it follows that TS patients score higher on the University of Sao Paulo Sensory Phenomena Scale (USP-SPS), a test that assesses abnormal sensory experiences, and that these scores correlated positively with the intensity of the premonitory urges reported by the patients [44].

Neurobiology

The pathological basis of TS remains partially unclear due to the high rate of comorbid disorders, the effects of treatments, and small clinical sample sizes. However, strong evidence implicates functional and/or morphological impairments in cortico-striato-thalamo-cortical (CSTC) pathways [45], which connect subcortical structures to the frontal cortex. Of these circuits, three are heavily implicated in the pathology of TS. These include the circuits from the premotor cortex to the putamen (the habitual behavioral circuit), the ventral medial prefrontal cortex to the caudate nucleus (the goal directed circuit), and inputs to the ventral striatum from the hippocampus, amygdala, prefrontal cortex and anterior cingulate gyrus (the emotion-related limbic circuit). These circuits are implicated by the behavioral data presented above and morphological data. For example, adults and children with tics have a significant, although slight, decrease in caudate and putamen volume [46]. In further support, it has been demonstrated in post mortem tissue that there is a deficit in interneurons within these same regions; these interneurons include the cholinergic tonically active neurons and the GABAergic interneurons expressing either parvalbumin or neuronal nitric oxide synthase (nNOS) [47-49]. Functional data also points toward

dysfunction in these circuits. Specifically, studies in this field have found activity in the prefrontal cortex and caudate during tic suppression [50].

Further studies on the neurobiology of TS are limited by the nature of TS: 1) small sample sizes; 2) the numerous comorbid disorders, which make it difficult to parse out structures contributing to TS; 3) the patient's treatment for TS or comorbid disorders, which can further confound clinical results; 4) subject movement during tic emission, which further hinders functional imaging. It is also feasible that discrepancies between studies may arise from a heterogeneous etiology. In other words, different neurobiological defects that ultimately lead to general imbalances of the inhibitory and excitatory inputs within the CSTC and produce multiple disorders that all physically manifest as TS. Regardless of the possible heterogeneous neurobiological underpinnings, TS, as a whole, is the result of insufficient inhibitory tone from the striatal interneurons, as suggested by the work of Dr. Vaccarinno [47-49] and/or excess striatal activation from the cortex or other brain regions [51, 52]. The imbalance between inhibitory and excitatory inputs may lead to disproportionate striatal stimulation and the activation of ectopic foci, as a result of inadequacy of center-surround interactions within this brain region [53]. The formation of ectopic foci then manifests as the premonitory urge and subsequent tic.

Dopamine in TS

Several neurotransmitters have been implicated in TS, including dopamine, serotonin, norepinephrine, acetylcholine, glutamate and GABA [45]. However, the most studied of these in relation to TS and the primary neurotransmitter studied in this dissertation has been the involvement of dopaminergic dysfunctions. Dopamine is directly involved in the CSTC circuits described above. The frontal cortex and ventral striatum receive dopaminergic inputs from the ventral tegmental area. In addition, outputs from the substantia nigra par compacta connect within

the striatum to glutamatergic cortical projections and on direct and indirect GABAergic striatal projections. The direct and indirect projections are populated by excitatory dopamine D1 receptors, and inhibitory dopamine D2 receptors respectively. Dysfunctions within the dopaminergic system that contribute to TS have been posited to involve presynaptic, intrasynaptic, and postsynaptic alterations [54]. For example, developmental hypofunction of dopaminergic neurons would result in hyperinnervation and an increased number of presynaptic dopamine transporters. Postsynaptic alterations, on the other hand, may include an increased number of striatal and cortical dopamine receptors. In addition, alterations in the phasic release of dopamine could lead to rapid variations in synaptic dopamine and contribute to the ectopic foci activation and induce tics.

These hypotheses and the overall contribution of dopamine to TS pathogenesis are supported by several key clinical observations. First, D1-like and D2-like receptor family antagonists are commonly used in the treatment of TS [55-58]. In addition, high doses of dopamine-elevating psychostimulants have been demonstrated to increase tics in both patients [59-61] and animal models [62-65]. The dysregulation in the dopaminergic system may be reflected in neuroimaging and post-mortem studies that have shown excessive activity and/or innervation of the cortex and basal ganglia of TS patients [66-70]. In addition, perhaps some of the strongest evidence for the prominent role of dopamine comes from positron emission tomography (PET) studies. While there are some discrepancies, these studies indicate an increased tonic striatal dopamine level in adult TS patients [71, 72]. Importantly, this elevation in dopamine has been seen in patients with TS, but not those with OCD without comorbid TS [59], indicating a specific role of dopamine in TS compared to a common comorbid disorder. In addition, these PET studies suggest that tics may be underpinned by rapid variations in synaptic DA content, leading to a prominent activation of D1 receptors in the striatum. Since the D1 receptors regulate the direct pathway projections to the

globus pallidus and substantia nigra pars reticulate, this may be directly related to the stimulation of ectopic foci. TS patients display alterations in several key behavioral domains regarding the orchestration of critical behavioral functions, such as habit formation, incentive motivation, configuration of salience maps and sensorimotor gating, indicating a key role for dopamine in the ventral striatum as well [37, 38, 73-76].

Etiology of TS

Genetic contributions. Despite several genome-wide association studies aimed at identifying candidate genes for TS, no single genetic mutation has been identified to be the causal factor of the disorder. Nevertheless, twin and family studies have revealed that TS is a highly heritable disorder with a population-based heritability of approximately 0.77, where 1 is equivalent to 100% heritability [77-79]. The findings of the genome-wide association studies and rare, familial forms of TS have identified several possible risk genes. Some of the most noteworthy genes identified include SLITRK1, which is involved in axonal targeting and neuronal differentiations [80-84], and HDC, the gene which converts histadine to histamine [85, 86]. The genes that have been identified are commonly involved in either dopamine regulation, for example histamine regulates striatal dopamine, or in the development of CSTC neuronal pathways. However, these mutations are not widespread throughout the clinical population and animal studies aimed at determining the contribution of these genetic mutations capture aspects of TS but not the complete disorder. Further discussion of these mutations and the preclinical research aimed at determining the specific contributions of these genes will be discussed in the following chapters. Still, these findings indicate that TS is highly polygenic and likely the result of gene x environment x sex interactions.

Environmental factors. Several environmental factors have been identified to contribute to TS pathogenesis. In the pre- and perinatal stages studies have reported that maternal contributions, including psychosocial stress, severe nausea and vomiting in the first trimester, smoking and consumption of medications, have been associated with a higher occurrence of TS [87-91]. Exposure of the child to infections, in particular Group A β-hemolytic streptococcus, has also been identified as a possible risk factor for TS [92-96]. These studies have further led to a classification of a new subgroup of TS patients, PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection) following the finding of antibodies for Group A β-hemolytic streptococci in a subgroup of children with acute-onset tic disorders [96-100]. However, further research has challenged this classification and so PANDAS is not currently recognized as a distinct entity be either the DSM or the ICD.

When combined with a genetic predisposition, such as those described above, pre- and perinatal insults may trigger the priming of microglia. Further environmental hits could activate these microglia, influence synaptic plasticity at critical points in development (such as the age of TS symptom onset), and enhance peripheral immune and inflammatory responses [101, 102]. For example, the influence of stress on tic fluctuations becomes three-times stronger with the co-occurrence of an infection [93].

In addition to these highlighted risk factors, TS patients demonstrate dysregulated immune-mediated mechanisms later in life. Post-mortem striatal transcriptome analyses has demonstrated that transcripts of genes involved in the activity of microglia are upregulated [49]. Furthermore, in vivo studies in children with TS have revealed activated microglia in the caudate nucleus [103]. Within the periphery, studies have revealed upregulated genes that encode for proteins involved in pathogen recognition and cell-mediated innate and adaptive responses within the peripheral

lymphoid and myeloid immune cells of young TS patients [104]. A chronic hyperactive innate and adaptive immune response has also been demonstrated through clinical studies that have described dysgammaglobulinaemia, decreased numbers of regulatory T cells and increased antibody response to pathogens in TS patients [105-107]. While not the focus of this dissertation, further research on the role of inflammation in TS pathogenesis will be critical to understanding this disorder.

Sex. Several key observations of TS patients have indicated a strong role for sex steroids, and specifically androgens in the pathogenesis of TS. This was first published in the late 1980s in articles describing alterations in the secretions of luteinizing hormone, the main regulator of gonadal androgen synthesis [108, 109]. Furthermore, clinical observations revealed that anabolic androgens exacerbated tics in TS patients [110]. In addition to these studies, other characteristics of this disorder point to a role for androgens. First, as mentioned earlier, there is a prominent sex difference in the presentation of this disorder. Males are more prone to being diagnosed with TS at a ratio of approximately 4:1 [111], which points toward a role of both sex specific steroids and sex dimorphic brain structures. In addition, the progression of this disorder further implicates a role for sex steroids. Symptoms often begin to manifest at the age of 6-7, which coincides with adrenarche, the stage in sexual maturation where the zona reticularis of the adrenal gland expands and begins to produce dihydroepiandrosterone (DHEA). Males with TS tend to display an increase in the frequency and severity of tics up to puberty and then often show a remittance of symptoms following puberty into adulthood, which further points to a key role in sex steroids and sex dimorphic brain maturation. However, females with TS do not display this remission of symptoms as young adults, but often continue to display severe tics as they age [112]. Finally, behavioral

characteristics of TS-affected children demonstrate that tic severity correlates with their preference for masculine play [113].

Current treatment options

The appropriate treatment strategy for patients with TS should consider the severity of tics and the effect on patient's daily functioning and quality of life. It is also critical to consider any comorbid disorders as these may be more problematic and disrupting than the core TS symptoms. In most cases the first course of action is to provide psychoeducation to parents, teachers and peers; this may be sufficient to aid patients with mild to moderate tics that are not harming social functioning. Should further intervention be required the first line of treatment is currently considered to be behavioral interventions [114-117] which include habit reversal therapy (HRT), comprehensive behavioral intervention for tics (CBIT) and exposure and response prevention (ERP). HRT involves three components: 1) awareness training to identify the premonitory urge and tic onset 2) competing response training where the patient is instructed to do an action that is at odds with the tic movement and 3) social support to give the patient praise and reminders [118]. CBIT takes this therapy further by the addition of function-based interventions, relaxation training, psychoeducation, and rewards for treatment compliance [29]. While HRT and CBIT address one tic at a time, ERP focuses on all tics at once and has shown promising results in pilot studies. ERP instructs patients to actively suppress all tics during therapeutic sessions while recognizing and fully experiencing the urge to tic [119].

The second line of treatment for TS patients are pharmacological interventions. These treatments are used only for patients that do not respond to behavioral therapy, do not have access to these resources, are not of an appropriate age, or in the cases where pharmacological treatment is the family's or patient's preference. The classes of pharmacological intervention fall into 3 broad

categories: dopamine receptor antagonists, vesicular monoamine transporter-2 (VMAT2) inhibitors, and non-dopaminergic agents. Dopaminergic blockade was one of the first demonstrated effective treatments [120-122]. The typical antipsychotics pimozide, haloperidol, and fluphenazine, and the atypical antipsychotics aripiprazole, risperidone, olanzapine, ziprasidone, and quetiapine have all been demonstrated to be effective at treating TS [123, 124]. However, only haloperidol, pimozide and aripiprazole have been approved by the FDA for this usage. In addition, the D1 antagonist, ecopipam, has shown promising results in initial trials [56] and the benzamides, tiapride and sulpiride, are first-line agents for TS treatment in Europe [125]. The next class of TS treatments are vesicular monoamine transporter-2 (VMAT2) inhibitors, which inhibit the transport of dopamine, norepinephrine, and serotonin from synaptic vesicles to the synapse via VMAT2. The VMAT2 inhibitors that are currently being studied for use in TS are benazine, deutetrabenazine, and valbenazine; these are all currently FDA approved to treat other disorders such as Huntington disease and tardive dyskinesia [124]. Finally, non-dopaminergic agents include the α-2 adrenergic receptor antagonists clonidine and guanfacine [126-128], and the GABAergic agents baclofen and topiramate [129, 130]. All of these therapies, however, have inconsistent efficacies, and often lead to serious side effects such as sedation, dizziness, weight gain/loss, and extrapyramidal symptoms that can reduce the quality of life and therapeutic compliance of TS patients [131, 132]. In part due to these challenges, alterative agents have been studied as well. These include local injections of botulinum toxin [133, 134], cannabinoids [135] and Chinese herbal medicines [136], which have shown promising evidence but further research on the mechanism of action and long-term safety is warranted on these therapeutic options.

2. Animal models

Sections of this chapter have been adapted from:

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Introduction

Animal models provide a powerful tool to delve deeper into the biological substrates of neuropsychiatric disorders and to test hypotheses in a controlled experimental setting. In the case of neuropsychiatric disorders such as TS that exhibit a high degree of complexity, mammalian species, and, in particular, rodents, are often the model of choice given their acceptable degree of neurobiological similarities with humans and cost-effectiveness. This review will introduce behaviors of relevance to TS and discuss the benefits and limitations of studying TS in rodent models.

Animal models of tic disorders: validity criteria and endophenotypes

The validation of animal models is based on three major criteria [137]:

1. Face validity: The degree of similarity between the behavioral performance of the animal models and the signs and symptoms in tic disorders.
2. Construct validity: The evaluation of the congruence between the etiological and pathophysiological processes in tic disorders and the neurobiological basis of the behavioral manifestations in the animal models.
3. Predictive validity: The responsiveness of an animal model to treatments validated for tic disorders.

It is important to analyze all three criteria of validity when drawing conclusions from animal models; however, this can be extremely challenging given the different behavioral repertoire and

cognitive abilities between rodents and humans. For instance, in the clinical population, tics are akin to purposeful movements that manifest as repetitive or out of context behaviors. However, these behaviors may be quite different in rodents. For example, over grooming has often been compared to tic-like behaviors since it is a purposeful movement that has manifested into a highly repetitive behavior; however, this behavior can result from multiple heterogenous alterations in the brain that may or may not be related to TS. Therefore, it is important not to over anthropomorphize animal behaviors and to apply the other criteria to the models as well. This is highlighted in a report by Swerdlow and Sutherland [138], which describes several instances where animal models displayed spontaneous motor jerks, akin to tic-like behaviors, but the underlying neurobiological causes were not related to TS. In addition, tic-like behaviors can be induced pharmacologically; however, Proietti Onori et al [139] has demonstrated that these behaviors vary across different murine strains, indicating a need for further caution when drawing conclusions from these studies and when choosing appropriate models. These examples demonstrate that while face validity is an important consideration when evaluating an animal model, it by no means can stand alone. Furthermore, premonitory urges and other internal experiences in animals are impossible to ascertain, making face validity even more challenging in the context of TS. Predictive validity can be an effective complement to face validity; however, a percentage of TS patients do not respond to any available treatment so excessive reliance on this parameter should be avoided as well.

Construct validity is a challenging criterion to verify in an animal model of tic disorders due to the limited knowledge of the pathophysiological bases of these conditions. This is compounded by the diagnostic classification of tic disorders, which are based solely on symptomatic parameters, not quantitative, measurable indices. Tic-disorders are further classified

into subtypes, of which TS is only one, by the severity and pervasiveness of tics, as well as the ontogeny of symptoms, but not on their neurobiological bases [25]. This system is likely to result in potentially heterogeneous conditions that share similar symptomatic aspects but differ in pathophysiological mechanisms being mixed in the same category. This is an issue for several reasons. One, these patients are likely to respond optimally to different therapies. Two, capturing the disorders with an appropriate animal model is further compounded when multiple conditions may be clinically categorized as one.

To overcome these issues, researchers have begun to dissect complex neuropsychiatric conditions down to more elementary "building blocks". Rather than attempt to capture the entire disorder in a single animal model, researchers have focused on intermediate phenotypes [140], defined as measurable indices that reflect a more elementary set of neuroanatomic, functional or psychological deficits. One such group of intermediate phenotypes are known as endophenotypes, which are heritable features corresponding to elements of vulnerability to a given disorder [141]. Endophenotypes encompass behavioral, neuroanatomical, biochemical, neurophysiological, and cognitive traits related to specific genetic factors [142-144]. While endophenotypes are not inherently pathological, they may facilitate the development of the disorder in the presence of other critical abnormalities or risk factors. As such, the study of endophenotypes and other such intermediate phenotypes can greatly enhance and further the field of TS.

Neurobehavioral phenotypes relevant to tic disorders

Stereotyped behaviors. Stereotyped behaviors are considered motor and behavioral sequences that are repeated purposelessly [145]. Stereotypies are considered like tics in that they are repetitive, habit-forming motor patterns, which typically mimic purposeful behaviors. Examples of these behaviors include oro-facial and head-bobbing stereotypies. These can be

induced by several pharmacological agents that activate neurotransmitter systems of relevance to TS, including agonists for dopamine. More specifically, dopamine agonists injected directly into the striatum induce stereotyped behaviors in rodents, with a mechanism that involves both D1-like and D2-like receptors, which captures construct validity, as well as, face validity. Furthermore, predicative validity is confirmed by the ability of antipsychotic agents to fully suppress these manifestations [146-148].

However, it should be noted that stereotyped behaviors likely encompass a broader set of phenomena than tics in human. The DSM-V has defined a separation between tics and stereotypies in humans [149]. Stereotypies are defined as more severe and pervasive than tics, and are typically associated with intellectual disabilities. In addition, stereotypies are characterized by greater rhythmicity, fewer temporal fluctuations, and no relation to premonitory antecedents. Therefore, while stereotypies may be related to tics they cannot be used alone to study TS in animal models.

Other behaviors that capture tic-like movements. To narrow the focus to movements that more closely replicate TS symptoms other tic-like behaviors can also be observed. These include jerks of the head, limbs or torso as is observed in several pharmacological models of TS and one genetic model (to be discussed below). However, another homonym of tics in patients is found in the eye blink response of preclinical models. Tics that include exaggerated eye blinks or other tics of the eyes (such as eye rolling) are usually the first and most common tic observed in TS patients [150, 151]. Furthermore, it has been documented that TS patients display increased eye blink rates over age matched controls [152-154]. These findings are likely a reflection of the hyperdopaminergic state underlying TS, as spontaneous eye blinks are posited to reflect central dopaminergic activity [155, 156]. In preclinical animal models it has been found that dopamine agonists, and specifically D1 receptor agonists, increase eye blink rates in rats and monkeys. These

responses are sensitive to typical TS medications indicating that high face and predictive validity for this model [157-161]. There is also demonstrated construct validity because the basal ganglia structures regulate the eye blink pathways in the brain, specifically the spinal trigeminal complex [156, 162-169].

Prepulse inhibition (PPI) of the startle reflex. PPI is the reduction of the startle reflex elicited by a strong sensory stimulus that occurs when the stimulus is preceded by a weaker signal [170-172]. This parameter is widely considered to be a highly dependable measure of sensorimotor gating, the cognitive function that enables the formation of salience maps by filtering out non-relevant information. Deficits in PPI indicate that these cognitive functions are impaired. Several studies have documented PPI deficits in TS patients [37, 38, 173, 174] and in OCD [175, 176] but have not found correlations between the severity of tics and obsessive-compulsive manifestations and PPI impairments [38, 173, 176]. Despite this limitation, PPI deficits are still considered a key intermediate phenotype for TS, although it should be noted that PPI deficits are found in several other neuropsychiatric disorders. PPI and sensorimotor gating are posited to reflect sensory alterations, which may underpin the enhanced sensory feedback and somatic sensitivity in TS [17, 24, 177, 178]. PPI is also a highly translational paradigm of study and captures all three aspects of animal model validation. Face and construct validity are captured through the very nature of sensorimotor gating. PPI is highly conserved though out the animal kingdom in both behavioral presentation and neurobiological mechanisms, and can be studied in species from fish to humans [171, 179-182]. In relation to construct validity, sensorimotor gating processes are largely contributed to by dopamine systems in the CSTC, which highly overlap with brain regions that are hypothesized to be involved in TS pathophysiology. Zeberdast et al [174] demonstrated that PPI deficits in TS patients are associated with altered caudate activation patterns. In support of high

face and construct validity, PPI deficits can be induced by pharmacological agents that stimulate dopamine receptors, as well as, by lesions in the CSTC loops in rodents [183-185]. Furthermore, these deficits in rodents can be ablated by pretreatment with TS medications [183]. However, it should also be noted that the efficacy of antipsychotic agents in reversing PPI alterations in clinical patients has not be validated [186-190].

Relevant animal models of tic disorders

There have been several approaches to studying TS in animal models including models based on clinical observations, which include genetic mutations and observed neurobiology; models of environmental risk factors, such as early infections; and models addressing the abnormal dopamine system, which are approached through both genetic and pharmacological methods. While none of these models capture the complete disorder they provide important insights into the syndrome. A few of these models that are of relevance to the dissertation will be discussed below.

Animal models of based on genetic screenings. As highlighted in the introduction, genetic mutations have been identified in subpopulations of TS patients; however, these mutations are not widespread and tend to only account for the predisposition found in certain families. Those with the most extensive pairing of preclinical research include the contactin-associated protein-like 2 (CNTNAP2), the SLIT and NTRK-like 1 (SLITRK1), and the gene encoding for L-Histidine decarboxylase (HDC).

Rare, familial forms of TS highlight the importance of genetic influences in TS and have led to the identification of several genes of interest to TS. For instance, CNTNAP2 has been identified in one such family [191] and plays a key role in the cell-adhesion pathways and cortical development [192-194]. While the CNTNAP2 mutant mice did not display high face validity, they did display aspects of construct and predictive validity. Most of the behaviors more closely

resembled that of autism mouse models than TS models; however, they did exhibit repetitive behaviors and excessive grooming behaviors that were reduced by the antipsychotic risperidone and haloperidol respectively [195, 196]. At a neurobiological and neurochemical level, these mice displayed abnormalities in neuronal migration, reduced number of interneurons (including GABAergic interneurons) and abnormal neuronal network activity, in addition to increased levels of dopamine released into the striatum and altered inhibitory signaling [195, 196] which are reminiscent of some clinical and postmortem studies of TS patients discussed in chapter 1 [47-49, 59, 71, 72].

Another gene family identified through familial studies is the SLIT and NTRK-like (SLITRK) family which consists of 6 genes that encode leucine-rich transmembrane proteins that are involved in axonal targeting and neuronal differentiations. SLITRK1 has been identified in several studies [80-84] as a candidate gene associated with TS, although there have been a few contrasting reports [197-200]. This gene has also been implicated in OCD and trichotillomania [80, 201, 202], two common comorbid disorders. In relation to the neuropathology of TS, some studies have shown that SLITRK1 is associated with CSTC circuits [203]; although the function is still unclear, there is evidence that this molecule regulates neurite growth [204]. However, despite promising clinical data, mouse models of SLITRK1 mutations have not been promising. The overexpression of SLITRK1 induces neuronal outgrowth, but alterations in behavior have not been reported [205]. Conversely, SLITRK1 knockout (KO) mice exhibit high norepinephrine levels and anxiety-like behaviors that are subject to reversal by clonidine, but these mice do not exhibit tic-like or stereotyped behaviors [206]. Interestingly, mice with null-allele mutations for SLITRK5 display excessive autogrooming and high anxiety-like behaviors. The altered neurobiology reveals that these behaviors are underpinned by over activation of the orbitofrontal

cortex and abnormal morphology of striatal cells [207]. These studies indicate that the SLITRK family is involved in the development of behavioral phenotypes related to TS and other neuropsychiatric disorders.

A mutation in the gene for L-Histidine decarboxylase (HDC), which encodes for the enzyme that facilitates the conversion of histidine into histamine [208] has been found in a familial type of TS [85, 86]. Interestingly, histamine can modulate striatal dopaminergic transmission [209], which makes it a prime candidate in TS pathogenesis. Studies by Dr. Chris Pittenger on *Hdc*-deficient mice have also revealed some interesting findings in relation to the role of HDC in TS pathology. While these mice do not exhibit spontaneous tic-like behaviors or stereotypies, they display a marked increase in amphetamine-induced stereotypies and fear-induced repetitive grooming [64, 210]. Haloperidol treatment and intracerebral histamine infusion were both sufficient to ablate the amphetamine-induced stereotypies [64]. Follow-up studies in TS individuals carrying the *Hdc* W317X mutation revealed that these patients exhibited PPI deficits like other TS patients and this is mirrored in the HDC KO mice [64]. Furthermore, these same studies revealed elevated substantia nigra D2/D3 binding in both *Hdc* W317X TS patients and HDC-KO mice. In addition, it has been demonstrated that this mutation leads to an increase in dopamine in the striatum [211] and dysregulated intracellular dopamine signaling [212]. Another study by this group demonstrated that silencing histaminergic neurons in the tuberomammillary nucleus of the hypothalamus induced activation of the dorsal striatum neurons and resulted in pathological grooming [213]. The critical histamine receptor in these processes has been identified to be the H3 receptor, which has demonstrated to be essential for dopamine receptor-mediated signaling via extracellular signal-regulated kinase 1/2 (ERK1/2) [214-216]. In addition, H3 receptor KO mice displayed PPI deficits whereas H1 receptor KO and HDC KO mice displayed

normal PPI levels [64, 217]. However, HDC KO mice do not display brain morphological alterations reminiscent of TS such as the number of striatal interneurons [218].

Animal models of observed neurobiology in TS. Several animal models of TS have been generated to reproduce brain morphological changes in TS. The most prominent of these address the observed deficit in GABAergic and cholinergic interneurons within the basal ganglia [47-49]. This has been undertaken through several approaches that include pharmacologically inactivating GABA receptors with picrotoxin or bicuculine, as well as, ablating the interneurons surgically.

Early studies focusing on disrupting GABAergic transmission in the basal ganglia found that administering $GABA_A$ antagonists directly to the striatum in rats resulted in abnormal movements [219, 220]. This was also found in monkeys when $GABA_A$ antagonists were injected into the putamen [221-223]. The abnormal movements observed in these experiments were reminiscent of TS in appearance and importantly could be ablated by administration of haloperidol [224]. Interestingly, localized injections into different functional regions of the striatum and globus pallidus externa resulted in behaviors similar to common co-morbid disorder of TS, including compulsivity, hyperactivity, and attention deficit symptoms [220, 225, 226].

Similar results have been achieved through targeted ablation of interneurons in mice. Tic-like behaviors were not observed at baseline; however, the mice did display TS-like tic behaviors if challenged with acute stress or d-amphetamine, as well as behaviors common of co-morbid disorders depending on the region and interneuron population targeted. For instance, ablation of cholinergic interneurons in the dorsolateral striatum produced tic-like stereotypies after acute stress or administration of d-amphetamine [227] but did not produce alterations in sensorimotor gating. Alternatively targeting fast-spiking interneurons within the same region produced stress-triggered stereotyped grooming but no increased stereotyped behaviors following d-amphetamine

administration; however, the mice displayed higher levels of anxiety compared to controls [228]. Reminiscent of clinical observations, further studies reported that conjoint depletion of both cholinergic and fast-spiking interneurons resulted in spontaneous stereotyped behaviors and deficits in social interaction that were observed in male, but not female, mice [229].

Animal models of environmental insults. As discussed in chapter 1, environmental risk factors for TS include early stress and/or adverse events in the prenatal and perinatal stages [87-90]. Preclinical studies that focus on obstetric complications and maternal smoking have been conducted; however, these studies have largely focused on the link to other mental disorders, given that these insults have been linked to multiple neuropsychiatric disorders later in life [230, 231]. It should be pointed out that these disorders share commonalities with TS including alterations in dopaminergic signaling, stereotyped behaviors and PPI deficits, so further examination of these models may reveal further insights into TS as well [230]. For instance treatment of pregnant female mice with IL-2 during mid-gestation resulted in behavioral alterations in the offspring that included increased self-grooming and rearing [232]; conversely IL-6 treatment resulted in decreased PPI in the offspring [233].

Models of early neuroinflammation have been more revealing in the context of TS. In relation to infection with group A streptococci infection, mice immunized with these bacteria were found to exhibit TS-related manifestations, which included increased grooming and rearing [234-236]. The mice also displayed anti-brain antibodies in their serum, as well as increased IgG concentrations in several brain regions, such as the striatum, cerebellum, and hippocampus [234, 235]. Interestingly, experiments where the investigators injected serum from TS patients into the striatum of rats resulted in the manifestation of motor and oral stereotypies, episodic vocalizations and increased genital grooming [237-239]. The rats also exhibited increased levels of dopamine

and reduced DAT expression [240]. Taken together these animal models support a contribution of environmental insults and neuroinflammatory events in the pathogenesis of TS. Nonetheless, further studies are warranted to determine the exact nature of these insults in the pathogenesis of TS versus other mental disorders.

Models of dopamine hyperactivity. Investigators have employed multiple means of inducing TS-like symptoms in animal models through manipulation of the dopamine system. These include genetic manipulations of dopamine receptors and pharmacological means.

A few genetic animal models have been created to focus on the role of excessive dopamine or hyperactive dopamine signaling in the CSTC circuits. Of these the most prominent are the dopamine transporter (DAT) KO and knockdown mutant mice and the D1CT-7 mouse. The main function of DAT is to facilitate the reuptake of dopamine into the presynaptic terminal. Therefore, a reduction in DAT activity would result in an increase in synaptic levels in the striatum and so studies have been undertaken to determine if there exists a relationship between variants of the gene encoding DAT, *DAT1,* and TS. While not conclusive, there have also been studies implicating *DAT1* as a potential risk factor for TS susceptibility [241-244]. DAT KO mice display perseverative behaviors, hyperlocomotion, attentional alterations, and PPI deficits [245-252] Furthermore, the PPI deficits and perseverative patterns observed in these mice are ablated by dopamine receptor antagonists [249]. Notably, DAT-deficient mice display lower surface expression and binding of D_1 receptors [253, 254].

The D1CT-7 mouse line was created to investigate the contribution of D1 containing neurons to psychiatric disorders. It was generated via the attachment of the cholera toxin intracellular enzymatic subunit A1 to the human dopamine D1 receptor promoter [255]. This construct resulted in the chronic potentiation of the activity of a subset of D1-containing neurons

located in layer II of the piriform cortex, layers II and III of the somatosensory cortex and intercalated nucleus of the amygdala [255]. Despite not harboring this transgene in the D1-harboring neurons of the striatum, as other data suggests is a critical region for TS pathology (however, see the discussion on HDC KO mice, as an example of mutations outside the basal ganglia inducing TS-like behaviors), D1CT-7 mice exhibit a number of TS-related phenomena. This mutant line is one of the few to exhibit spontaneous tic-like manifestations, consisting of sudden axial jerks, from the third week of postnatal life, which roughly coincides with the age of onset of TS in humans. These behaviors are attenuated by several TS treatments, including antipsychotic drugs and clonidine [256](Nordstrom and Burton, 2002). Furthermore, D1CT-7 mice exhibit sexual dimorphism in that males display tic-like behaviors of greater severity and complexity [256](Nordstrom and Burton, 2002). Other behavioral alterations include hyperlocomotion, leaping and other psychomotor abnormalities, and perseverative responses (such as overgrooming), which have been likened to compulsive manifestation of OCD. However, outside of and prior to the work presented in this dissertation the construct validity of the D1CT-7 mouse has been questioned, despite the high face and predictive validity observed. This is due in part to the artificial nature of the line and the anatomical localization of their transgene [138]. Clinical data suggests cortical hypoactivation in TS, which conflicts with the implications from this animal model. Pyramidal cells in layer II and III are the primary source of intracortical horizontal projections [257] and receive abundant input from dopaminergic neurons [258, 259], which enhance excitatory post-synaptic currents through D1 receptor activation [260] and suggests that tic-like manifestations may result from corticostriatal hyperactivity. In addition, the role of the intercalated nucleus of the amygdala in TS is unknown. However, the D1 potentiation in this region may underpin the OCD-like manifestations in D1CT-7 mice [261, 262]. Further research into this

model is needed to determine the true validity with respect to TS and will be discussed in further length in the experiments presented in later chapters.

Pharmacological models of dopamine hyperactivity. A multitude of studies have been undertaken using dopamine receptor agonists and antagonists to replicate certain phenotypes of TS. Both indirect (i.e., d-amphetamine) or direct (apomorphine) dopamine receptor agonists have been shown to induce stereotypies and PPI deficits [183, 249, 263-266]. Further research has also demonstrated that the contribution of D_1-like and D_2-like receptor agonists to PPI disruptions varies depending on the species and strain of the experimental animals [267]; however, for most cases these phenotypes are contributed to by both receptor families. The mechanism by which these compounds elicit these TS-like behaviors is in part the result of the disequilibrium of activation in the striosomes (which display abundant D1 receptors on striatonigral neurons of the direct pathway) with respect to the matrix (which features high levels of D2 receptors on the striatopallial neurons of the indirect pathway) [63, 268-271]. In contrast, the PPI deficits induced by dopaminergic agonists have been demonstrated to reflect the activation D1 and D2 receptors in the nucleus accumbens [272-274]. These behavioral alterations are highly sensitive to antipsychotic agents, signifying high predictive validity [56, 146, 183].

The purpose of chapters 3 and 4.

There are several large gaps in the knowledge concerning TS and other tic disorders. For instance, the contribution of different dopamine receptors is largely unknown. It has largely been assumed that this disorder is primarily due to abnormalities concerning the D2 receptors since D2 antagonists are the first line of pharmacological therapy prescribed to patients. However, there is repeated evidence that these therapies are not always efficacious and at the most effective doses they result in significant sedation or risk of extrapyramidal side effects. Initial clinical studies have

been conducted to treat TS patients with D1 antagonists as well, but these are still in preliminary trials and only one study has been published [56]. In addition, the approved treatments rarely address stress-induced fluctuations in symptom severity, indicating a need for further research on the underlying mechanisms of these symptoms. As suggested by the genetic studies and brain imaging results discussed in chapter 1, this disorder may be highly heterogeneous. As such, it will be important to uncover other receptors and therapeutic targets to give physicians more options in treating TS and to treat tic fluctuations due to stressful life events. Therefore, we have undertaken experiments to study the contribution of D1 receptors to TS-related endophenotypes and the induction of these endophenotypes by stress to expand the field of animal models with which to study novel therapeutics related to D1 receptors and stress.

To address these experiments, we have chosen two complementary experimental models. As discussed above, there is not a single animal model that completely captures all aspects of TS; therefore, we have chosen to address the problem with multiple animal models. The first model is the D1CT-7 mouse model. We chose this model because it is the only animal model to spontaneously display tic-like behaviors without further manipulations. Despite the questions regarding the validity of this model, there are several important aspects of TS that can be addressed using this model. For starters, it has not been demonstrated that they display PPI deficits, which calls into question aspects of the validity of this model. Furthermore, the response of these mice to stress, compared to wildtype littermates has not been determined. Since TS patients exhibit a heightened sensitivity to stress this is potentially an important aspect to study in animal models as well. We will present in the next chapter our findings that these mice exhibit stress-exacerbated phenotypes of TS, including an increase in tic-like behaviors and PPI deficits. To better support these findings and to overcome the challenges and weaknesses posed by the D1CT-7 model we

also addressed these issues in an animal model of TS that relies on observing tic-like behaviors in a wildtype mouse, namely eye blinks. As discussed above eye blink tics are one of the most common tics observed in TS patients and both clinical and preclinical studies have indicated that eye blink responses are heavily regulated by dopamine. Therefore, we measured eye blink responses to specific D1 receptor agonists and stress to determine the nature of these behaviors in relation to TS in mice.

3. The D1CT-7 mouse model of Tourette syndrome displays sensorimotor gating deficits in response to spatial confinement

This chapter has been adapted from:

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Introduction

To further explore the validity of the D1CT-7 mutant mouse line and determine the extent of its construct validity, the present study was designed to assess the sensorimotor gating and stress susceptibility of D1CT-7 mice, given the importance of these phenomena in the pathophysiology of TS. In particular, we examined the behavioral responses of D1CT-7 and wild-type (WT) littermates subjected to a naturalistic environmental stressor, which consisted of a 20 min spatial confinement (SC) within a cylindrical enclosure placed in their home cages (Figure 3.1A). The advantage of SC over other common modalities of experimental stress (such as foot shock or restraint) is that it does not lead to marked anxiety-like behaviors, which may mask or interfere with tic-like responses or other spontaneous behaviors.

Methods

Animal welfare and ethical statement. We used 3- to 4-month-old, experimentally naïve male Balb/c mice weighing 20–30 g. Animals were purchased by Jackson Labs (Bar Harbor, ME, USA) and bred and genotyped as reported by Campbell et al. [255]. Because the pattern of inheritance of D1CT-7 mice is autosomal dominant, WT females were bred with heterozygous D1CT-7 sires; this breeding scheme was selected to standardize maternal behavior. Animals were housed in group cages with ad libitum access to food and water. The room was maintained at 22°C,

on a 12:12 h light/dark cycle from 0800 to 2000 h. Animals were tested during their light cycle between 1200 and 1600 h to minimize any potential circadian effects. All experimental procedures were in compliance with the National Institute of Health guidelines and approved by the Institutional Animal Use Committee of the University of Kansas. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [275, 276].

Experimental design. The first experiment (n = 8 per group) was carried out to validate the stressful effects of SC, by testing plasma corticosterone levels at the end of the 20 min manipulation, as compared with non-confined (NC) counterparts. We then tested the effects of SC on spontaneous behaviors of D1CT-7 and WT mice (n = 8 per group), with a particular focus on tic-like manifestations and repetitive digging responses. The third, fourth, fifth and sixth studies were performed to verify the effects of SC exposure on startle and PPI, locomotor behavior, novel object exploration and social interaction respectively. All experiments were performed with groups of eight mice per genotype, with the exception of the assessment of the correlation between startle parameters and tic-like behaviors, which was conducted with 15 SC-exposed D1CT-7 mice to confer sufficient statistical power for regression analyses. The final series of experiments was performed to test the efficacy of haloperidol, clonidine and SCH23390 on tic-like behaviors and PPI deficits in D1CT-7 mice. These experiments were conducted with 12 mice per group for each pharmacological assessment.

All experiments were conducted by trained observers unaware of the treatments using a randomized design for treatment assignment. Mice were not used for more than one experiment to avoid stress carry-over effects. The numbers of animals for each test were based on preliminary power analyses based on pilot studies.

Space confinement. Animals were confined within a clear, bottomless Plexiglas cylinder (10 cm in diameter × 30 cm in height), which was placed in their home cages, deeply embedded in bedding to ensure stability. A schematization of this experimental setting is provided in Figure 3.1. Space confinement (SC) lasted 20 min, and behaviors were video-recorded for the last 10 min so as to allow animals to avoid potential behavioral alterations caused by neophobia induced by the exposure to the unfamiliar enclosure. Tic-like manifestations were defined as rapid (<1 s) twitches of the head and/or body. Observations were obtained by trained observers, blinded to the genotype and treatment, as previously indicated [256]. For each experiment, one cohort was subjected to 20 min SC prior to behavioral testing. Another cohort consisting of NC control mice remained in their home cages. In order to avoid potential carry-over effects of SC stress, each animal was used only once in our experimental design.

Corticosterone measurements. Animals were exposed to SC or NC conditions for 20 min between 1200 and 1600 h, then rapidly killed via decapitation. Trunk blood was collected at 1200 and 1600 h. Serum corticosterone was measured in triplicate using an enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA). Intraassay precision and inter-assay precision was calculated as 4.6 and 8.3% respectively. Limit of detection was determined as 16.9 pg·mL−1.

Acoustic startle reflex and PPI. Startle testing was conducted as previously described [277]. Briefly, the apparatus used for detection of startle reflexes (SR-LAB; San Diego Instruments, San Diego, CA, USA) consisted of five Plexiglas cages (diameter: 5 cm) in sound attenuated chambers with fan ventilation. Each cage was mounted on a piezoelectric accelerometric platform connected to an analogue digital converter. The response to each stimulus was recorded as 65 consecutive 1 ms readings. A dynamic calibration system was used to ensure comparable sensitivities across chambers. The startle testing protocol featured a 70 dB background

white noise, and consisted of a 5 min acclimatization period, followed by three consecutive blocks of pulse, prepulse + pulse and 'no stimulus' trials. During the first and the third block, mice received only five pulse-alone trials of 115 dB. Conversely, in the second block mice were exposed to a pseudorandom sequence of 50 trials, consisting of 12 pulse-alone trials, 30 trials of pulse preceded by 73, 76 or 82 dB pre-pulses intensities (10 for each level of prepulse loudness) and eight no stimulus trials, where only the background noise was delivered. Intertrial intervals were selected randomly between 10 and 15 s. Sound levels were assessed using an A-scale setting. Percent PPI was calculated with the following formula:

$$100 - \frac{mean\ startle\ amplitude\ for\ prepulse\ pulse\ trials}{mean\ startle\ amplitude\ for\ pulse\ alone\ trials} \times 100$$

The first five pulse-alone bursts were excluded from the calculation. As no interaction between prepulse levels and treatment was found in the statistical analysis, %PPI values were collapsed across prepulse intensity to represent average %PPI.

Open-field locomotor behavior. Spontaneous locomotor behaviors to novel environments were tested for 60 min in a square force plate actometer (side: 42 cm; height: 30 cm) as previously described [278]. Each force plate actometer consisted of four force transducers placed at the corners of each load plate. Transducers were sampled 100 times s−1, yielding a 0.01 s temporal resolution, a 0.2 g force resolution and a 2 mm spatial resolution. Custom software directed the timing and data-logging processes via a LabMaster interface (Scientific Solutions Inc., Mentor, OH, USA). Additional algorithms were used to extract macrobehavioural variables, such as distance travelled, number of low-mobility bouts etc. Distance travelled was calculated as the sum of the distances between coordinates of the location of center of force recorded every 0.50 s over the recording session. Low-mobility bouts were defined as periods of 5 s during which mice confined their movements to a 15 mm radius virtual circle. Time spent in the center was measured

in a central quadrant (side: 21 cm) over the first 5 min block. Rotation bias was calculated by summing the locomotor turn direction over time using the center of the actometer floor as a reference point. Wall leaps were identified based on specific force–time waveform (required to have minimum force below −90% of body weight for 0.03 s or longer, and at no more than 3 cm from the wall), using custom scrolling graphics software.

Novel object exploration. Novel exploration was measured by placing foreign objects in the animal's home cage for 15 min as previously described [279]. The number and duration of exploratory approaches towards the objects were scored from video recordings and quantified. Exploratory activity was defined as sniffing or touching the objects with the snout, but not climbing or sitting on the objects.

Social interaction. Social behaviors were tested for 10 min in an unfamiliar cage and video recorded as previously described [280]. Behavioral measures consisted of the number and duration of interactions towards foreign age- and weight-matched male Balb/c WT conspecifics, as well as the number and duration of fighting behaviors (attacks and fighting episodes). Care was taken in differentiating fighting episodes from compulsive biting or allogrooming in D1CT-7 mice, which have been shown to result in occasional harm to cage mates. Social interaction was defined as sniffing or touching the conspecific with the snout.

Statistical analyses. Normality and homoscedasticity of data distribution were verified using Kolmogorov–Smirnov and Bartlett's tests. Statistical analyses of parametric data were performed with one-way or two-way ANOVAs, followed by Newman–Keuls' test for post hoc comparisons. Locomotor behaviors were analyzed using a two-way ANOVA design for repeated measures with genotype, condition and time as the factors. Correlations were performed between

tic-like outbursts and PPI, as well as between tics and startle amplitude by means of linear regression analyses. Significance threshold was set at 0.05.

Drugs. The following drugs were used: clonidine and SCH23390 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in saline. Haloperidol (Sigma-Aldrich) was dissolved in a single drop of 1 M HCl and diluted with saline. The doses of each drugs were selected so as to yield ≥80% occupancy of their targeted receptors [281, 282] without reducing spontaneous activity in the SC paradigm (as verified in pilot studies). Different cohorts of animals were used for each drug treatment group. The nomenclature of all receptors and drug targets mentioned in this article conforms to the guidelines indicated in British Journal of Pharmacology's Concise Guide to Pharmacology [283].

Results

SC increases corticosterone levels. The efficacy of SC as a stressor was verified in WT and D1CT-7 mice by testing the changes in plasma corticosterone levels induced by this manipulation. A two-way ANOVA revealed that SC induced a marked increase in corticosterone concentrations in both genotypes (main effect of SC); furthermore, D1CT-7 mice displayed higher corticosterone levels than WT, irrespective of the environmental conditions (main effect of genotype) (Figure 3.1B). Nevertheless, no significant interactions between SC and genotype were found.

SC enhances tic-like responses and digging in D1CT-7 mice. We then tested the behaviors of D1CT-7 and WT littermates in response to SC, as compared with NC counterparts kept in their home cages. In D1CT-7 mice, SC induced a dramatic increase in tic-like responses (Figure 3.2B) and digging behavior (Figure 3.2C). In contrast, SC did not significantly alter the behaviors of WT mice. Furthermore, tic-like and digging behaviors in SC-exposed D1CT-7 mice

were significantly greater than those observed in SC WT controls. These data suggest that this SC elicits a pronounced enhancement of TS-related behaviors in D1CT-7, but does not significantly affect WT mice.

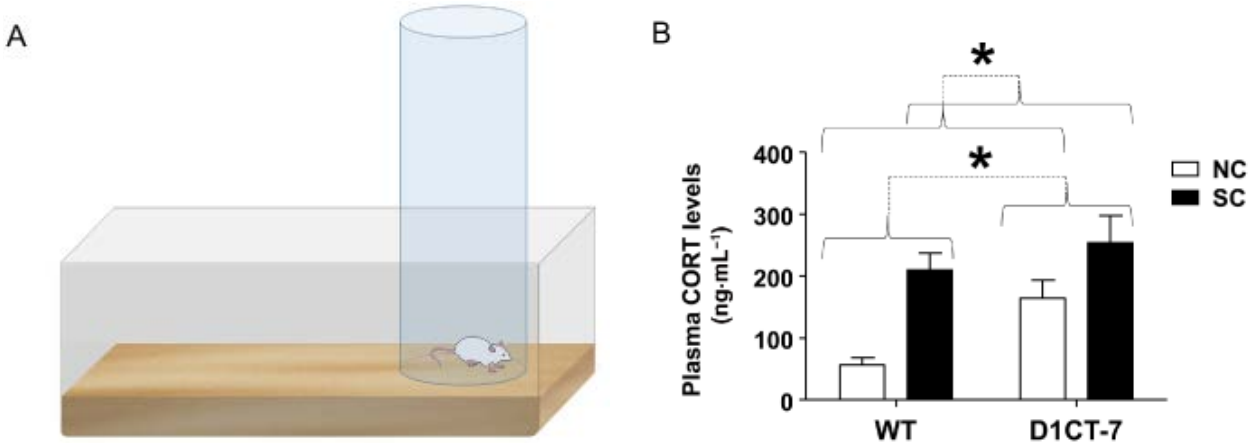

Figure 3.1 Effect of SC on plasma corticosterone. (A) Graphical representation of the experimental setting used for SC. (B) Effects of SC on corticosterone levels. Data are shown as means ± SEM. *P < 0.05 for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. n = 8 per group. For more details, see text.

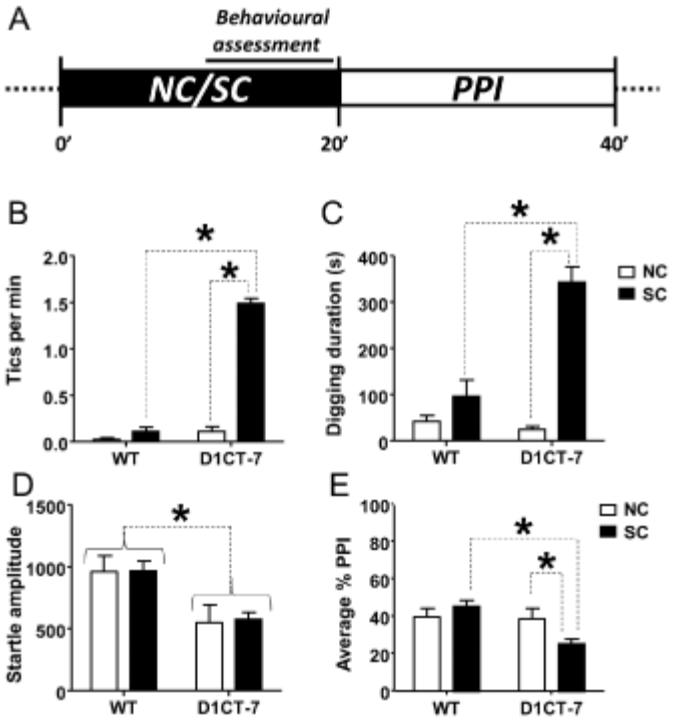

Figure 3.2 SC elicits a robust increase in tic-like behavior, repetitive digging behavior and deficits in PPI in D1CT-7, but not WT mice. (A) Experimental timeline. (B) Effects of SC on tic-like responses. (C) Effects of SC on repetitive digging. (D) Effects of SC on startle amplitude. (E) Effects of SC on PPI of the startle. Data are shown as means ± SEM. *$P < 0.05$ for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. $n = 8$ per group. For details, see text.

D1CT-7 mice exhibit sensorimotor gating deficits following SC. In a separate experiment, we investigated whether SC exposure led to alterations in sensorimotor gating, as assessed through the PPI of the acoustic startle. D1CT-7 mice exhibited a reduction in startle amplitude (Figure 3.2D), irrespective of exposure to SC. Furthermore, SC exposure elicited a significant reduction in PPI (Figure 3.2E) in D1CT-7, but not WT mice. Linear regression analyses revealed that the number of tic-like behaviors was not correlated with either startle amplitude [$F(1, 14) = 1.19$; NS; $R2 = 0.08$] or %PPI values [$F(1, 14) = 2.69$; NS; $R2 = 0.16$] (data not shown).

SC exposure does not affect hyperactivity in D1CT-7 mice in a novel open field. Next, we investigated whether SC exposure can alter other behavioral parameters related to TS symptoms. Accordingly, we tested the locomotor responses in SC-exposed D1CT-7 and WT mice using a novel open field on a force–plate actometer. Three-way ANOVA (with genotype, environmental condition and time as factors) revealed that D1CT-7 mice exhibited a hyperactive phenotype compared with WT mice (Figure 3.3B). Furthermore, SC was found to increase overall locomotor activity. Conversely, no significant time-dependent differences were found. A significant genotype × condition interaction was also detected, and post hoc analyses revealed that SC significantly increased locomotor behavior in WT mice, but failed to affect the hyperlocomotion of D1CT-7 mice. The analysis of low-mobility bouts (Figure 3.3C) revealed significant main effects for genotype and condition, as well as their interaction. Post hoc comparisons showed that D1CT-7 animals engaged in fewer low-mobility bouts than WT mice pre-exposed to the same conditions. In addition, SC-exposed WT mice showed fewer low-mobility bouts than NC-exposed WT counterparts; however, no differences were detected in the time spent in the center (Figure 3.3D). NC D1CT-7 mice were also found to display a significantly higher rotation bias than both NC WT and SC D1CT-7 mice (Figure 3.3E). D1CT-7 mice also exhibited

a genotype-specific increase in the number of wall leaps (Figure 3.3F) compared with WT counterparts.

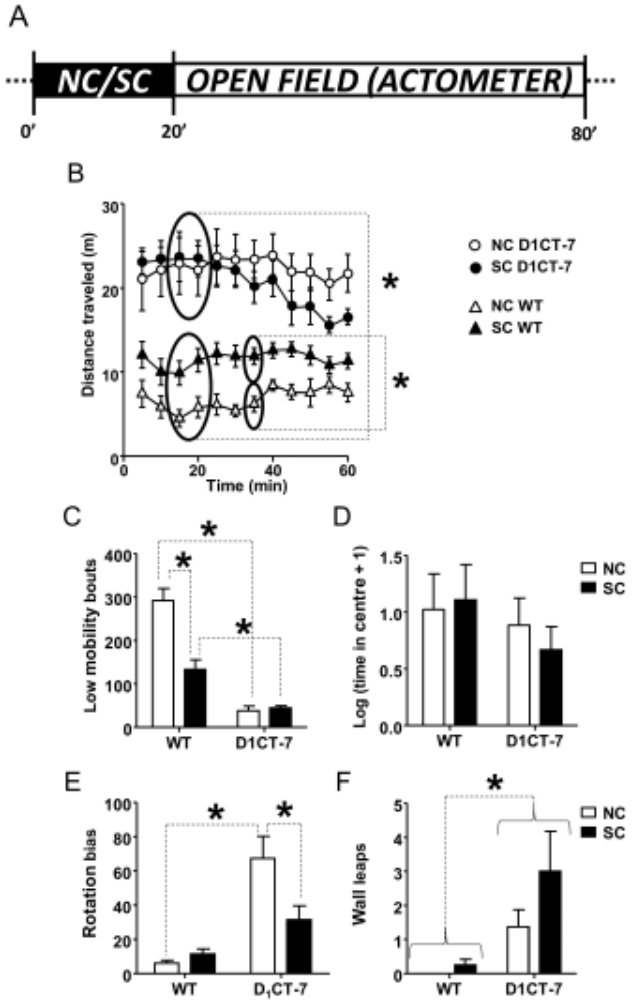

Figure 3.3 D1CT-7 mice exhibit open-field locomotor hyperactivity regardless of SC. (A) Experimental timeline. (B) Effects of SC exposure on locomotor activity of D1CT-7 and WT mice. (C) Effects of SC exposure on low-mobility bouts. (D) Effects of SC exposure on rotation bias. (E) Effects of SC exposure on wall leap behavior. Data are shown as means ± SEM. **P* < 0.05 for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. *n* = 8 per group. For details, see text.

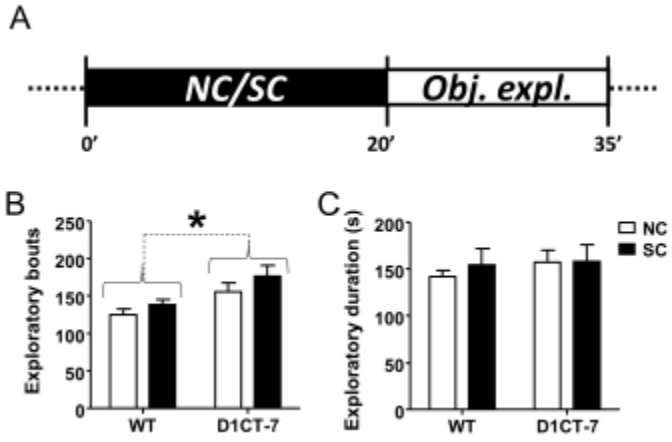

Figure 3.4 D1CT-7 mice show increased exploratory approaches towards novel objects. (A) Experimental timeline. (B) Effects of SC exposure on the number of exploratory approaches. (C) Effects of SC exposure on total exploratory duration. Data are shown as means ± SEM. **P* < 0.05 for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. *n* = 8 per group. For details, see text.

SC does not affect novel object exploration in D1CT-7 mice. We next examined whether SC evoked anxiety-related behaviors towards novel objects. D1CT-7 mice exhibited an increase in novel object exploratory approaches (Figure 3.4B), but no differences were detected for condition (i.e. SC vs. NC) or genotype × condition interactions. Two-way ANOVA analyses of exploratory duration (Figure 3.4C) revealed no significant effects of genotype, condition or their interaction.

SC increases aggressive behavior in D1CT-7 mice. To determine whether SC modified behavioral responses to foreign conspecifics, animals were tested in the social interaction paradigm. We found that social exploratory approaches were significantly reduced following SC (Figure 3.5B), irrespective of the genotype. The duration of the social interaction was equivalent among all groups (Figure 3.5C). Notably, D1CT-7 mice exhibited a higher number of aggressive episodes during social encounters compared with their WT counterparts (Figure 3.5D), but no differences were detected for condition or genotype × condition. SC exposure increased aggressive behaviors in D1CT-7 mice, but not in WT animals. (Figure 3.5E). Notably, fighting behaviors were characterized by defensive postures (such as tail rattling) and typical aggressive manifestations, and were clearly distinct from harmful tic-like manifestations, such as compulsive biting and exaggerated allogrooming.

Tic-like behaviors and PPI deficits in D1CT-7 mice are sensitive to haloperidol, clonidine and SCH23390. Because D1CT-7, but not WT mice, exhibited tic-related behaviors and PPI deficits and only following exposure to SC, we limited our pharmacological testing to this group in order to avoid potential floor effects. Next, we tested the predictive validity of D1CT-7 mutants as animal models of TS by evaluating the effect of standard anti-tic agents on the number

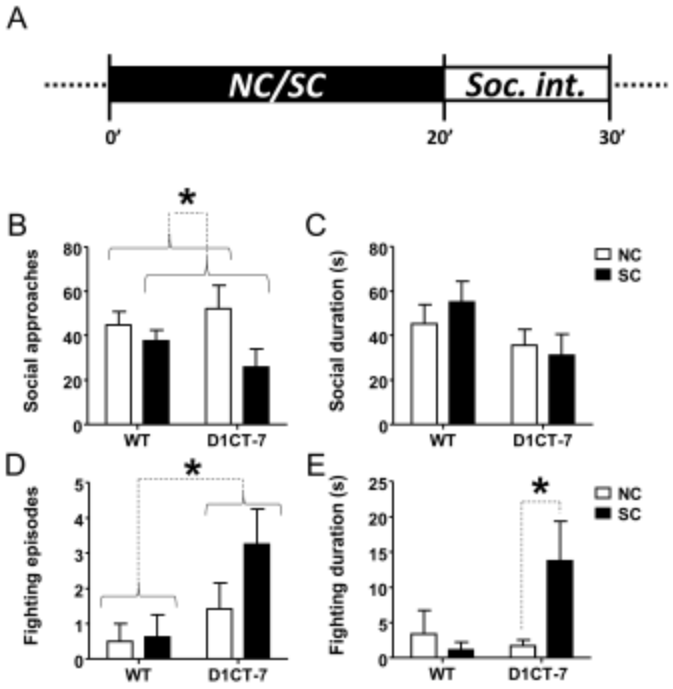

Figure 3.5 SC increases aggression in D1CT-7 mice in the social interaction paradigm. (A) Experimental timeline. (B) Effects of SC exposure on the number of social approaches. (C) Effects of SC exposure on total duration of social interaction. (D) Effects of SC exposure on number of fighting episodes. (E) Effects of SC exposure on total fighting duration. Data are shown as means ± SEM. **P* < 0.05 for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. *n* = 8 per group. For details, see text.

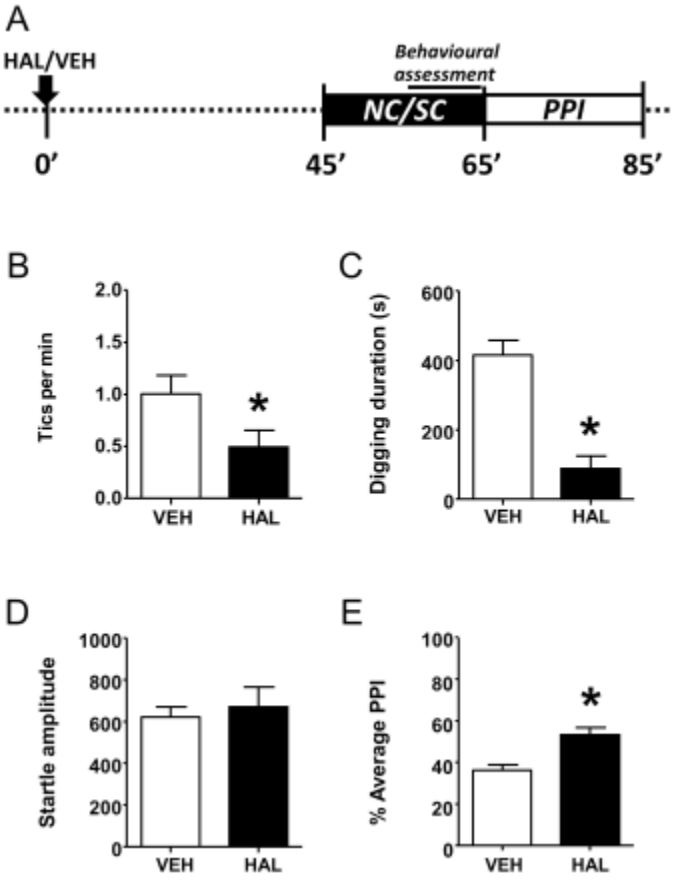

Figure 3.6 Haloperidol (HAL; 0.3 mg/kg, i.p.) reduced repetitive (perseverative) and tic-like behaviors, as well as PPI deficits in D1CT-7 mice exposed to SC. (A) Timeline of treatments and experiments. (B) Effects of HAL on tic-like responses. (C) Effects of HAL on perseverative digging. (D) Effects of HAL on startle amplitude. (E) Effects of HAL on PPI. All data refer to SC-exposed D1CT-7 mice. Data are shown as means ± SEM. **P* < 0.05 compared with vehicle (VEH). *n* = 12 per group. For more details, see text.

of tic-like behaviors (during SC) and sensorimotor gating disruptions (following SC). Haloperidol (0.3 mg/kg, i.p., injected 45 min before testing) elicited a reduction in the number of tic-related manifestations (Figure 3.6B) and reduced digging activity (Figure 3.6C) in D1CT-7 mice. Although haloperidol attenuated PPI disruptions (Figure 3.6E), it did not affect overall startle amplitude (Figure 3.6D) or latency to peak startle (data not shown). Similar to haloperidol, clonidine (0.2 mg/kg, i.p., administered 30 min before testing) decreased the expression of tic-like behaviors (Figure 3.7B) and digging activity (Figure 3.7C). Clonidine also significantly reduced startle amplitude (Figure 3.7D) and countered PPI disruptions (Figure 3.7E), but did not affect latency to peak startle (data not shown).

Finally, we examined whether the D1 receptor antagonist SCH23390 (1 mg/kg, s.c., injected 20 min before testing) prevented the TS-related features in SC D1CT-7 mice. We found that D1 receptor blockade decreased tic-like behaviors (Figure 3.8B) and digging (Figure 3.8C) during SC; furthermore, SCH23390 reduced startle amplitude (Figure 3.8D) and restored PPI (Figure 3.8E) following SC. In contrast, no differences were observed in the latency to peak startle (data not shown).

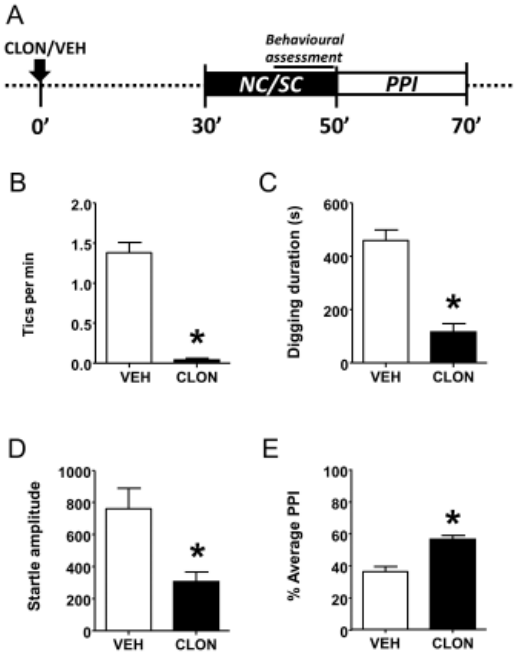

Figure 3.7 Clonidine (CLON; 0.2 mg/kg, i.p.) reduced repetitive (perseverative) and tic-like behaviors, as well as PPI deficits in D1CT-7 mice exposed to SC. (A) Timeline of treatments and experiments. (B) Effects of CLON on tic-like responses. (C) Effects of CLON on perseverative digging. (D) Effects of CLON on startle amplitude. (E) Effects of CLON on PPI. All data refer to SC-exposed D1CT-7 mice. Data are shown as means ± SEM. **P* < 0.05 compared with vehicle (VEH). *n* = 12 per group. For more details, see text.

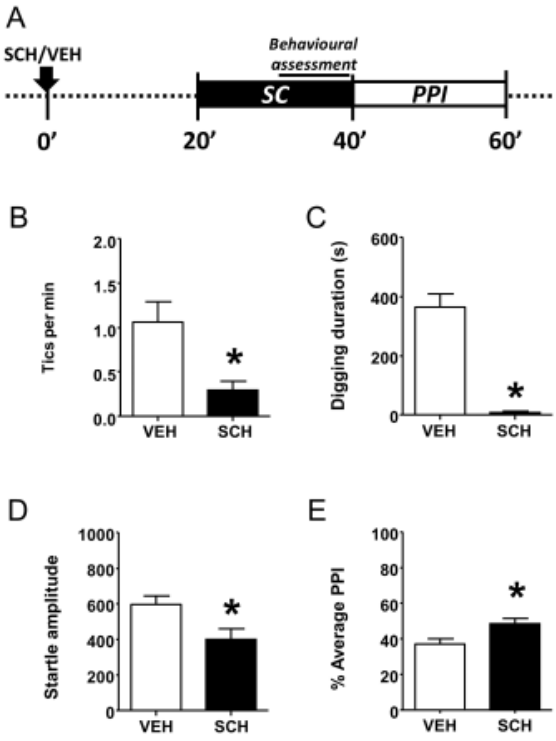

Figure 3.8 The D1 receptor antagonist SCH23390 (SCH; 1 mg·kg−1, s.c.) reduced repetitive (perseverative) and tic-like behaviors, as well as PPI deficits in D1CT-7 mice exposed to SC. (A) Timeline of treatments and experiments. (B) Effects of SCH on tic-like responses. (C) Effects of SCH on perseverative digging. (D) Effects of SCH on startle amplitude. (E) Effects of SCH on PPI. All data refer to SC-exposed D1CT-7 mice. Data are shown as means ± SEM. *P < 0.05 compared with vehicle (VEH). n = 12 per group. For more details, see text.

Discussion

The results of the present study show that the environmental stress caused by SC, triggered a number of TS-related phenotypes in D1CT-7, but not WT mice, including a robust exacerbation of tic-like and repetitive responses, as well as PPI deficits and aggressive behaviors. These effects are in line with previous reports documenting exacerbation of leaping and climbing compulsions in D1CT-7 mice exposed to predator urine odor [284]. We also found that SC-induced tic-like responses and PPI deficits were significantly inhibited by standard TS therapies, such as the antipsychotic haloperidol and clonidine, as well as the D1 dopamine receptor antagonist SCH23390. Although D1CT-7 mice displayed lower startle amplitude than their WT litter-mates, this parameter was not affected by SC, suggesting that the observed reduction of %PPI did not result from computational artefacts.

As mentioned earlier, the exacerbation of tic behavior in response to stress and PPI deficits are essential elements of TS pathophysiology [37, 38, 285], which are posited to reflect perceptual and information-processing alterations. In particular, PPI deficits have therefore been highlighted as key parameters to assess construct validity of animal models of TS [138, 286]. Based on this background, our findings collectively support the construct validity of this TS model. The pathophysiological relevance of D1CT-7 transgenic mice to TS symptoms is also supported by the recent discovery that TS patients display hyperactivity of the same sensorimotor circuit that is neuropotentiated in these mice [255, 287-289]; furthermore, it is worth noting that recent data indicated that in rodents the optogenetic stimulation of the cortex has been shown to produce features similar to symptoms of OCD – a highly co-morbid syndrome with TS [290].

There is ample evidence showing that behavioral stereotypes are a common sign of discomfort in animals subjected to prolonged SC [291-294]. The responsiveness of D1CT-7 mice to short-term SC may signify their high sensitivity to the spatial restriction and low contextual stimulation imposed by this manipulation, which contrast with the environmental requirements for their high spontaneous locomotor and exploratory activity. In addition, the higher plasma levels of corticosterone in D1CT-7 mice subjected to baseline conditions suggest that these mice display an intrinsically elevated stress response. The results of our experiments suggest that the summation of the baseline stress levels of D1CT-7 mice and the effect of SC may lead these animals to reach a critical threshold of activation, which may trigger the exacerbation of tic-like behaviors and the reduction in PPI. Irrespective of this issue, future studies are warranted to explore the basis of the higher baseline corticosterone plasma levels in D1CT-7 mice.

The fact that tic-like behaviors and PPI deficits were prevented by the modulation of dopamine and noradrenaline receptors suggests that the effects of SC in D1CT-7 mice are probably

caused by an enhanced catecholamine release in critical areas of the CSTC circuit, which is the anatomical substrate of TS pathophysiology, stereotyped behaviors and PPI regulation [38, 54, 289] Accordingly, acute stress has been shown to stimulate dopamine and noradrenaline release in the striatum and in the prefrontal cortex, [295, 296]. Further microdialysis studies are needed to evaluate the variations in dopamine and noradrenaline release during SC in D1CT-7 and WT mice.

In baseline conditions, D1CT-7 mice were shown to express the transcript of their neuropotentiating transgene in a subset of D1 receptor-containing neurons, including glutamatergic pyramidal projection neurons in somatosensory and piriform cortex as well as GABAergic interneurons in the intercalated nucleus of the amygdala [255]. Although the somatosensory cortical areas potentiated in the mice similarly hyperactivate to trigger premonitory urges in human TS, to the best of our knowledge these regions have not been directly implicated in the regulation of PPI. Thus, the observed sensorimotor deficits in D1CT-7 mutants may be due to the effect of potentiated glutamatergic projections from these regions to limbic circuits. Given that the elevation of the transgene's cAMP in the CT potentiates the responsiveness of neurons to their own endogenous excitatory neurotransmitters [255], it is possible that these neuropotentiated cortical and amygdala neurons in the D1CT-7 mice may respond to stress-triggered endogenous fast-acting neurotransmitter input by subsequently 'stepping up' their own neurotransmitter output, which could aggravate any baseline, sub-threshold symptoms. In this respect, it is worth noting that the increase in glucocorticoids produced by acute stress has been shown to enhance glutamate release from the cortex and amygdala [297], highlighting the possibility that changes in corticolimbic glutamate output may participate in the effects of SC in these animals.

Alternatively, SC may affect the expression of the D1 receptor-associated neuropotentiating effect in CSTC areas; indeed, previous studies have shown that acute stress

elicits rapid changes in the expression and transcription of dopaminergic genes [298]. Thus, it is possible that SC may lead to the expression of the D1CT-7 construct in areas of the CSTC circuits, which may remain undetected under baseline conditions. Future studies are warranted to examine the effects of SC on neurochemical changes in CSTC in D1CT-7 mice.

The effects of SC on tic-like behaviors and PPI in D1CT-7 mice were antagonized by haloperidol and clonidine, in line with previous findings on the effectiveness of both drugs at reducing spontaneous tic-like responses in this context [256]. In addition, the effectiveness of the selective D1 receptor antagonist SCH23390 suggests that D1 receptors are involved in the tic-like behaviors and PPI deficits and is in agreement with previous findings indicating that these molecular targets contribute to both the PPI and stereotyped behaviors in mice [277, 299, 300]. D1 receptors play a key role in the processing of informational salience and the enactment of behavioral stereotypies in rodents. For instance, D1 receptor agonists disrupt sensorimotor gating in mice [277, 300] and striatal D1 receptor activation induces locomotor hyperactivity and stereotyped behavior through the reinforcement of ongoing behaviors [51, 299]. It is worth noting that ecopipam, a selective D1 receptor antagonist, is currently under investigation as a potential therapeutic agent for the treatment of tics [56].

In parallel with clinical reports, we did not detect any correlation between the severity of the tic-like outbursts and PPI deficits in D1CT-7 mice [37]. Because both tic-like responses and PPI deficits were triggered by SC and responded to anti-tic therapies, it is likely that these two parameters may depend on converging, but not identical, anatomical substrates.

Another remarkable effect of SC in D1CT-7 mice was a significant enhancement in aggressive behaviors towards foreign conspecifics in the social interaction paradigm. These manifestations were characterized by typical aggressive and hostile behavior, including tail rattling

and aggressive chasing of the conspecific, and were clearly distinct from compulsive biting during allogrooming, as previously reported [255, 256]. The observed stress-induced aggressiveness in a model of TS is in line with clinical reports documenting disruptive behaviors, rage outbursts and anger control problems in TS patients, which are often preceded by stressful feelings of tension similar to sensory phenomena [301, 302].

In agreement with previous studies, we found that D1CT-7 mice displayed hyperactive locomotor behavior [255], which was demonstrated as fewer low-mobility bouts and greater distances travelled. The hyperlocomotion displayed by D1CT-7 mice was paralleled by an increase in the number of approaches, but not overall duration, of exploratory activity directed towards unfamiliar objects. Neither phenomenon was significantly affected by SC; however, this manipulation significantly increased the exploratory activity of WT mice. D1CT-7 mutants also showed a greater locomotor rotational bias, which probably reflects elevated brain dopamine levels and its tendency to induce repetitive, compulsive behavior [255] in this transgenic line. Indeed, rodents treated with indirect-acting dopamine agonists, such as amphetamine or cocaine also show large rotational biases [278, 303-305], which probably signify behavioral stereotypy [303]. In accord with previous reports [255], D1CT-7 mice displayed more wall leaps than WT mice, a finding consistent with the hypothesis that the D1CT-7 mice express an increased tendency to engage in repetitive, short-duration topographically distinct motor behavior. Notably, rotational bias was strikingly reduced by SC in D1CT-7 mice; although the specific cause of this phenomenon remains unclear, it is possible that this effect may reflect the higher occurrence of other repetitive behaviors, such as rearing or grooming, which may have partially over-ridden their locomotor responses.

SC D1CT-7 mice failed to exhibit open-field thigmotaxis, a response that has been associated with anxiety-related responses [306]. Although this finding may appear counterintuitive, given the increased stress levels in SC D1CT-7 mice, it should be noted that the extrapolation of anxiety-related phenomena (and particularly thigmotaxis) in hyperactive mice is generally considered to be unreliable, in view of the high risk of false-positive and false-negative findings [307].

Although the SC-exposed D1CT-7 mice exhibited several behavioral features that closely mirror phenotypic traits found in TS patients, several limitations of the study should be recognized. Firstly, our study focused solely on males, in view of the high predominance of TS in this gender. Although males have a higher severity of tic-like manifestations, mutants of both sexes display these behavioral abnormalities [256], and further studies on the sex differences in this line are warranted. Secondly, we found that D1CT-7 mice exhibited fewer spontaneous tic-like outbursts than previously described [256] and more sensitivity to the D1 receptor antagonist, SCH23390, than previously described [308], both of which may be due to a different penetrance of the gene mutation in our colony. Thirdly, the evaluation of the pharmacological effects of haloperidol, clonidine and SCH23390 was only performed in SC-exposed D1CT-7 mice, but not in NC counterparts (or in WT controls). This design was made necessary by the small number of D1CT-7 mice available, in view of their suboptimal reproductive efficiency, which allowed us to obtain no more than 10–12 males per generation (with an equivalent number of breeders), as well as our specific experimental decision of testing each animal only once (in order to avoid potential carry-over effects). Fourthly, although the effects of all drugs were observed to produce no overt alterations in the activity of the mice, we could not measure the locomotor behavior in these mice subjected to SC, and therefore cannot fully rule out the possibility that the observed amelioration

in tic-like responses is partially due to subtle changes in locomotion. Finally, in spite of the analogy between SC-induced behavioral responses and symptoms of TS, our analyses revealed that the D1CT-7 mutants do exhibit some behavioral responses not directly related to this disorder, such as reduced startle acoustic reflex, and hyperlocomotor activity (although the latter activity can be likened to manifestations of ADHD or perseverative traits in OCD, both conditions that are often co-morbid with TS).

In spite of these limitations, our findings showing that SC exacerbated the tics and PPI deficits in D1CT-7 mice, and that these deficits were sensitive to validated therapies for TS, appear to confirm the translational relevance of D1CT-7 mice as a valuable animal model that may replicate the influence of environmental stress on TS symptoms. Future studies are needed to elucidate the specific neurobiological changes induced by SC in this model, and their role in the symptoms of TS.

Acknowledgements

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Author contributions

S. C. G., S. C. F. and M. B. designed the research study and wrote the manuscript. S. C. G., L. J. M., S. C. F. and M. B. analyzed the data. S. C. G., L. J. M., H. J. S., C. M. J. and A. M. G. conducted the experiments.

Conflict of interest

None.

4. Studying tics in wild-type rodents: examining eye blinks as a proxy for tics

Introduction

Often, the first tic observed in TS patients involves exaggerated or increased eye blinking [150, 151]. In addition, patients have also been reported to display an increase in spontaneous eye blink rates [152-154], which is likely a reflection of the hyperdopaminergic state underlying this disorder, as spontaneous eye blinks are posited to reflect central dopaminergic activity[155, 156]. Several preclinical studies have been undertaken to investigate the contribution of dopamine receptors in eye blink responses in nonhuman primates and rats [157-161, 309]; however, to date there have not been any reports addressing eye blink responses in mice or the contribution of stress in any animal model. Here we analyze the eye blink response of freely moving or restrained mice following pharmacological challenge with the dopamine D1 receptor agonist SKF 82958 to study the contributions of stress and the D1 receptor on these responses. We also verify the specificity of the eye blink response by pretreating with the D1 receptor antagonist, SCH 23390 and the Tourette syndrome therapeutics, haloperidol and clonidine.

Methods

Animals. We used 3-4-month-old, experimentally naïve male C57BL/6 mice (n = 8-10 per treatment group) weighing 20-30 g. Animals were purchased by Jackson Labs (Bar Harbor, ME). Animals were housed in group cages with *ad libitum* access to food and water. The room was maintained at 22°C, on a 12 h: 12 h light/dark cycle from 8 am to 8 pm. Animals were tested during their light cycle between 12 and 4 pm to minimize any potential circadian effects. All experimental procedures were in accordance with the NIH guidelines and approved by the IACUCs of the Universities of Kansas and Utah.

Drugs. The following drugs were used: SCH 23390, haloperidol, clonidine, and SKF 82958 (Tocris Bioscience, Bristol, UK; Sigma-Aldrich, Saint Louis, MO). SCH 23390 was dissolved in distilled water. Haloperidol was dissolved in 10% acetic acid buffered with sodium hydroxide and diluted with saline. Clonidine and SKF 82958 were both dissolved in saline.

Eye blink observations. Eye blinks were scored by trained observers blinded to the treatment. Observations were made for 5 minutes either while the mouse was allowed to move freely on the underside of a 10 cm diameter beaker or while restrained in a clear 5 cm diameter tube.

Experimental design.

Experiment 1. Mice were treated with SKF 82958 (0.3mg/kg, IP) or saline 20 minutes before eye blinks were recorded while the mice moved freely on the bottom of a beaker.

Experiments 2-4. A total of 12 groups of mice were used for these experiments. 8 groups of mice were treated with SCH 23390 (1mg/kg, SC), clonidine (0.2 mg/kg, IP) or the corresponding vehicle 10 minutes before a second treatment of either SKF 82958 or saline. Eye blinks were observed in freely moving mice 20 minutes after the last injection. 4 more groups of mice were treated with haloperidol (0.3mg/kg, IP) or vehicle 25 minutes before SKF 82958 or saline treatment. These mice were also observed for eye blinks while freely moving.

Experiment 5. Mice were exposed to a 20-minute restraint in a clear Plexiglas tube (5cm diameter) or left in a clean standard mouse cage for 20 minutes. Eye blinks were then recorded for five minutes while the mice remained restrained in the tube or were allowed to move freely on the bottom of a beaker. *Experiments 6-9.* 16 groups of mice were tested in these experiments. 8 of the groups were treated with SCH 23390, clonidine or the corresponding vehicle 10 minutes before being placed in the restrainer or clean standard mouse cage. The next 4 groups were treated with

haloperidol or vehicle 25 minutes before exposure to restraint or left freely moving. The final 4 groups were treated with SKF 82958 immediately before being place in the restrainer or mouse cage for 20 minutes. In all experiments eye blinks were recorded at the end of the 20-minute experimental manipulation for five minutes while the restrained subject remained in the restrainer and freely moving mice were allowed to move about on the bottom of a beaker.

Statistical analyses. Normality and homoscedasticity of data distribution were verified by using Kolmogorov-Smirnov and Bartlett's tests. Statistical analyses of parametric data were performed with one-way or multi-way ANOVAs, followed by Tukey's T-test for *post-hoc* comparisons. The significance threshold was set at 0.05.

Results

Dopamine D1 receptors regulate the eye blink response. We first investigated the contribution of D1 receptors to normal eye blink responses by treating the mice with the D1 agonist SKF 82958 (Fig. 1A). Mice displayed an increased rate of eye blinks following SKF 82958 treatment ($P < 0.05$). To verify that these responses were specific to the D1 receptor we next pre-treated the animals with the D1 receptor antagonist SCH 23390 (Fig 1B). As expected SKF 82958 treatment increased eye blinks/min [pre-treatment x treatment interaction: $F(1,30) = 11.84$, $P < 0.01$; vehicle + vehicle vs vehicle + SKF 82958, $P < 0.001$)]. Furthermore, SCH 23390 pre-treatment significantly reduced the number of eye blinks in the SKF 82958 treated mice ($P < 0.001$). We also tested the possible contribution of D2 receptors by pre-treating the mice with the D2 antagonist haloperidol (Fig 1C). A main effect of both haloperidol pretreatment [$F(1,28) = 15.16$, $P < 15.16$, $P < 0.001$] and SKF 82958 treatment [$F(1,28) = 18.16$, $P < 0.001$], but no interaction was found. Finally, we tested clonidine, a noradrenergic α2 receptor agonist that is commonly used to treat TS. We found that while SKF 82958 produced a significant increase in

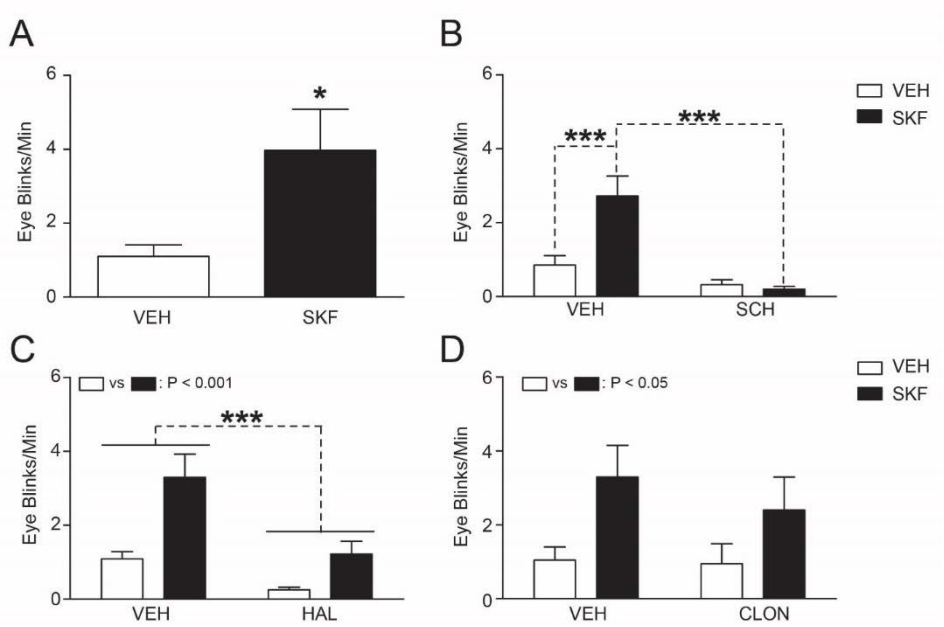

Figure 4.1 Eye blink responses are regulated by dopamine D1 receptors in mice. (A) The D1 agonist SKF 82958 (SKF; 0.3mg/kg) increased eye blinks in wildtype C57bl/6 mice. (B) The D1 antagonist SCH 23390 (SCH; 1 mg/kg) ablated eye blink responses induced by SKF. (C) The D2 antagonist haloperidol (HAL; 0.3mg/kg) non-selectively reduced eye blink responses in mice following SKF treatment. (D) The noradrenergic α_2 receptor agonist Clonidine (CLON; 0.2mg/kg) did not alter eye blink responses in mice treated with SKF. *$P < 0.05$ and ***$P < 0.001$ for comparisons indicated by dashed line. Date are shown as mean ± SEM. N=8/group. Abbreviations: VEH, vehicle.

eye blinks [main effect of SKF 82958: $F(1,28) = 7.04$, $P < 0.05$)], clonidine did not ablate these effects.

Eye blink responses are sensitive to stress in a D1 dependent fashion. We next examined the response of mice to an acute restraint stress of 20 minutes. We found that restraint increased eye blinks over freely moving mice ($P < 0.001$). To determine if D1 receptors mediated this response we treated the mice with SCH 23390 prior to restraint. We found that vehicle treated restrained animals displayed increased eye blinks over vehicle treated controls [treatment x condition interaction: $F(1,29) = 5.76$, $P < 0.01$; Freely moving + vehicle vs restrained + vehicle, $P < 0.001$]. Importantly, we also found that SCH 23390 decreased eye blinks in restrained animals ($P < 0.001$). The TS therapeutic haloperidol did not have an effect on restraint induced eye blinks [main effect of condition: $F(1,29) = 83.36$, $P < 0.001$). However, another TS therapy, clonidine, did ablate the effects of restraint on eye blink responses [treatment x condition interaction: F(1,28)

= 18.05, $P < 0.001$; Freely moving + vehicle vs restrained + vehicle $P < 0.01$; restrained + vehicle vs restrained + clonidine, $P < 0.001$]. We also tested the combined effects of both restraint and SKF 82958 treatment on these mice. We found that both restraint [F(1,30) = 17.86, $P < 0.001$] and SKF 82958 treatment [F(1,30) = 13.19, $P < 0.01$] increased eye blinks, but there was no significant interaction between the two.

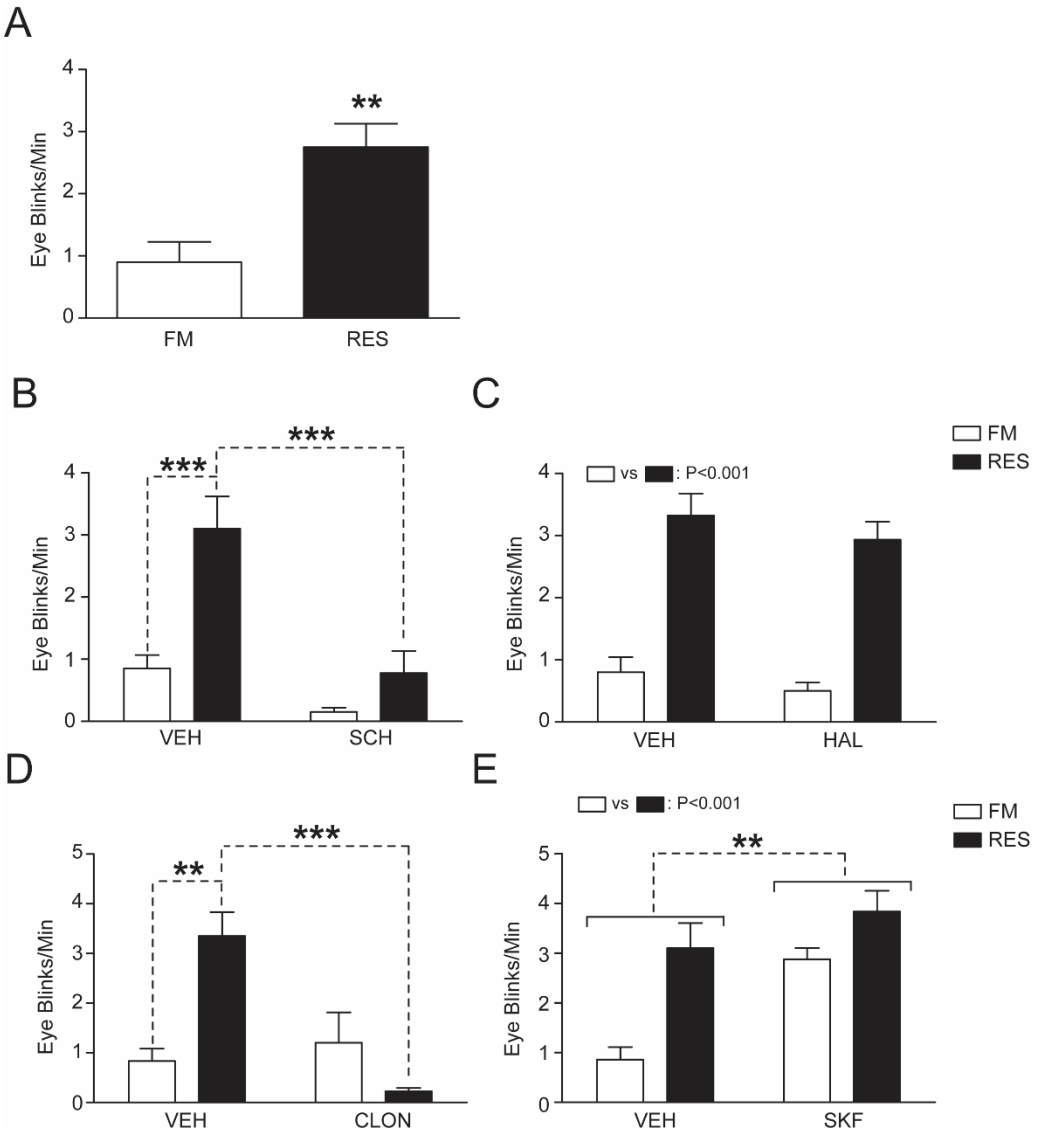

Figure 4.2 Stress increases the eye blink rate in mice. (A) 20 minutes of restraint stress increases eye blinks in wildtype C57bl/6 mice. (B) The D1 antagonist SCH 23390 (1mg/kg) attenuated the stress-induced eye blink response in mice. (C) The D2 antagonist haloperidol (HAL; 0.3mg/kg) did not alter eye blink responses. (D) The noradrenergic α2 receptor agonist Clonidine (CLON; 0.2mg/kg) selectively ablated stress-induced eye blink responses. (E) The D1 agonist, SKF 82958 (0.3mg/kg) did not further exacerbate eye blink responses in conjunction with stress. **P < 0.01 and ***P < 0.001 for comparisons indicated by dashed line. Date are shown as mean ± SEM. N=8/group. Abbreviations: VEH, vehicle.

Discussion

In this study, we found that dopamine D1 receptors contribute to eye blink responses in mice. Specifically, we showed that the D1 agonist SKF 82958 increased eye blinks and this response was selectively inhibited by the D1 receptor antagonist SCH 23390. The TS therapeutic haloperidol decreased both spontaneous eye blinks and those induced by SKF 82958; however, this result may be due to the overall sedative effect of haloperidol and not specific to D2 mechanisms [310]. Another TS therapy, clonidine was also investigated but it did not decrease eye blinks following SKF treatment. These studies are in agreement with previous research on Sprague-Dawley rats [157] and primates [158-161, 311] that have all demonstrated that D1 receptor specific agonists increase eye blinks in the species of study. The results presented here extend this work to include mice in the group of experimental models to study the contribution of dopamine D1 receptors to eye blink responses. In addition, this is the first study to measure eye blinks in unrestrained rodents. This is a very important methodological consideration since we also found that eye blinks in mice are sensitive to restraint stress. Interestingly, an acute restraint stress of 20 minutes was sufficient to drastically increase eye blink responses. This response was ablated by the D1 receptor antagonist SCH 23390 and the alpha-2 adrenergic receptor agonist clonidine. Haloperidol had no effect on eye blinks in this experiment.

The relevance of these data to TS are multifold. It has been demonstrated that alterations in dopaminergic activity directly alter spontaneous eye blink rates. For example, an increase in eye blinks is often the first reported symptom of TS [150, 151] and further studies have shown that the spontaneous blink rate in TS patients is higher than in controls [152-154]. Since spontaneous eye blinks is considered a measure for central dopaminergic activity, the increase of spontaneous eye blinks in TS patients is hypothesized to reflect the hyperdopaminergic state of these patients. It has

also been observed that patients with other disorders of the dopamine system have alterations in spontaneous blinking rates. For example, schizophrenic patients, another group of patients considered to have increased dopamine levels also display increased blinking [312-314]. Alternatively, patients with Parkinson's disease, which is characterized by low dopamine levels, have decreased eye blinking [313-315]. These clinical observations have been mirrored in the MPTP primate model of Parkinson's disease [316, 317].

TS symptoms are underpinned by hyperdopaminergic systems in the CSTC loop [45]. While the basal ganglia do no directly regulate blinking, this region can modulate input to the spinal trigeminal complex through the superior colliculus and nucleus raphe magnus [156, 162-169]. The spinal trigeminal complex has been posited to be directly involved in the spontaneous blink generator circuit and furthermore, it has been proposed that basal ganglia dopamine projections inhibit this complex through the inhibition of the superior colliculus, and subsequent excitation of the nucleus raphe magnus [162, 163, 167, 309, 318]. It has been demonstrated that reduced dopamine levels and dopamine antagonists increase the trigeminal reflex blink amplitude and excitability, which results in a decrease in the spontaneous eye blink rate [164, 165, 309]. Alternatively, apomorphine, a dopamine receptor agonist decreases the trigeminal reflex blink and spontaneous eye blink amplitude and is associated with an increase in the eye blink rate [165, 309]. Therefore, an increase in dopamine activity in TS patients may increase blinking rates through this system.

In addition, these data, along with previously published data investigating D1 receptor agonists on eye blink responses, underline a fundamental role of D1 receptors in eye blink responses and ultimately eye blink tics. The contribution of dopamine D1 receptors in the pathogenesis of TS is poorly understood, although the D1 receptor antagonist ecopipam has shown

efficacy in clinical trials [56] suggesting a critical role for D1 receptors. Research on animal models further supports D1 receptors as a key therapeutic target for TS as outlined in these and other animal studies on Tourette syndrome [277, 319, 320].

These data are also in agreement with clinical findings that tics in TS patients are exacerbated by stress. Furthermore, it may in part explain the lack of efficacy of some TS therapeutics to address stress-induced fluctuations of symptoms. As seen here, haloperidol is a poor therapeutic to address stress-induced TS-like eye blink responses. However, the D1 receptor antagonist SCH 23390 decreased eye blinks both in groups challenged with the D1 receptor agonist SKF 82958, as well as mice exposed to restraint stress. In addition, while clonidine did not ablate SKF 82958 induced eye blinks, the drug did attenuate the eye blink response to stress. These results suggest that targeting the D1 receptor or the adrenergic system could be a more appropriate therapeutic options to address stress- induced Tourette syndrome symptom fluctuations. However, more research on these fluctuations are necessary to better understand the contributions of stress to this disorder and develop more appropriate therapeutics.

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5. Neurosteroids in TS pathogenesis

Sections of this chapter has been adapted from:

Bortolato, M., Frau, R., Godar, S.C., Mosher, L.J., Paba, S., Marrosu. F., Devoto, P., *The implication of neuroactive steroids in Tourette's syndrome pathogenesis: A role for 5α-reductase?* J Neuroendocrinol, 2013. **11**: p. 1196-208

Introduction

The research presented in the previous chapters identifies stress as a key component to exacerbate TS-like behaviors or replicate dopamine induced TS-like behaviors. This has been highly corroborated by clinical observations that have identified stress as a factor in the waxing and waning course of symptom severity. However, the molecular underpinnings of this observation remain poorly understood. The possible contributions of stress to TS symptoms are numerous and will be discussed in the upcoming chapter.

Clinical studies associating psychosocial stress and tics

While it has been assumed that stressful life events may impact both the onset of TS and tic exacerbations later in the course of the disorder, the clinical research has not fully supported these assumptions. This is especially the case where TS onset is concerned and is highlighted in two conflicting reports published almost 20 years apart. Bornstein et al [321] published a large survey that identified several adverse events as common occurrences in the year prior to TS onset, which included fever, undergoing operations using general anesthesia and emotionally tense events such as divorce of parents or moving to a new home. However, a subsequent publication by Horesch et al [322] did not find a greater number of stressful events leading up to the onset of symptoms. Therefore, further research is necessary before conclusions can be drawn on these studies.

The research on the impact of psychosocial stress in relation to tic exacerbations has been more thoroughly researched. Psychosocial stress or anxiety has been reported repeatedly to worsen tics [321, 323], with greater correlation between the severity of tics and daily life stressors rather than global stress level [324]. Interestingly, TS patients also reported a higher number of stressful life events, as well as an increased level of perceived stress [324]. Similar findings, although not all to the same degree of significance have been repeatedly reported [325-330]. Finally, as mentioned in chapter 1 it has been reported that maternal psychosocial stress during pregnancy is a strong predictor of tic severity [88, 90].

At a more acute level, there have also been limited reports on the effects of psychosocial or physiological stress on tic fluctuations through the course of the day. One series of studies reported that thermal stress increased the frequency of tics [331, 332]. Studies on short-term effects of psychosocial stress have indicated that activities that elicit feelings of tension can result in an increased frequency of tics. These activities include socializing and watching movie clips that provoked anticipation [333-335]. In addition, activities that require intense concentration, such as mental math, have been demonstrated to decrease the patient's ability to suppress tics, indicating that psychosocial stress may not directly influence tic frequency but rather reduce the ability to suppress tics [336, 337]. In line with this finding, it has been posited that stressful events may increase cortex excitability that thereby compromise motor control which is needed for tic suppression [336, 338, 339].

The interactions between stress and dopamine.

In line with the clinical observations described above, TS patients exhibit a more pronounced activation of the hypothalamic-pituitary-adrenal (HPA) axis than controls. The HPA axis is a multistep biochemical pathway that is activated in response to a perceived stress. In brief,

the prefrontal cortex and/or the amygdala perceive an environmental stressor and transmit this information to the paraventricular nucleus (PVN) of the hypothalamus. The PVN releases corticotrophin-releasing hormone (CRH), which acts on receptors on corticotropic cells in the pituitary gland to trigger the release of adrenocorticotropic hormone (ACTH) into the bloodstream. In the adrenal gland, ACTH induces the secretion of glucocorticoids (cortisol in humans and corticosterone in rodents) from the cortex. Through the steroid hormone receptors in the brain, these periphery-synthesized steroids are able to regulate signaling pathways and long-term remodeling of dendrites and synapses [340, 341]. In addition to the glucocorticoids, there is also an increase in the synthesis of other neurosteroids, namely 3α, 5α-tetrahydroprogesterone (allopregnanolone, AP) and 3α, 5α tetrahydrodeoxycorticosterone (alloTHDOC) [342-344]. AP and alloTHDOC are among the most potent allosteric modulators of $GABA_A$ receptors and through this receptor they aide in closing the stress cascade by suppressing the endocrine response to stress. In this manner they restore the HPA axis to homeostatic functioning [343-349]. The adrenal gland and the gonads are the primary sources for AP in the periphery; however, the brain also synthesizes neurosteroids to mediate the stress cascade. In fact, AP levels increase in response to stress within the brain even in animals that have been adrenalectomized or gonadectomized [350].

Clinical evidence highly supports that TS patients have a stronger activation of the HPA axis than controls. In fact, several studies have shown increased salivary cortisol levels, plasma ACTH, and CRH following various stressors, including mock MRI scans and lumbar puncture [351-353]. A few conflicting studies have not found increases in cortisol levels; however, these studies took blood samples in the morning so differences may have been masked by the circadian rise in plasma cortisol levels early in the day [353, 354]. To date there have not been reports on the levels of AP or alloTHDOC following acute stress in TS patients.

There is a plethora of evidence indicating that stress impacts the dopaminergic system. In humans it has been demonstrated that psychosocial stress induces dopamine release in the ventral striatum and prefrontal cortex [355-357]. Furthermore, in rodent studies, stress has been shown to increase extracellular dopamine concentrations in several brain regions of interest to TS and comorbid disorders. These include the prefontal cortex [358-362], the amygdala [363] and the nucleus accumbens [364, 365]. In addition, Pat et al [366] demonstrated that ovine CRH stimulated dopaminergic neuron terminals in the nucleus accumbens of rats. These findings are very interesting to TS pathogenesis for several reasons. 1) Enhanced dopamine activity following stress may lead to a imbalance of the direct and indirect pathways of the striatum and thus lead to an increase in tics; 2) The prefrontal cortex is highly involved in the patient's ability to suppress tics; therefore, abnormalities in dopamine signaling in this brain region may lead to a decreased ability to suppress tics and lead to an enhancement of tic frequency. This may in turn explain how stressors that require intense concentration, such as the mental math exercise, can reduce the ability of patients to suppress tics [336, 337].

The reciprocal of these observations has also been found, or in other words, altered dopaminergic systems might induce alterations in the physiological stress response. Despite findings that TS patients exhibit enhanced HPA responses to stressful stimuli, clinical data assessing a direct relationship between altered dopamine levels or signaling pathways and stress responsivity is lacking [336, 351-353]. However, in animal studies there has been extensive research that indicates modulation of physiological stress responses from dopamine [367-370].

These clinical and preclinical data suggest that there are many points of interaction between the stress cascade and the dopamine system. However, the exact nature of these interactions remains unclear. Based on the current research, there are several possible hypotheses that can be

stated [336]: 1) psychosocial stress initiates and/or exacerbates tics through the activation of dopaminergic neurotransmission; 2) dysfunctions within the dopaminergic system leads to the elevated physiological stress response: 3) abnormalities in both the physiological stress response and underlying hyperdopaminergic state of TS feed off each other in a feedforward loop that aggravates and exacerbates both issues. It is outside the scope of this dissertation to fully determine which of these hypotheses may be most correct; however, some parts of these questions will be addressed.

Neurosteroids and dopamine: how do neurosteroids affect the course of TS and interact with dopamine?

Several avenues of potential research and unanswered questions arise from the clinical and preclinical data discussed. However, the research presented here will focus on the neurosteroids that are upregulated following acute stress, specifically AP and to a lesser extent alloTHDOC. This research has followed clinical and preclinical findings from our laboratory that have pinpointed a key role for neurosteroids to modulate dopamine-mediated responses. In addition, our previous data has indicated a therapeutic potential for targeting the synthesis of AP and alloTHDOC, along with other potent neurosteroids, in the treatment of TS. Since this is a largely understudied aspect of TS, these studies will be especially valuable in understanding the waxing and waning nature of TS and in identifying novel therapeutic targets.

Acute stress increases the synthesis of AP and alloTHDOC (Fig 5.1) via an increase in the enzyme 5α-reductase (5αR) [371-373]. There are two isoenzymes of 5αR that are capable of mediating this reaction, which consists of the saturation of the 4,5 double bond of the A ring of Δ4-3-ketosteroid substrates, including deoxycorticosterone, progesterone, androstenedione and testosterone [374]. This step in steroidogenesis forms the precursors to AP and alloTHDOC, 5α-

dihydroprogesterone and 5α-dihydrodeoxycorticosterone, respectively. While these isoenzymes carry out identical reactions and are both found in the membrane of the endoplasmic reticulum, 5αR type 1 (5αR1) and 5αR type 2 (5αR2) vary in several key characteristics. The most important of these are the localization throughout the body, substrate affinity, and pH optima [374]. 5αR1 was the first isoenzyme to be isolated and is encoded by the gene *SRD5A1*. This enzyme is expressed in cells and structures originating in the ectoderm, including epidermal keratynocytes, melanocytes, sebaceous and sweat glands, neurons of the central and peripheral nervous system and adrenal glands [375-379]. 5αR1 has also been found in fibroblasts, hepatocytes and various organs including the prostate, lung, colon and kidney [376, 380-384]. Furthermore, 5αR1 is the predominant isoenzyme in the adult brain [383, 385-391]. The substrate affinity (Km) of the substrates, testosterone and progesterone are 1.7uM and 1.3uM respectively. This enzyme also functions optimally at a neutral pH range of 6-8.5. In contrast 5αR2, encoded by *SRD5A2*, is found primarily in organs and tissues of the male urogenital tract (prostate, epididymis, testicles, and seminal vesicles), genital skin, hair follicles and liver [381]. Within the brain, the 5αR2 transcript has been mainly identified in early development stages [392]; however, this isoenzyme has also been found in the adult brain [393]. Interestingly, the brain regions where 5αR2 is found is also more distinct than 5αR1, which is found throughout the brain. 5αR2 is most highly expressed in the hypothalamus, prefrontal cortex, and nucleus accumbens [372, 392, 394]. In further contrast to 5αR1, 5αR2 has a higher affinity for testosterone (Km = 0.2uM) and progesterone (Km = 0.2uM) and the optimal pH range is much narrower, 5-5.5 [374]. These characteristics indicate that the role of 5αR2 is much more heavily regulated than 5αR1 and the two isoenzymes may function in phasic and tonic manners respectively. It is also worth noting that due to the localization of 5αR2, the primary physiological function of 5αR2 is the development of male genitalia and androgen

synthesis. In humans, mutations in 5αR2 lead to normal urogenital tracts but underdeveloped external male genitalia and prostate [395].

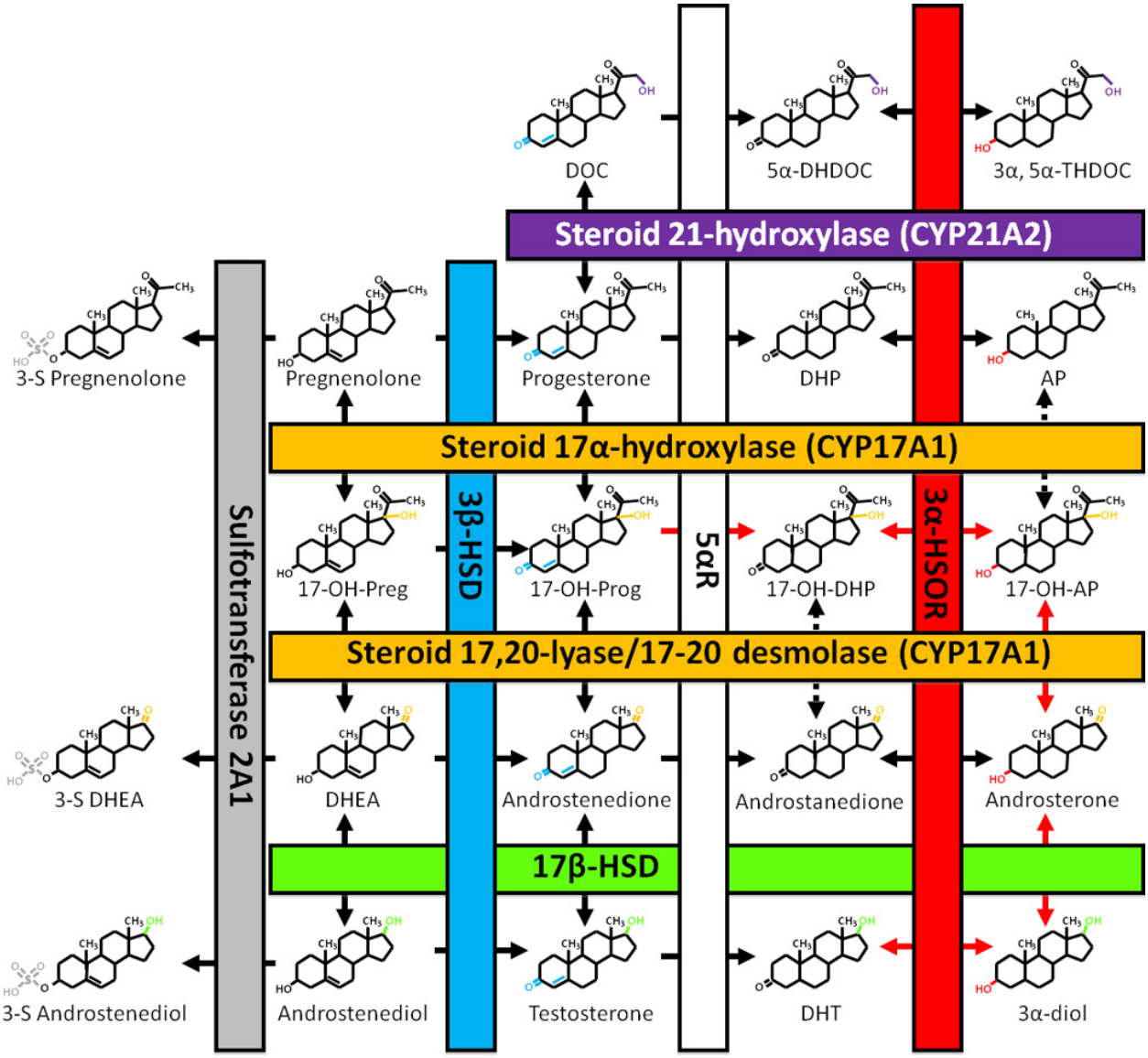

Figure 5.1 Schematization of major neurosteroidogenic pathways.
Metabolic changes in steroid configurations are represented in the same color as the enzymes (boxes) catalyzing the reactions. Red arrows represent the major reactions corresponding to the "backdoor" pathway of DHT synthesis. Dotted arrows represent reactions that have been hypothesized, but not fully ascertained in the brain. Enzymes: 3β-HSD; 3β-hydroxysteroid dehydrogenase; 5αR, 5α-reductase 17β-HSD: 17β-hydroxysteroid dehydrogenase; 3α-HSOR: 3α-hydroxysteroid oxidoreductase; CYP21A2: Steroid 21-hydroxylase; CYP17A1: cytochrome P450 17A1. Steroids: DOC, deoxycorticosterone; 5α-DHDOC, 5α-dihydro deoxycorticosterone; 3α,5α-THDOC, 3α,5α-tetrahydrodeoxycorticosterone; 3S- pregnenolone, pregnenolone sulfate; DHP, 5α-dihydroprogesterone; AP, 3α,5α-tetrahydroprogesterone (allopregnanolone); 17-OH-Preg, 17-hydroxypregnenolone; 17-OH-Prog, 17-hydroxyprogesterone; 17-OH-DHP, 17-hydroxydihydroprogesterone; 17-OH-AP, 17-hydroxyallopregnanolone; 3-S DHEA dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone; 3S- androstenediol, androstenediol sulfate; DHT, 5α-dihydrotestosterone; 3α-diol, 5α-androstane-3α,17β-diol.

Since 5αR mediates the rate limiting step in steroidogenesis it has been the target of several pharmacological therapies. In particular, the 5αR inhibitors, finasteride and dutasteride, are prescribed for benign prostate hypoplasia and male-pattern alopecia; however, research is being conducted to utilize these compounds for other disorders [374]. One of the most commonly prescribed 5αR inhibitors is the compound finasteride. This compound completely inhibits 5αR2 at clinical doses but not 5αR1; however, due to the chronic nature of finasteride treatment there is significant inhibition of both isoenzymes [396-398]. Importantly, finasteride has a very low dissociation constant for 5αR2 (K_i = 3-5 nM), which results in a half-life of about 30 days making this inhibition almost irreversible in the short term [383]. The side effects of finasteride are very limited and are most commonly described are decreased libido, ejaculatory disorders and erectile dysfunction [399-402]. Of note, however, a subset of patients has developed a severe untreatable depression following treatment with finasteride for the FDA approved uses (alopecia and benign prostate hypoplasia) which, did not remit upon termination of finasteride treatment and while rare, has resulted in instances of suicide [403-406]. Therefore, further research is warranted concerning the role of finasteride and 5αR in the mediation of depressive behaviors.

The research surrounding the use of finasteride as a therapy for TS started with several key clinical observations:

1) The stress sensitivity described in the introduction and further expanded on in this chapter highlights a possible role for AP and alloTHDOC
2) The age of onset of TS coincides with adrenarche. This is the time period when the innermost zone of the adrenal gland, the zona reticularis develops and begins producing DHEA, DHEA-sulfate (DHEAS) and small amounts of testosterone [407-410].

3) Following puberty, tics and other symptoms remit in many male patients. Although, in contrast it has been noted that there is a high occurrence of tic disorders in familial male precocious puberty [411], which may imply that steroidal changes involved in puberty may be conducive to tic exacerbation, rather than remission. This is in line with the increase in symptoms up through puberty and the remittance being observed in late adolescence. Other neurodevelopmental aspects during this time frame may also account for the decrease in symptoms following puberty, such as maturation of the dopaminergic system and sexually dimorphic brain structures.
4) This disorder is highly sex dimorphic. Female patients often do not exhibit a remission in symptoms as they age and tics can actually worsen as they enter young adulthood [112]. Furthermore, males are diagnosed with this disorder at a much higher rate (4:1) over females. The severity of TS symptoms is also correlated with a preference for masculine play and some of the core features of TS are reminiscent of androgen-mediated behaviors such as impulsivity, aggressiveness, rage, increased sex drive and premature erotic urges [113, 301, 412-415]. These observations indicate that androgens may play an important role in the pathogenesis of this disorder. Alternatively, as suggested in #3, this dimorphism may also result in the contribution of sexually dimorphic brain structures. However, it has also been noted that exogenous androgens exacerbate TS symptoms [110] suggesting a more direct role for androgen steroids.

Addressing all of these possible lines of research is outside the scope of this dissertation; however, these observations in conjunction with promising preclinical results led to clinical trials conducted by our laboratory. The subjects were limited to adult males with refractory TS; however, the findings indicated that finasteride would be a promising therapeutic option [416, 417]. In these

studies, finasteride reduced the severity and frequency of both motor and vocal tics and did not induce side effects associated with the typical anti-dopaminergic therapeutic options. In addition, other therapies have been investigated that target the androgen receptors; however, these therapies had short-lived efficacy and a marked potential for severe hepatic side effects [418-421]. Despite these promising results there are drawbacks to the use of finasteride as a TS therapy. For one, the induced depression is a critical issue that must not be ignored. Furthermore, this drug cannot be used on male children, who represent the clear majority of patients, due to the demasculinizing effects of inhibiting androgen synthesis during this critical stage in development. Therefore, we have expanded our research to explore finasteride's mechanisms of action to identify novel targets and therapeutic options for children. We have focused primarily on targeting the stress induced tic fluctuations since this is a therapeutic avenue that many drugs do not target.

Our preclinical research to the point of this dissertation was focused on the interaction between steroids and dopamine modulation. This research has led to several important findings and paved the way for the research presented in this dissertation. The first study conducted by Bortolato et al. in 2008 [422] demonstrated that both finasteride and dutasteride could ablate the PPI deficits induced by the nonspecific dopamine receptor agonist apomorphine and amphetamine. In subsequent studies it was identified that finasteride infusion directly into the nucleus accumbens core or shell replicated the effects of the systemic administration of finasteride on apomorphine-induced PPI deficits [423]. These studies were both conducted in rats using general dopamine receptor agonists; in contrast, finasteride did not affect the reductions of PPI produced by apomorphine in mice. However, finasteride did attenuate the PPI deficits induced by the D1 receptor agonist SKF 82958. Interestingly, whereas the D2 receptor agonist quinpirole is not capable of inducing PPI deficits in mice alone, the combination of quinpirole and finasteride

produced significant PPI deficits [277]. The replication of these results in rats has been limited by the assumption that while mice are susceptible to PPI disrupting effects from D1, but not D2, receptor agonists, the opposite is true for rats. However, further investigation has found that this is a strain, rather than species, specific difference [267], and so part of the research presented in the remainder of this dissertation will focus on translating these findings from mice to rats.

In summary this evidence points to a critical role for stress steroids in the mediation of TS symptom fluctuations throughout the course of the disorder. In the following chapters, research that aims to address the mechanism of these observations will be discussed. Whether or not psychosocial stress activates dopamine neurotransmission to exacerbate tics, abnormal dopamine sensitizes the HPA axis to elevate the patient's response to stress, or a combination of the two, it is clear that TS patients have a stronger HPA axis activation over controls. This elevated HPA response would also increase 5αR activity and drive an increase in AP and alloTHDOC that could promote the exacerbation of TS symptoms. This hypothesis will be explored in the presented research by investigating the responses of the TS animal models described in chapters 3 and 4 to neurosteroids and/or the inhibition of steroidogenesis. The molecular mechanisms underpinning these observations will also be investigated through genetic knockout and pharmacological means. In addition, we will address the issue of 5αR1 versus 5αR2 as they contribute to this disorder and parse out the separate roles of each isoenzyme. Based on the specific characteristics outlined above, such as the role of 5αR2 to mediate androgen synthesis, the distinct pH optima and localization in the brain (areas that mediate emotional responses), we hypothesize that targeting 5αR1 specifically may be a therapeutic option to reduce levels of AP and reduce increases in tics as a result of stress, without causing demasculinization or depression in the patients. These

experiments will focus on mice with genetic deficiencies of either isoenzyme to determine the separate roles of each in PPI regulation and depressive-like behaviors.

Mechanisms of AP action in the brain

Following the research described above, we also aimed to determine the molecular mechanisms through which AP mediates dopamine-stress interactions. As highlighted above, AP is a positive allosteric modulator for the $GABA_A$ receptor at nanomolar concentrations [424, 425] and is capable of directly opening $GABA_A$ at micromolar concentrations [426, 427]. In the capacity of a positive allosteric modulator, AP enhances the actions of $GABA_A$ by prolonging the opening time of the chloride ion channels [428, 429]. It has been demonstrated that the subunit composition of $GABA_A$ alters the effectiveness of AP [426]. The five subunits of $GABA_A$ consist of two α subunits, two β subunits, and either a δ or γ subunit [430]; $GABA_A$ receptors containing the δ subunit are modulated by AP to a greater extent than those containing the γ subunit [431-433]. The contribution of these subunits to the effectiveness of AP is unknown and binding sites involving these subunits have not been identified. The two known binding sites for AP consist of binding to the trans-membrane domain of α-subunits (to potentiate GABA) and the interfacial residues between α and β subunits (to directly activate $GABA_A$) [427]. Through this receptor AP acts to shut down the stress cascade to protect the brain against excitotoxity following stress [434]. This is accomplished by increasing the GABAergic inhibitory neurotransmission in the CRH-releasing parvocellcular neurons of the hypothalamus PVN which results in the downregulation of CRH and ACTH gene transcripts [349, 435-437]. AP also modulates GABAergic neurotransmission in other parts of the limbic system and prefrontal cortex [345, 438-441].

Another receptor of interest is the pregnane x receptor (PXR). PXR is a nuclear hormone receptor that has largely been studied in the periphery and especially in the liver where it has been

found to been a key regulator in drug clearance through the transcriptional activation of drug detoxification genes [442]. In addition, PXR regulates genes involved in cholesterol and bone homeostasis [443, 444]. A diverse array or endogenous and exogenous compounds and chemicals activate PXR, which include pregnanes (such as AP), glucocorticoids, bile acids, vitamin E and vitamin K2, and drugs such as rifampin and protease inhibitors, in addition to environmental contaminants [443, 445-450]. The expression of PXR is widespread throughout the body of both humans and rodents [443, 447, 448, 451], including several regions of the brain [452-455]. Of note to steroidogenesis and dopamine modulation, it has been found that PXR knockdown in rats decreased the levels of AP in the midbrain, striatum and hippocampus and interferes with dopamine motivated behaviors such as mating [453, 454, 456-460]. These findings suggest that PXR is both a receptor for AP and acts as part of a feedforward mechanism to produce more AP. In addition, these data support the hypothesis that AP modulates dopamine mediated behaviors. To further this research and apply it to our studies we will be studying the effects of AP in PXR KO mice.

Finally, we investigated contributions of the purinergic receptor P2X4R [461]. A large portion of the research on P2X4R in the brain has focused on the role of P2X4R in inflammation and microglia, which may contribute to some of the observed risk factors for TS, including β-streptococcus infection, although this possible connection has not been fully explored. The P2X4R is part of the purinergic ionotropic P2X family of receptors, which are homotrimeric cation-permeable channels that are activated by extracellular adenosine 5’-triphosphate (ATP) [462, 463]. P2X4R is the most abundant of these in the central nervous system [464, 465], where this receptor is expressed in both neurons and microglia [466, 467]. Preclinical research has found that ivermectin, a positive allosteric modulator of P2X4R induced anxiolytic-like effects and PPI

deficits [468]. Interestingly, knockout of P2X4R also induces PPI deficits, as well, as sociocommunicative impairments [469]. These findings are in agreement with other reports that P2X4R modulate N-methyl-D-aspartate (NMDA) glutamate receptors [470] that are known to be involved in emotional and cognitive responses [471] and have been implicated in autism-spectrum disorder (ASD). Furthermore, NMDA receptor antagonists have been shown to induce PPI deficits in rodent models [472] so modulation of these ion channels by P2X4R may explain the alterations in sensorimotor gating capabilities in the mouse studies described above. P2X4R have also been shown to regulate striatal dopamine homeostasis, which would also contribute to sensorimotor gating functions [473]. To date there have not been any studies exploring the role of P2X4R in AP mediated effects outside of the original study identifying AP as a P2X modulator [461]. Therefore, we will begin characterizing the effect of AP in the P2X4R KO mice as an initial step in determining these interactions.

6. Allopregnanolone mediates the exacerbation of Tourette-like responses by acute stress in mouse models

This chapter has been adapted from:

Mosher, L.J., Godar, S.C., Nelson, M., Fowler, S.C., Pinna, G., Bortolato, M., *Allopregnanolone mediates the exacerbation of Tourette-like responses by acute stress in mouse models.* Sci Rep, 2017. **7**(1): p. 3348

Introduction

As previously discussed tics are highly variable in intensity and frequency. Among other factors, these symptom fluctuations are posited to reflect the impact of select physical and psychological stressors [17, 39, 474]. The neurobiological mechanisms whereby tics are exacerbated by contextual triggers, however, are poorly understood; as a result, no pharmacological interventions are currently available to prevent or mitigate tic aggravation in response to stress. Therefore, to further study the role of stress and stress steroids in an animal model of TS we expanded on our findings presented in chapter 3 where we documented that D1CT-7 mice display exacerbated tic-like responses and PPI deficits in response to a naturalistic environmental stressor, consisting of a 20-min spatial confinement (SC) within a 10-cm diameter cylindrical enclosure in their home cages. In particular, we focused on the stress steroid, AP and how this steroid contributes to the stress-induced fluctuations observed in the D1CT-7 mouse. We limited our analysis to the prefrontal cortex (PFC), given that rich evidence has established that this region is particularly sensitive to the effects of acute stress on neurosteroid synthesis [342] and plays a fundamental role in the control of tics (and its modulation by stress) in TS [43, 336].

Methods

Animals. We used 3–4-month-old, experimentally naïve male Balb/c mice (n = 8–15 per genotype and treatment group) weighing 20–30 g. Animals were purchased from Jackson Labs (Bar Harbor, ME) and genotyped as previously described [255]. Since the inheritance pattern of D1CT-7 mutation is autosomal dominant, we bred WT females with heterozygous D1CT-7 sires. This breeding scheme was selected to standardize maternal behavior. Animals were housed in group cages with ad libitum access to food and water. The room was maintained at 22 °C, on a 12 h: 12 h light/dark cycle from 8 am to 8 pm. Animals were tested during their light cycle between 12 and 4 pm to minimize any potential circadian effects. All experimental procedures were in accordance with the NIH guidelines and approved by the IACUCs of the Universities of Kansas and Utah.

Drugs. The following drugs were used: progesterone, DHP, AP (Tocris Bioscience, Bristol, UK), finasteride (Astatech, Bristol, PA) and haloperidol (Sigma-Aldrich, Saint Louis, MO). Finasteride, progesterone, DHP and AP were suspended in 5% Tween 80, diluted with 0.9% saline, and administered by IP injection in a 10 ml/kg volume. Haloperidol was dissolved in 10% acetic acid buffered with NaOH and diluted with saline.

Dissection of brain regions. Immediately after decapitation, brains were frozen and the frontal portion cut into 1-mm-thick slices using a Jacobovitz brain slicer (Zivic Miller, Portersville, PA). The slices obtained from 1.18 to 0.14 anterior to bregma were mounted on a coverslip kept at 4 °C and disks (1.5-mm diameter) were punched out from these slices.

Measurement of neurosteroid content. Extraction, derivatization, and GC-MS analyses of neurosteroids were performed with minor modifications as described [475, 476]. The steroid measurements included progesterone, 5α-dihydroprogesterone, AP, and pregnanolone (3α, 5β-tetrahydro-progesterone). Supernatants were extracted with ethyl acetate and,

after lyophilization, neurosteroids were purified and separated by HPLC. Tritiated neurosteroids (American Radiolabeled Chemicals, St. Louis, MO) were added to monitor retention time through HPLC while deuterated internal standards (CDN Isotopes, Pointe-Claire, QC, and Steraloids, Newport, RI) were added to allow quantification of the compound of interest. The HPLC fractions containing progesterone, AP, and pregnanolone were derivatized with heptafluorobutyric acid anhydride (HFBA) (Supelco, Bellefonte, PA). 5α-dihydroprogesterone was derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA)-ammonium iodide (NH_4I)/1,4-dithioerythritol(DTE)/ acetonitrile(CH_3CN) (Sigma-Aldrich) in a ratio of 1,000/2/5/1,000, and subjected to GC-MS. Mass fragmentography analysis of derivatized hormones was performed in the standard electron impact mode with a detection limit of ≈10 fmol and intra-assay coefficients of variation less than 5%. Neurosteroids were identified based on their GC/MS retention time characteristics; the definitive structural identification of each neurosteroid was provided by its unique mass fragmentation pattern. To calculate the quantity of the neurosteroid of interest in each fraction, the area under the peak of the neurosteroid in the sample was divided by the area under the peak of the deuterated internal standard. Only peaks with a signal-to-noise ratio greater or equal to 5:1 were integrated.

Western Blot. Samples were homogenized on ice in a buffer containing 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 320 mM sucrose, protease and phosphatase inhibitor cocktail. Homogenates were centrifuged for 5 min at 3000 x g to precipitate nuclei; supernatant fractions were collected and centrifuged at 20,000 x g for 60 min. The resulting pellet (P2) was solubilized in T-PER lysis buffer (Tissue Protein Extraction Reagent, Pierce, Rockford, IL) supplemented with protease and phosphatase inhibitor cocktail. Small aliquots of the homogenate were used for protein determination by a modified Lowry protein assay method (DC protein assay, Bio-Rad

Laboratories, Hercules, CA). Equal amounts of proteins were separated on a 4–15% Criterion TGX Precast Gel (Bio-Rad Laboratories) by electrophoresis and transferred to a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer system (Bio-Rad Laboratories). Membranes were then blocked with 3% BSA (Sigma-Aldrich) in TRIS-buffered saline supplemented with 0.1% Tween 20 for 2 h at room temperature and then incubated overnight with primary antibodies (1:1000) at 4 °C. Primary antibodies used in this study include the following: anti-actin (ab3280, Abcam, Cambridge, MA); anti-$GABA_A$ receptor α1 (NB 300-191, Novus Biologicals, Littleton, CO); anti- $GABA_A$ receptor α4 (NB 300-194, Novus Biologicals); anti-$GABA_A$ receptor δ (ab111048, Abcam); and anti-$GABA_A$ receptor π (ab26055, abcam). These $GABA_A$ receptor subunits were chosen as they express a high affinity for neurosteroids and have been indicated to regulate the binding of AP and neurosteroids to $GABA_A$ receptors [477-479]. After washing, membranes were incubated with HRP-conjugated secondary antibodies. Antibody binding was detected using Clarity ECL substrate (Bio-Rad Laboratories) and proteins were analyzed by the ChemiDoc Touch system and the Image Lab software (Bio-Rad Laboratories). Membranes were stripped and re-probed with an anti-actin antibody for normalization.

Behavioral Studies. Tic-like manifestations were scored by trained observers blinded to the treatment, as previously indicated [256]. Tic-like manifestations were defined as rapid (<1 second) twitches of the head and/or body. SC, PPI and locomotor analyses were carried out as previously described in chapter 3.

Statistical analyses. Normality and homoscedasticity of data distribution were verified by using Kolmogorov-Smirnov and Bartlett's tests. Statistical analyses of parametric data were

performed with one-way or multi-way ANOVAs, followed by Newman-Keuls' test for post-hoc comparisons. The significance threshold was set at 0.05.

Results

SC increases neuroactive steroid levels in the prefrontal cortex (PFC). The first study was aimed at the measurement of steroid levels in the PFC of D1CT-7 and wild-type (WT) littermates following SC (Fig. 6.1). The analysis of progesterone content (Fig. 6.1A) revealed a significant genotype × confinement interaction [$F(1,17) = 8.15$, $P < 0.01$], reflecting significant differences between confined D1CT-7 mice and either WT controls ($P < 0.001$) or non-confined D1CT-7 mice ($P < 0.001$; Newman-Keuls). In contrast, DHP levels (Fig. 6.1B) were higher in D1CT-7 mice than WT controls [Main effect for genotype: $F(1,21) = 6.03$, $P < 0.05$]; however, SC had no significant effect on the content of this neurosteroid. Furthermore, no significant interactions between genotype and stress were found. The PFC concentrations of AP (Fig. 6.1C) in D1CT-7 mice were higher than those detected in their WT littermates [$F(1,22) = 14.97$, $P < 0.001$]. The same neurosteroid was found to be enhanced by SC [$F(1,22) = 6.38$, $P < 0.05$]. No

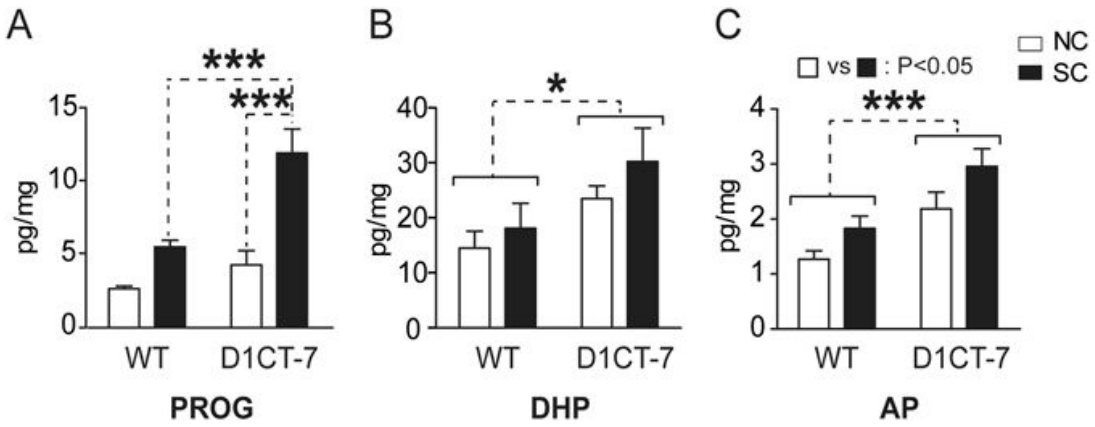

Figure 6.1 Neurosteroid levels in the prefrontal cortex of D1CT-7 and wild-type mice following space confinement. (A) progesterone, (B) 5α-dihydroprogesterone (DHP) and (C) allopregnanolone were measured after 20 min of SC. Data are shown as means ± SEM. *P < 0.05, and ***P < 0.001 for comparisons indicated by dashed lines. Main effects for genotype are indicated as comparisons between brackets. Main effects for SC are indicated as comparison between symbols. N = 4–7/group. NC, no spatial confinement. For further details, see text.

significant genotype × stress interaction was detected. Finally, no significant differences were found in the content of pregnanolone, the 3α,5β-reduced derivative of progesterone (Table 6.1).

NC WT	4.77 ± 4.12
NC D1CT7	0.7 ± 0.18
SC WT	2.38 ± 1.13
SC D1CT7	1.5 ± 0.34

Table 6.1 Levels of pregnanolone in the prefrontal cortex of D1CT-7 and wild-type (WT) littermates following spatial confinement (SC). Data are shown as means ± SEM (in pg/mg). NC, non-confined. N=4-7/group.

AP selectively increases tic-like responses and induces PPI deficits in D1CT-7, but not WT mice. Given that our previous results showed that SC was associated with increased levels of progesterone, DHP and AP, we next tested whether the administration of any of these steroids may reproduce the adverse effects of this stressor on tic-like responses and PPI deficits (13). Behavioral testing began 10 min after steroid injections. Neither progesterone (15 mg/kg, IP) (Fig. 6.2A–D) nor DHP (15 mg/kg, IP) (Fig. 6.2E–H) elicited any significant behavioral change in D1CT-7 mice. The only significant effect detected by these analyses was a significant reduction in startle amplitude in D1CT-7, in comparison with WT counterparts [Fig. 6.2C: $F(1,28) = 45.65$, $P < 0.001$; Fig. 6.2G: $F(1,28) = 44.92$, $P < 0.001$], as previously reported (13). However, this effect was not modified by any treatment.

In contrast with these findings, AP (5–15 mg/kg, IP) significantly increased tic-like responses in D1CT-7 mice (Fig. 6.2I) [$F(2,30) = 4.27$, $P < 0.05$; 2-way ANOVA]. Specifically, the 15 mg/kg dose significantly increased the frequency of tic-like responses between 10 and 20 min after the injection ($P < 0.01$; Newman-Keuls) (Fig. 6.2I). Conversely, AP did not affect digging behavior in either genotype (Fig. 6.2J). The analysis of startle magnitude (Fig. 6.2K) showed only statistical trends with respect to main effects of genotype [$F(1,57) = 3.62$, $P = 0.06$] and AP treatment [$F(2,57) = 2.53$, $P = 0.09$]; however, no significant interactions between these two factors were detected. Notably, a significant genotype × treatment interaction was found for the

mean PPI values [$F(2.57) = 3.25$, $P < 0.05$] (Fig. 6.2L). Post-hoc analyses showed that the 15 mg/kg dose of AP induced a significant reduction in mean PPI values as compared with WT counterparts treated with the same dose ($P < 0.01$), as well as D1CT-7 treated with either vehicle ($P < 0.01$) or 5 mg/kg of AP ($P < 0.05$).

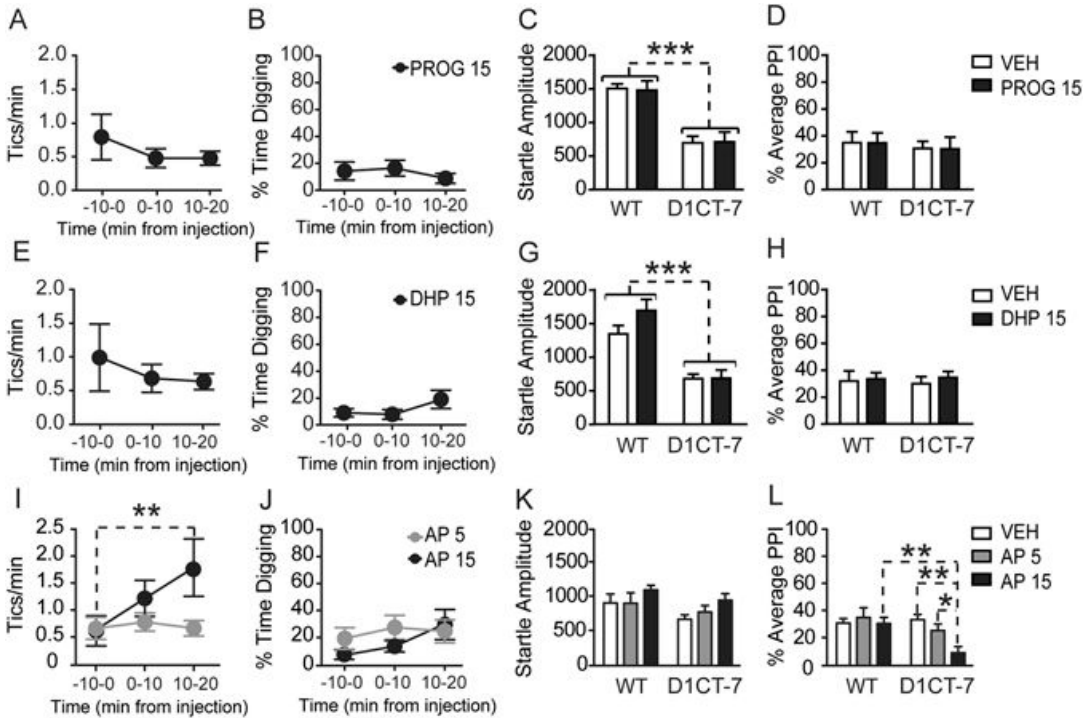

Figure 6.2 Allopregnanolone exacerbates TS-like behaviors in D1CT-7 mice. Effects of (A–D) progesterone (PROG), (E–H) 5α-dihydroprogesterone (DHP) and (I–L) allopregnanolone (AP) on behavioral phenotypes related to Tourette syndrome. Data are shown as means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 for comparisons indicated by dashed lines. Main effects for genotype are indicated as comparisons between brackets. Doses are indicated in mg/kg (IP). N = 8–13/group. VEH, vehicle; WT, wild-type; PPI, prepulse inhibition of the startle. For further details, see text.

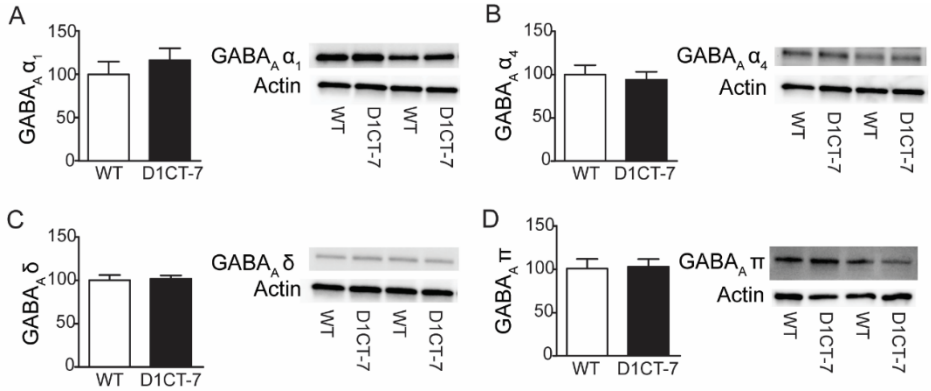

Figure 6.3 D1CT-7 mice do not exhibit alterations in $GABA_A$ receptor subunit expression. Expression of $GABA_A$ receptor (A) α_1, (B) α_4, (C) δ and (D) π subunits in the prefrontal cortex of D1CT-7 and wild type (WT) mice. Data are shown as means ± SEM. Representative gels for each subunit and actin controls are presented. Gel images have been cropped to the band of interest for each protein. N=6/group. For further details, see text.

D1CT-7 mice do not exhibit alterations in the levels of key neurosteroid-binding $GABA_A$ receptor subunits in the PFC. To explore whether the observed effects of AP may reflect changes in $GABA_A$ receptors, we measured the levels of key $GABA_A$ subunits that are posited to modulate the activity of AP and other neuroactive steroids, namely α1, α4, δ and π. Nevertheless, the expression of these proteins in the PFC (as captured by western blotting) was equivalent in WT and D1CT-7 littermates (Fig. 6.3), indicating that the actions of AP were not supported by apparent changes in $GABA_A$ stoichiometry.

AP increases locomotor activity in D1CT-7, but not WT mice. Next, we examined whether the behavioral changes induced by 15 mg/kg of AP were accompanied by variations in locomotor activity. As previously observed [286], D1CT-7 mice exhibited significantly higher locomotion [Main effect of genotype: $F(1,23) = 58.42$, $P < 0.001$]. In addition, a significant genotype × treatment interaction [$F(2,46) = 5.34$, $P < 0.01$] revealed that AP selectively increased the locomotor activity between 10 and 20 min after injection in D1CT-7 mice ($P < 0.01$ in comparison with baseline), but not WT controls (Fig. 6.4A). The analysis of rotation bias (Fig. 6.4B) and velocity (Fig. 6.4C) revealed that these indices were significantly elevated in D1CT-7 mice [Main genotype effects: Rotation bias: $F(1,23) = 19.91$, $P < 0.001$; Velocity: $F(1,18) = 2.39$, $P < 0.001$], but were not altered by AP administration in either genotype. Conversely, D1CT-7 and WT mice showed similar thigmotactic behavior (Fig. 6.4D), but AP increased the average distance from the walls of the arena in both genotypes [Main effect: $F(1,19) = 10.08$, $P < 0.01$]. In confirmation of previous data [480], D1CT-7 mice displayed a decreased stride length [$F(1,18) = 22.81$, $P < 0.001$] (Fig. 6.4E) and an increased stride rate [$F(1,18) = 82.33$, $P < 0.001$] (Fig. 6.4F) compared to WT mice. Neither parameter, however, was affected by AP treatment.

Haloperidol countered the enhancement in tic-like responses induced by AP. We previously documented that haloperidol (0.3 mg/kg, IP) suppressed the increase in tic-like jerks and PPI deficits induced by SC in D1CT-7 mice. Given that our previous resulted showed that AP treatment led to similar effects as those caused by SC, we tested whether its effects may be countered by haloperidol. D1CT-7 mice were pretreated with haloperidol (0.3 mg/kg, IP) 20 min

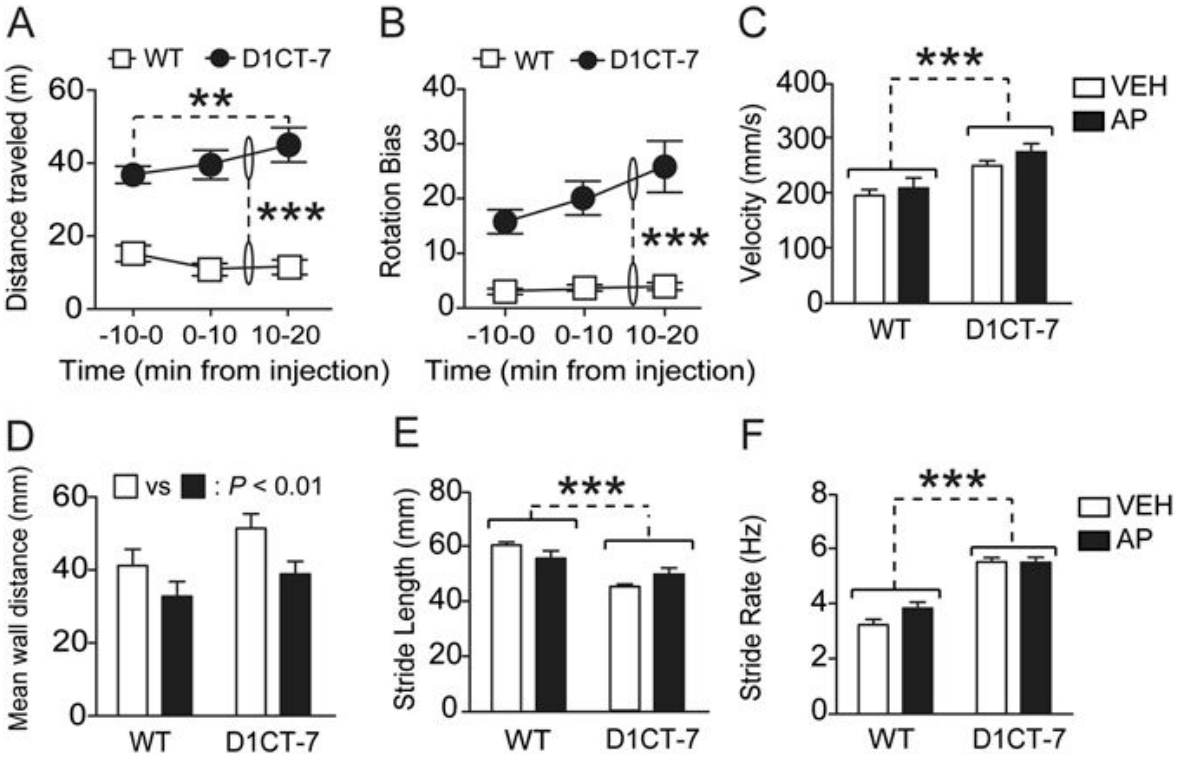

Figure 6.4 Effects of allopregnanolone (AP; 15 mg/kg, IP) on the locomotor activity of D1CT-7 and wild-type (WT) mice. Data are shown as means ± SEM. **P < 0.01 and ***P < 0.001 for comparisons indicated by dashed lines. Main effects for genotype are indicated as comparisons between brackets. Doses are indicated in mg/kg (IP). N = 12–13/group. VEH, vehicle. For further details, see text.

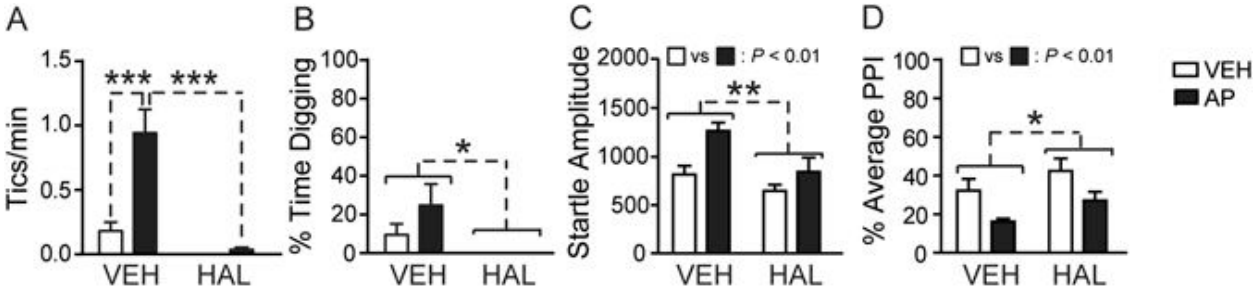

Figure 6.5 Combined effects of haloperidol (HAL; 0.3 mg/kg, IP) and allopregnanolone (AP; 15 mg/kg, IP) on behavioral phenotypes related to Tourette syndrome in D1CT-7 mice. Data are shown as means ± SEM. **P < 0.01 and ***P < 0.001 for comparisons indicated by dashed lines. N = 8–9/group. VEH, vehicle; PPI, prepulse inhibition. For further details, see text.

prior to AP (15 mg/kg, IP) administration. Analysis of tic-like behaviors (Fig. 6.5A) revealed a significant haloperidol × AP interaction [$F(1,31) = 12.92$, $P < 0.001$]. This effect indicated that, while AP increased tic-like responses, pre-treatment with haloperidol suppressed this response (Ps < 0.001). Conversely, digging (Fig. 6.5B) was suppressed by haloperidol, irrespective of AP administration [Main effect of haloperidol: $F(1,31) = 7.02$, $P < 0.05$]. Startle analysis (Fig. 6.5C) revealed that haloperidol reduced the mean amplitude of this parameter [Main effect of haloperidol: $F(1,31) = 8.63$, $P < 0.01$], while AP increased it [Main effect of AP: $F(1,31) = 10.38$, $P < 0.01$]; however, no significant interaction between these two treatments was detected. As expected, PPI was reduced by AP [Main effect of AP: $F(1,31) = 9.58$, $P < 0.01$] and increased by haloperidol [Main effect of haloperidol: $F(1,31) = 4.41$, $P < 0.05$], but these two effects did not significantly interact (Fig. 6.5D).

Finasteride counters the behavioral and neuroendocrine effects of SC. To further assess whether AP mediated the effects of SC in D1CT-7 mice, we then verified whether the observed increase in tics and PPI deficits induced by SC could be countered by finasteride. While progesterone levels in the PFC of confined D1CT-7 mice were confirmed to be higher than those in wild type controls [Main effect of genotype: $F(1,20) = 7.51$, $P < 0.05$], the content of this steroid was not affected by finasteride treatment (Fig. 6.6A). The analysis of finasteride's effects on DHP revealed a statistical trend for a reduction in DHP levels in both wild type and D1CT-7 mice [$F(1,20) = 4.05$, $P = 0.06$] (Fig. 6.6B). Finally, ANOVA detected a significant genotype × treatment interaction on AP levels [$F(1,22) = 6.77$, $P < 0.05$]. Post-hoc analyses revealed that finasteride fully countered ($P < 0.001$) the enhancement in AP produced by SC in D1CT-7 mice ($P < 0.05$) (Fig. 6.6C).

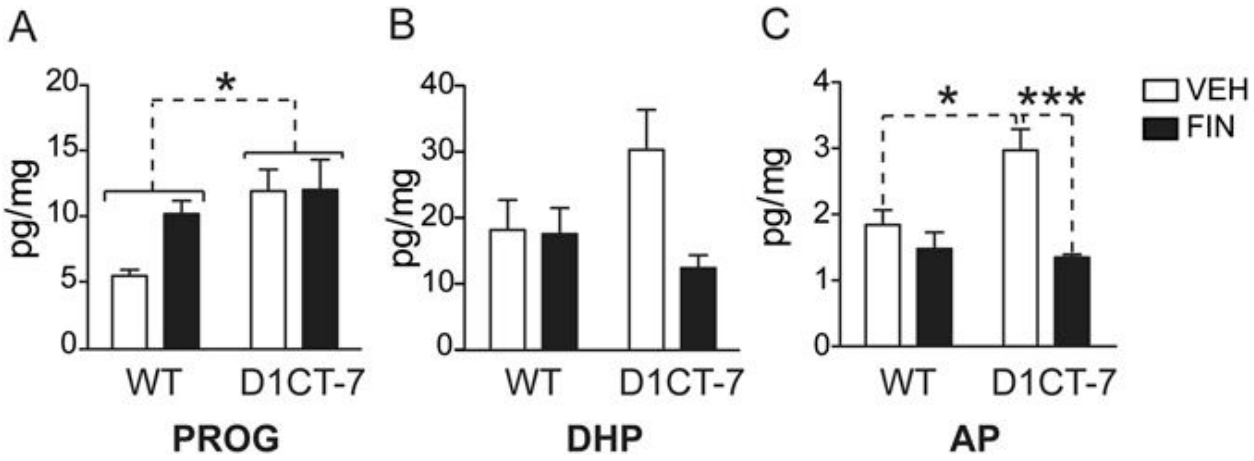

Figure 6.6 Finasteride reduces allopregnanolone in D1CT-7 mice following space confinement. Effects of finasteride (FIN; 50 mg/kg, IP) on levels of (A) progesterone, (B) 5α-dihydroprogesterone (DHP) and (C) allopregnanolone (AP) in the prefrontal cortex of D1CT-7 and wild-type (WT) littermates following spatial confinement (SC). Data are shown as means ± SEM. **P < 0.01, and ***P < 0.001 for comparisons indicated by dashed lines. Main effects for genotype are indicated as comparisons between brackets. Main effects for SC are indicated as comparison between symbols. N = 5–7/group. VEH, vehicle. For further details, see text.

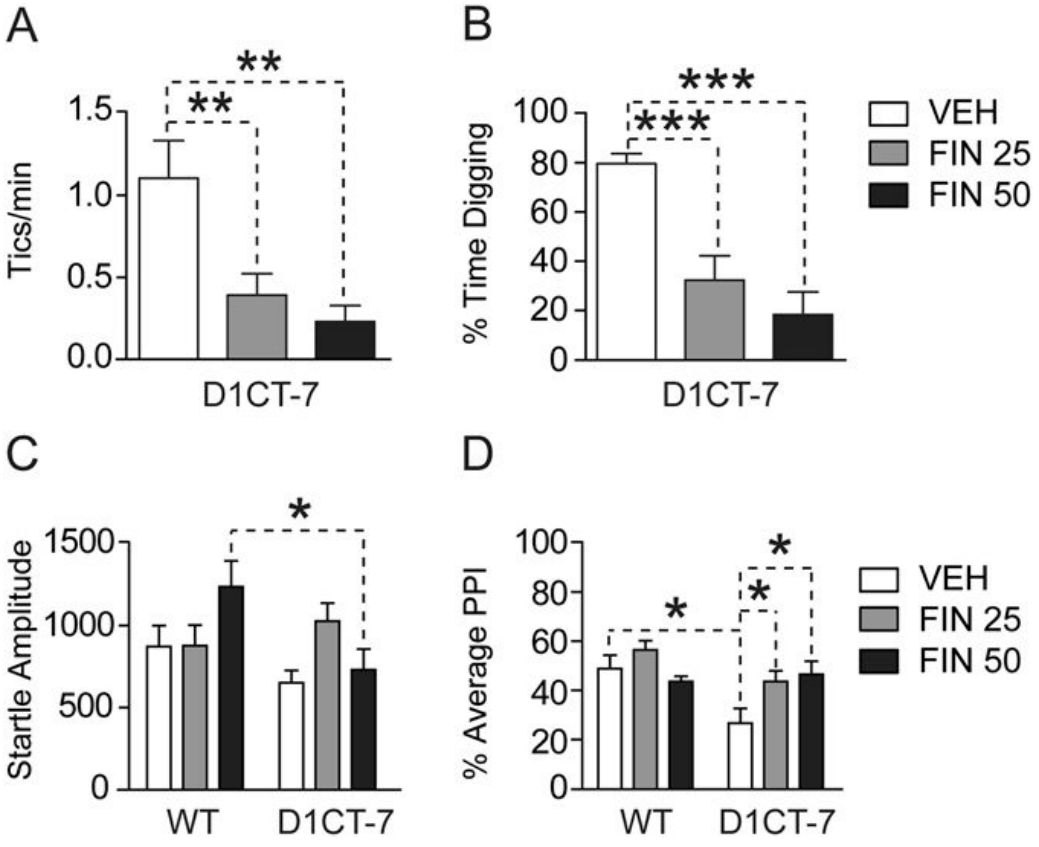

Figure 6.7 Effects of finasteride (FIN; 25–50 mg/kg, IP) on behavioral phenotypes related to Tourette syndrome. Data are shown as means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 for comparisons indicated by dashed lines. Doses are indicated in mg/kg (IP). N = 8/group. VEH, vehicle; WT, wild-type; PPI, prepulse inhibition of the startle. For further details, see text.

We next tested whether the reduction in AP levels induced by finasteride could be paralleled by the normalization of behavioral responses in spatially-confined D1CT-7 mice (Fig. 6.7). Finasteride dose-dependently reduced the tic-like bursts (Fig. 6.7A) induced by SC in D1CT-7 mice [$F(2,21) = 8.15$, $P < 0.01$; $P < 0.01$ for comparisons between vehicle and 25 mg/kg of finasteride; $P < 0.01$ for comparisons between vehicle and 50 mg/kg of finasteride]. Finasteride also reduced digging behavior (Fig. 6.7B) in D1CT-7 mice [$F(2,21) = 15.51$, $P < 0.01$; $P < 0.001$ for comparisons between vehicle and 25 mg/kg of finasteride; $P < 0.001$ for comparisons between vehicle and 50 mg/kg of finasteride]. The analysis of startle amplitude revealed a significant genotype × treatment interaction [$F(2,42) = 3.56$, $P < 0.05$], which reflected a greater startle-reducing effect of finasteride (50 mg/kg) in D1CT-7 than WT mice ($P < 0.05$) (Fig. 6.7C). PPI analyses (Fig. 6.7D) also revealed significant genotype × treatment interactions [$F(2,42) = 3.63$, $P < 0.05$]; post hoc scrutiny of this effect confirmed that SC significantly reduced PPI in vehicle-treated D1CT-7 mice ($P < 0.05$). In addition, finasteride ablated the gating deficits induced by SC in D1CT-7 mice at both the 25 mg/kg ($P < 0.05$) and 50 mg/kg ($P < 0.05$) doses.

Finally, the analysis of locomotor activity in both WT and D1CT-7 mice (Fig. 6.8) showed a significant genotype × treatment × time interaction [$F(10,210) = 2.37$, $P < 0.05$]. Overall, while D1CT-7 mice exhibited higher locomotor activity than their WT counterparts, the 50 mg/kg dose of finasteride exerted a profound hypolocomotive effect in both genotypes.

AP opposes the effect of finasteride on gating deficits, but not tic-like responses, in D1CT-7 mice. Lastly, we verified whether AP may reverse the ability of finasteride to attenuate the effects of SC in D1CT-7 mice. Finasteride (25 mg/kg, IP) and AP (15 mg/kg, IP) were administered at 40 and 10 min prior to SC, respectively. Only the 25 mg/kg dose of finasteride was used for these studies, since initial observations showed that the combination of AP with the higher

dose of finasteride had profound sedative effects in D1CT-7 mice. Finasteride was confirmed to reduce tic-like behaviors [Main effect: $F(1,28) = 16.23$, $P < 0.001$] (Fig. 6.9A) and digging responses [$F(1,28) = 23.21$ $P < 0.001$] (Fig. 6.9B); however, these effects were not countered by AP injection. In contrast with these findings, analysis of PPI revealed a significant finasteride × AP interaction [$F(1,28) = 7.10$, $P < 0.05$], which reflected the ability of AP to reverse ($P < 0.001$) the ameliorating effects of finasteride ($P < 0.001$) on this endophenotype (Fig. 6.9D).

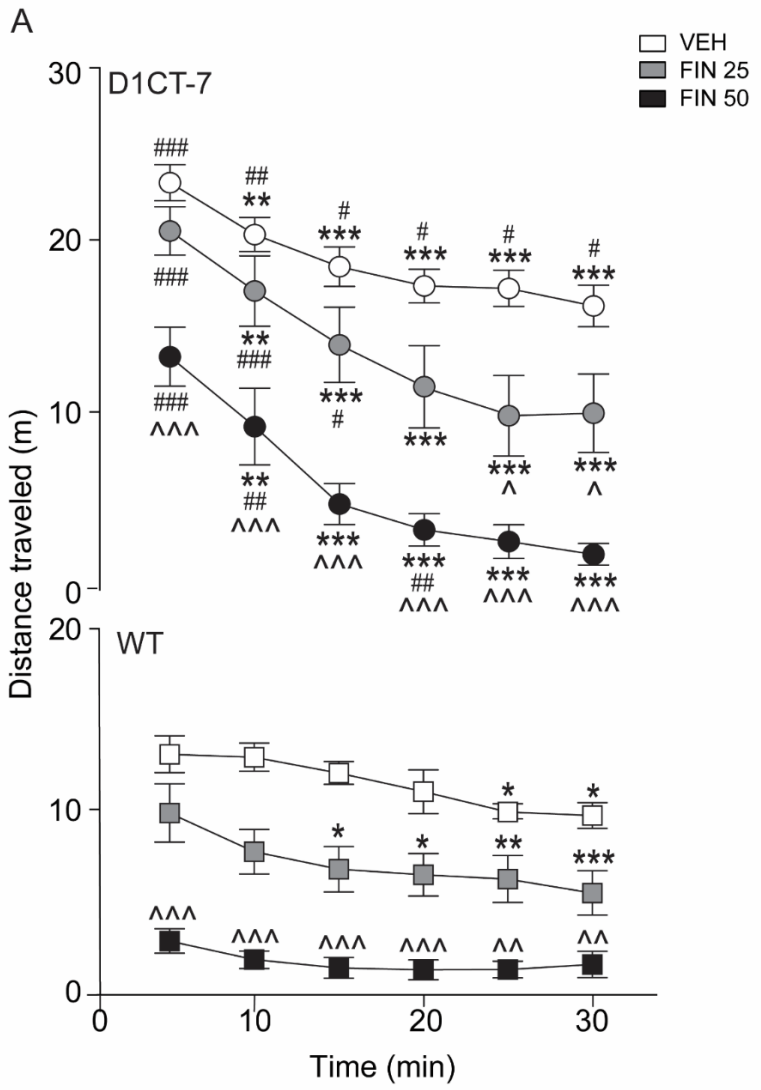

Figure 6.8 Effects of finasteride (FIN; 25-50 mg/kg, IP) on locomotor activity of D1CT-7 and wild-type (WT) littermates. Data are shown as means ± SEM. #P < 0.05, ##P < 0.01 ###P < 0.001 for WT vs D1CT-7 of the same time block and treatment. *P < 0.05, **P < 0.01 and ***P < 0.001 for indicated time point vs the 1st time block of the same genotype and treatment. ^P < 0.05, ^^P < 0.01 and ^^^P < 0.001 for comparisons between the indicated time point vs VEH treated of the same genotype and time block. N = 8/group. Abbreviations: VEH, vehicle.

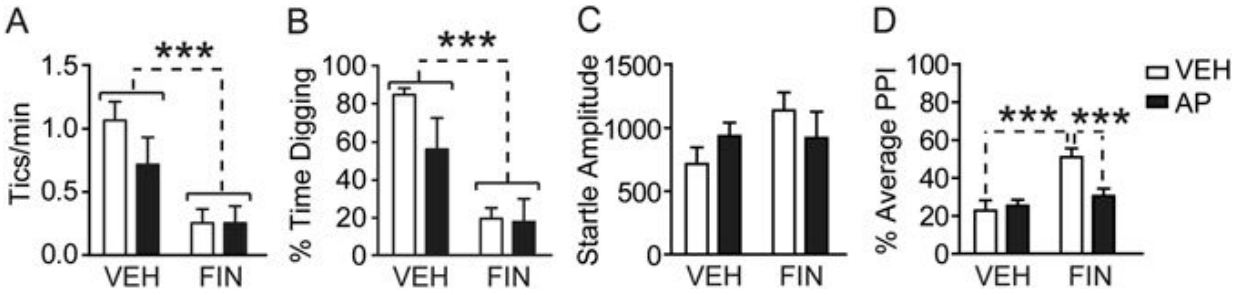

Figure 6.9 Combined effects of finasteride (FIN; 25 mg/kg, IP) and allopregnanolone (AP; 15 mg/kg, IP) on behavioral phenotypes related to Tourette syndrome in D1CT-7 mice. Data are shown as means ± SEM. ***P < 0.001 for comparisons indicated by dashed lines. Main effects for genotype are indicated as comparisons between brackets. N = 8/group. Abbreviations: VEH, vehicle; PPI, prepulse inhibition. For further details, see text.

Discussion

The present results show that, in the D1CT-7 mouse model of TS, acute stress exacerbates tic-like responses and impairs PPI by promoting the synthesis of the neurosteroid AP. Indeed, while these responses were associated with a generalized enhancement in progesterone, DHP and AP in the PFC, only the latter steroid elicited behavioral abnormalities akin to those observed following SC. Furthermore, the 5α-reductase inhibitor finasteride led to a normalization of stress-induced behavioral changes and of cortical AP levels in D1CT-7 mice, without eliciting any such effects in wild type littermates.

As mentioned above, tics are characterized by striking fluctuations in intensity and frequency, which are greatly contributed by the influence of select environmental triggers [39, 474]. Against this backdrop, the results of this study provide the first evidence of a mechanism that may be responsible for the adverse effects of stress on TS symptoms. The translational relevance of these results is underscored by our previous observations and open-label trials, documenting the therapeutic action of finasteride in adult male TS patients [394, 416, 417]. The present data are also in agreement with our finding that sleep deprivation, a common trigger for

tic exacerbation [39, 481], leads to sensorimotor gating deficits in rats via the enhancement of AP biosynthesis in the PFC [482].

The observed increase in neurosteroid concentrations in the PFC is in alignment with their well-documented role in the regulation of stress responses [454]. Our studies did not explore the mechanisms whereby SC increases cortical progesterone levels. A possible explanation for this effect may be the upregulation of the translocator protein 18KDa (TSPO), which has been observed following acute stress in other rodent models [483]. TSPO transports cholesterol into the inner mitochondrial membrane, thereby enabling the conversion of cholesterol into pregnenolone, the direct precursor of progesterone [484, 485].

Acute stress has been shown to increase 5α-reductase expression in the PFC [372]. Although the neurophysiological role of AP in humans in response to stress may be different from that recognized in rodents [486], preliminary observations suggest that acute stress may also increase AP biosynthesis in humans [487]. The notion that the stress-induced increase in AP is aimed at reducing anxiety is of particular interest in the context of TS. Indeed, tic execution in TS patients is generally regarded as a response to stressful stimuli that mitigates the discomfort associated with premonitory urges [23], and, indeed, tic severity has been found to be negatively correlated with cortisol levels [353]. This idea suggests that tic execution may be a by-product of the mechanisms of stress coping mounted by the cortex to offset the anxiogenic effects of stress itself [39]. Furthermore, tics are typically preceded by uncomfortable sensory phenomena [23, 488], which often reflect a psychological fixation on specific somatic cues and are typically exacerbated by environmental stress [17]. Although sensory phenomena are subjective in nature and cannot be captured in animal models, the underlying alterations in information-processing have been related to deficits in PPI of the startle. Accordingly, a reduction of this index has been

observed in TS patients [37, 38] and may be a key phenotypic marker to assess construct validity in TS models [138, 286]. Previous work from our group has provided support for a role of neurosteroids in the regulation of PPI and finasteride pretreatment can prevent PPI deficits, which are induced by dopaminergic agonists as well as environmental stressors [422, 482, 489]. Furthermore, our work has shown that these effects can be regulated by the PFC, as well as by the nucleus accumbens, but not the dorsal striatum [423].

The molecular mechanisms whereby AP leads to a robust exacerbation of tic-like behaviors and PPI deficits in D1CT-7 mice remain elusive. Although no alterations of cortical $GABA_A$ receptor subunit composition were detected in this mouse model, it is conceivable that the behavioral outcomes of AP may be contributed by the positive allosteric modulation of $GABA_A$ receptors in the PFC. Both $GABA_A$ receptors and neurosteroidogenic enzymes are expressed in the same cortical pyramidal neurons [490], providing a mechanism for excessive inhibition of glutamatergic neurons in the PFC; in turn, this process may result in the activation of striatum and other subcortical areas. Accordingly, stress has been shown to impair the function of the PFC [491]. In addition, the effects of AP may also be contributed by other mechanisms, such as the activation of membrane progesterone or pregnane X receptors. Alternatively, we cannot rule out that some of these actions may be due to the conversion of AP into its sulfo-conjugated derivative, AP sulfate, which acts as a negative allosteric modulator of NMDA glutamate receptors [492]. Irrespective of the specific receptors involved in the transduction of AP-mediated signals, exacerbation of Tourette-like manifestations may also be facilitated by AP-driven stimulation of dopamine release in the dorsal and ventral striatum [493]. In keeping with this concept, our data indicates that the dopamine receptor antagonist haloperidol reverses the enhancement in tics produced by AP, and countered - albeit not selectively – the PPI deficits induced by this

neurosteroid in D1CT-7 mice. These effects are in line with the effects of haloperidol on stressed D1CT-7 mice (chapter 3).

Several limitations of the study should be recognized. First, the translational value of these findings is partially limited by the evaluation of AP's effects only in one model of TS. Nevertheless, it is worth noting that, among the currently available animal models of TS [286], D1CT-7 mice feature unique characteristics with respect to face and predictive validity. For example, tic-like alterations in D1CT-7 mice are sex-dimorphic; furthermore, these responses are sensitive to all major TS therapies, such as haloperidol and clonidine ([255], chapter 3). While the construct validity of D1CT-7 mice with respect to TS was not apparent at the time of their development, their brain pattern of neuropotentiation was found to be restricted to the somatosensory/ insular cortex and amygdala [255, 256]; notably, all these regions, and in particular the insular cortex, have been recently shown to be particularly relevant in the regulation of premonitory sensory phenomena [39, 43, 494]. Nevertheless, D1CT-7 mice feature phenotypes that may not be directly related to TS, such as their hyperactivity; thus, future studies in other models of TS are necessary to confirm the role of AP in stress-elicited exacerbation of symptoms. Second, we have shown that the doses of finasteride used in our experiments can reduce locomotor activity in mice; this observation raises the possibility that the effects of finasteride may be due to non-specific sedative effects. While it is possible that the hypolocomotion induced by this drug may have contributed to some of the effects reported in this study, this possibility is substantially tempered by the finding that the doses that reduced tic-like behaviors in D1CT-7 mice did not lead to a suppression of startle responses, a common feature of sedative drugs [495]. Third, although our results point to a key role of AP in the regulation of TS-like responses in D1CT-7 mice, we cannot exclude that other neurosteroids may also participate in the behavioral effects of stress.

Further investigations will need to focus on other neurosteroids increased by stress such as tetrahydrodeoxycorticosterone or androgenic neuroactive steroids, as well as the role of the $GABA_A$ receptor and other AP-sensitive receptors. These analyses may prove essential to help clarify the male predominance of TS, as well as potential mechanisms of comorbidity with other neuropsychiatric problems, including ADHD and OCD. Fourth, our analyses were only limited to the PFC of adult mice; however, it is likely that the effects of other neurosteroids may differ with age; furthermore, other regions, such as the nucleus accumbens, may be involved in the effects of finasteride [496]. Given the limitations in the size of this region, however, further improvements in our ability to detect neurosteroid levels will be needed to address this issue.

Despite these limitations and caveats, the present findings are the first to suggest the potential involvement of AP in the adverse effects of acute stress on tics and related sensory correlates. Future studies will be essential to confirm these findings in TS patients and explore the therapeutic potential of neurosteroid-targeting therapies in tic disorders.

7. Neurosteroid contributions to eye blink responses in mice

Introduction

Restraint has been demonstrated in chapter 4 to increase the rate of eye blinks in mice. Since eye blinks are regulated by dopaminergic neurotransmission, this paradigm is ideal for continuing the research on dopamine and neuroactive stress steroid interactions. Therefore, we employed this method of studying TS-like tic behaviors in mice treated with finasteride, as well as those with genetic knockout of either 5αR1 or 5αR2.

Materials and Methods

Animals. We used 3-4-month-old, experimentally naïve male C57BL/6 mice (n = 8-10 per treatment group) weighing 20-30 g. Animals were purchased from Jackson Labs (Bar Harbor, ME). We also studied 5αR1KO and 5αR2KO mice along with the heterozygous (HZ) and WT littermates (strain: C57BL/6), obtained from breeding colonies at the Universities of Kansas and Utah. All mice were generated from HZ x HZ crosses. Progenitors were obtained by Dr. Mala Mahendroo (Southwestern University). Unless stated otherwise for specific experimental purposes, all mice were housed in groups of 4-5/cage, with at least 1 mouse/genotype, and had ad libitum access to food and water. Housing facilities were maintained at 22ºC with a reverse light/dark cycle (lights off at 08:00 AM hours and on at 08:00 PM). Whenever the same mice were used for multiple behavioral paradigms, the order of animals in each test was counterbalanced throughout the study. Experimental manipulations were carried out in the animals' dark cycle between 10:00 AM and 6:00 PM. All handling and experimental procedures were performed in compliance with the National Institute of Health guidelines and approved by the local Institutional Animal Care and Use Committees.

Genotyping. Mouse genotyping was performed by PCR. Samples of genomic DNA were extracted from tail biopsies acquired from mice at weaning (postnatal day 21). The following primers were used to identify 5αR1 KO mice: 1) GAT TGG GAA GAC AAT AGC AGG CAT GC 2) CCA GAC ACG AAC TTC CAC GCT TCT G 3) ATG GAG TTG GAT GAG TTG TGC. Reaction conditions were: 94°C x 1.5 min, 94°C x 20s, 55°C x 30s, 72°C x 2 min, 4°C x ∞. The following primers were used to identify 5αR2 KO mice: 1) GAT GAC CTC TCC GGG CTT CC 2) GAA TGT TCC AAG TCA CAG GC 3) CGC TTC TGA GGA GAG AAC TGA CTG A. Reaction conditions were: 94°C x 2 min, 94°C x 40s, 55°C x 40s, 72°C x 5 min, 4°C x ∞, as previously described [497].

Drugs. Finasteride (Astatech, Bristol, PA) was dissolved in 5% tween 80 and diluted with saline. Finasteride was administered 40 min before scoring. SKF 82958 (Sigma Aldrich, St. Louis MO) was dissolved in saline and administered 20 min before scoring.

Eye blink observations. Eye blinks were scored by trained observers blinded to the treatment as described in chapter 4.

Statistical analyses. Normality and homoscedasticity of data distribution were verified by using Kolmogorov-Smirnov and Bartlett's tests. Statistical analyses of parametric data were performed with t-tests, one-way, or multi-way ANOVAs, followed by Tukey's T-test for post-hoc comparisons. The significance threshold was set at 0.05.

Results

The first series of experiments was designed to determine if finasteride (50mg/kg, IP) had an effect on eye blink rates at baseline, in the context of restraint stress, or against SKF 82958 treatment. This was accomplished through three separate experiments. In the first experiment, finasteride was administered and baseline eye blink rates were determined. In this case, there was

no effect of finasteride on eye blinks (Fig 7.1A). Second, we pretreated the mice with finasteride prior to SKF 82958 (0.3mg/kg, IP) treatment. We demonstrated in chapter 4 that SKF 82958 induced an increase in eye blinks. Surprisingly, finasteride did not decrease the rate of eye blinks in the mice treated with the D1 antagonist. (Fig 7.1B). Finally we exposed mice to a 20 minute restraint stress prior to scoring the eye blinks. In this experiment, finasteride did decrease the eye blink frequency in mice exposed to restraint stress ($P < 0.001$).

The next series of experiments was aimed at determining if the effects of finasteride on eye blinks following restraint stress was contributed to by 5αR1 or 5αR2 inhibition. 5αR1 KO and 5αR2 KO mice were tested along with WT littermates. In the first experiments, the mice were exposed to restraint stress prior to counting the eye blink frequency to determine if there were alterations in the response of these mice to stress. However, both 5αR1 KO (Fig. 7.2A) and 5αR2 KO (Fig 7.2B) mice responded in a similar manner to restraint stress as their WT counterparts (5αR1 KO, main effect of restraint: $F(1,25) = 37.97$, $P < 0.001$; 5αR2 KO, main effect of treatment:

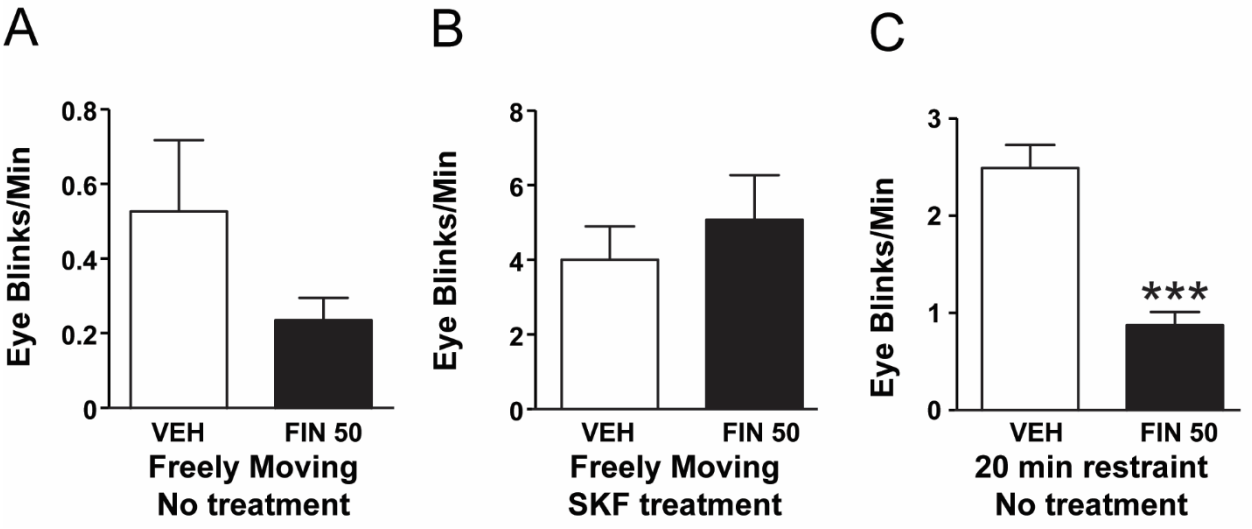

Figure 7.1 Finasteride reduces eye blinks in stressed mice.
(A) Eye blink responses from freely moving mice pretreated with finasteride (50mg/kg) or vehicle. N=11-12/group (B) Eye blink responses from freely moving mice pretreated with finasteride or vehicle prior to SKF 82858 treatment (0.3mg/kg). N=12/group (C) Eye blink responses from restrained mice pretreated with finasteride of vehicle. N=10/group Data are shown as means ± SEM. ***P < 0.001 compared to the corresponding vehicle treated group. Abbreviations: VEH, vehicle; FIN, finasteride. For further details, see text.

F(1,28) = 64.54, $P < 0.001$). In a subsequent experiment we pretreated the mice with finasteride prior to restraint to determine if either genotype was resistant to the effects of finasteride. However, we again found that the 5αR1 KO mice (Fig 7.2C) and 5αR2 KO mice (Fig 7.2D) responded the same as the WT littermates (5αR1 KO, main effect of treatment: F(1,28) = 28.68, $P < 0.001$; 5αR2 KO, main effect of treatment: F(1,28) = 82.47, $P < 0.001$).

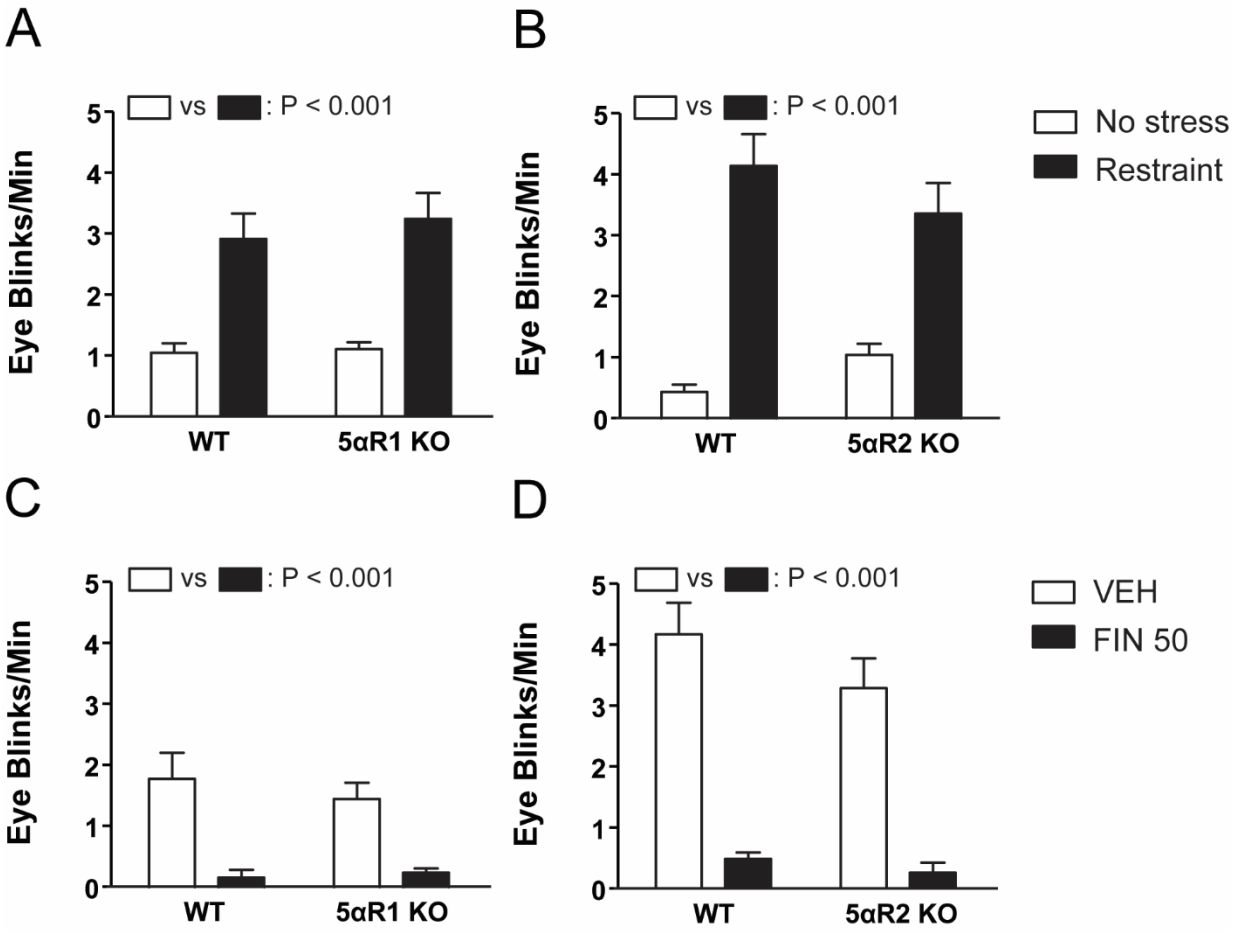

Figure 7.2 Both 5αR1 and 5αR2 mediate the effects of finasteride on eye blink responses.
5αR1 KO (A) and 5αR2 KO (B) mice along with their WT littermates were exposed to no stress or restraint stress for 20 minutes prior to scoring of eye blink responses. In a separate experiment 5αR1 KO (C) and 5αR2 KO (D) mice and the WT littermates were pretreated with finasteride or vehicle before they were all exposed to restraint stress for 20 minutes before eye blink observations. N=7-8/group. Data are shown as means ± SEM. Abbreviations: VEH, vehicle; FIN, finasteride; 5αR1 KO, 5α-reductase type 1 knockout; 5αR2 KO 5α-reductase type 2 knockout. For further details, see text.

Discussion

The experiments presented here demonstrate that finasteride reduces eye blinks in the context of stress but not against the D1 agonist SKF 82958. This implies that either D1 receptors and stress mediate different pathways to exacerbate eye blink frequencies (as demonstrated in chapter 4) or D1 receptors are functioning downstream of the effects from neurosteroids. In addition, we show that both 5αR1 and 5αR2 contribute to stress induced increases in eye blink frequency and that the loss of neither is sufficient to reduce the effect of stress. Furthermore, finasteride is capable of reducing eye blink responses from stress in both 5αR1 KO and 5αR2 KO mice indicating that acute inhibition of either isoenzyme ablates this stress response. However, these results also emphasize the need for specific inhibitors of mouse 5αR1 and 5αR2 or conditional KO animals that allow for the study of a reduction in these enzymes in adulthood or at specific developmental milestones rather than as a constitutive knockout. The results from these studies indicate that while acute inhibition of either 5αR isoenzyme is sufficient to attenuate the eye blink stress response, chronic inhibition is not, which indicates that compensatory mechanisms are likely at work to overcome the loss of either isoenzyme.

8. The neurosteroid enzyme 5α-reductase modulates the role of D1 dopamine receptors in rat sensorimotor gating

This chapter has been adapted from:

Frau, R., Mosher, L.J., Bini, V., Pillolla, G., Pes, R., Saba, P., Fanni, S., Devoto, P., Bortolato, M., *The neurosteroid enzyme 5α-reductase modulates the role of D1 dopamine receptors in rat sensorimotor gating.* Psychoneuroendocrinology, 2016. **63**: p. 59.67.

Mosher, L.J., Frau, R., Pardu, A., Pes, R., Devoto, P., Bortolato, M., *Selective activation of D1 dopamine receptors impairs sensorimotor gating in Long-Evans rats.* Br J Pharmacol, 2016. **173**(13): p. 2122-34.

Introduction

The therapeutic mechanism of finasteride has been previously demonstrated to be limited to dopamine D1 receptors in mice [277]; however, replication of these data and a full analysis of dopamine receptor antagonism by finasteride are complicated by the observations that most mouse strains are inherently unresponsive to PPI disrupting effects of D2 receptor agonists, while rats are observed to be sensitive to PPI deficits induced by D2 receptor agonists but not D1 receptor agonists. However, further research has demonstrated that these limitations are due to strain, not species differences [267]. Furthermore, we have previously reported that finasteride is effective at ablating the PPI disrupting effects of nonspecific dopamine agonists in Sprague Dawley (SD) rats, a strain of rats that respond to D2 but not D1 agonists. Nevertheless, D1 receptor activation has been shown to be directly involved in the PPI deficits induced by non-specific dopaminergic agonists, such as apomorphine (APO) [498] indicating that the therapeutic mechanism of finasteride may involve the D1 receptor in rats as well.

To test this hypothesis we first expanded on previous research by further investigating other strains of rats to determine if other strains are sensitive to PPI disrupting effects from D1 receptor agonists. We found that the selective and independent activation of D1 and D2 receptors produces PPI deficits in the hooded Long-Evans (LE) strain ([320]; see appendix for full paper). Expanding on these findings we investigated the effects of finasteride in both SD and LE rats treated with dopamine receptor agonists.

Materials and Methods

Animals. All animal care and experimental procedures were in compliance with the National Institute of Health guidelines and approved by the Institutional Animal Use Committees of the University of Kansas and Cagliari. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [275, 276]. The present study was conducted on male SD, Wistar (WIS) (Harlan, Italy) and LE rats (Charles River Laboratories, Raleigh, NC, USA). Rats (3–4 months old; 300–350 g of body weight) were housed 3–4 per cage in rooms maintained at a temperature of 22 ± 2°C and a humidity of 60%. Animals were given ad libitum access to food and water and held under an artificial 12/12 h light/dark cycle, with lights off from 10:00 a.m. to 10:00 p.m. In order to reduce stress during the experiment, each rat was handled gently for 5 min each day of the week preceding the behavioral testing. Each animal was used only once throughout the study and all efforts were made to minimize animal suffering. PPI and microdialysis studies occurred between 11:00 AM and 5:00 PM. Care was taken in ascertaining the uniformity of all husbandry conditions across the two facilities where the experiments were performed (University of Kansas, USA and University of Cagliari, Italy). All experimental procedures were in compliance with the National Institute of Health guidelines and approved by the Institutional Animal Use Committees of the University of Kansas and Cagliari.

Drugs. The following drugs were used in the present study: finasteride, (R)-(−)-apomorphine hydrochloride, SKF 82958 hydrobromide, (−)-quinpirole hydrochloride, sumanirole maleate, (+)-PD 128907 hydrochloride, SCH 23390 and GR 103691 (Sigma Aldrich, Milan, Italy). Finasteride was suspended in a vehicle solution containing 5% Tween 80 and 95% saline, while the other drugs were dissolved in saline solution. Drug doses are based on mg/kg of salts. All solutions were freshly prepared on the day of testing and administered subcutaneously (s.c.) and intraperitoneally (i.p.) in an injection volume of 1 and 2 ml/kg body weight, respectively. The doses and the latency time of the drugs used in these experiments were determined by our previous studies and in accordance with those commonly used in PPI studies on rats [181, 320, 422, 499].

Acoustic Startle Reflex and PPI. Startle and PPI testing were performed as previously described [500]. The apparatus used for detection of startle reflexes (Med Associates, St Albans, VT, USA) consisted of six standard cages placed in sound-attenuated chambers with fan ventilation. Each cage consisted of a Plexiglas cylinder of 9 cm diameter, mounted on a piezoelectric accelerometric platform connected to an analogue-digital converter. Two separate speakers conveyed background noise and acoustic bursts, each one properly placed so as to produce a variation of sound within 1 dB across the startle cage. Both speakers and startle cages were connected to a main PC, which detected and analyzed all chamber variables with specific software. Before each testing session, acoustic stimuli and mechanical responses were calibrated via specific devices supplied by Med Associates. Rats were first subjected to a pre-test session, during which they were exposed to a sequence of seventeen trials, consisting of 40-ms, 115-dB burst, with a 70-dB background white noise. Experimental groups were defined based on the average startle amplitude of the rats, so as to maintain comparable values of average startle response across all groups. Three days after the pre-test session, rats were treated and underwent

a test session. This session featured a 5-min acclimatization period, with a 70-dB background white noise, which continued for the remainder of the session. The acclimatization period was followed by three blocks, each consisting of a sequence of trials: the first and the third block consisted of five pulse-alone trials of 115 dB (identical to those used in the pre-test session). The second block consisted of a pseudorandom sequence of 50 trials, including 12 pulse-alone trials, 30 trials of pulse preceded by 74, 78, or 82 dB pre-pulses (10 for each level of pre-pulse loudness), and 8 no-pulse trials, where only the background noise was delivered. Inter-trial intervals (i.e, the time between two consecutive trials) were selected randomly between 10 and 15 s.

The % PPI was calculated only on the values relative to the second period, as well, using the following formula:

$$100 - \frac{mean\ startle\ amplitude\ for\ prepulse\ pulse\ trials}{mean\ startle\ amplitude\ for\ pulse\ alone\ trials} \times 100$$

For both the pre-test and the test session, the interstimulus interval (i.e., the duration between the prepulse and the pulse in each trial) was kept at 100 ms. The selection of this interstimulus interval was based on previously published experiments from our group (Mosher et al., 2015), which showed this parameter to be optimally suited to reveal PPI deficits in response to selective dopamine receptor agonists in LE and SD rats.

A major caveat in %PPI computation is that increases or reductions in startle magnitude can respectively lead to artifacts, due to “ceiling” or “floor” effects [501].

In consideration of finasteride’s ability to reduce startle magnitude [422], whenever finasteride was found to produce significant effects on both startle magnitude and %PPI, we performed confirmatory analyses of ΔPPI values. This parameter was calculated as the absolute differences between startle magnitudes on pulse-alone and prepulse+pulse trials [502].

Microdialysis. Microdialysis experiments were performed as previously described in Devoto et al. [423]. SD rats were deeply anesthetized with Equithesin (containing, per 100 ml, 0.97 g pentobarbital, 2.1 g MgSO4, 4.25 g chloral hydrate, 42.8 ml propylene glycol, 11.5 ml 90% ethanol; 5 ml/kg, i.p.) and placed in a Kopf stereotaxic apparatus. The skull was exposed and a hole was drilled for the implant of vertical microdialysis probes (membrane AN 69-HF, Hospal-Dasco, Bologna, Italy; cut-off 40,000 Daltons, 2 mm active membrane length), in the nucleus accumbens shell [AP +1.7, L ± 0.8, V −7.8 from the bregma, according to the coordinates of Paxinos and Watson [503]]. The probes were secured to the scull by means of two screw and cranioplastic cement. The day after probe implantation, artificial cerebrospinal fluid (147 mM NaCl, 4 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, pH 6–6.5) was pumped through the dialysis probes at a constant rate of 1.1 μl/min via a CMA/100 microinjection pump (Carnegie Medicine, Stockholm, Sweden) in freely moving animals, and dialysate samples were collected every 20 min. Dopamine and DOPAC were immediately analyzed by HPLC with electrochemical detection, by HPLC systems equipped with 3.0 × 150 mm C18 (3.5 μ) Symmetry columns (Waters, Milan, Italy), maintained at 40°C by Series 1100 thermostats (Agilent Technologies, Waldbronn, Germany), and ESA Coulochem II detectors (Chelmford, MA, USA). The mobile phase consisted of 80 mM Na2HPO4, 0.27 mM EDTA, 0.6 mM sodium octyl sulfate, 8% methanol, 3% acetonitrile, pH 2.8 with H3PO4, delivered at 0.3 ml/min; the Coulochem analytical cell first electrode was set at +200 mV, the second one at −200 mV. Quantification was performed recording the second electrode signal. Under these conditions, dopamine detection limit (signal to noise ratio 3:1) was 0.3 pg per injection on column. On completion of testing, rats were sacrificed by Pentothal overdose, the brains removed and sectioned by a cryostat (Leica CM3050 S) in 40 μm thick coronal slices to verify locations of dialysis probes. No animal was found with errant location of the device.

Data analysis. Normality and homoscedasticity of data distribution were verified by using the Kolmogorov-Smirnov and Bartlett's tests. Analyses were performed by multiple-way ANOVAs, as appropriate, followed by Student-Newman-Keuls' test for post hoc comparisons of the means. For %PPI analyses, main effects for prepulse levels were consistently found throughout all the analyses, showing loudness-dependent effects; since no interactions between prepulse levels and other factors were found, data relative to different prepulse levels were collapsed. Significance threshold was set at 0.05.

Results

Assessment of effects of D1 receptor agonists in SD and LE rats. We first investigated the responses of SD rats (n = 9 per group) to the full D1 agonist SKF 82958. This drug did not significantly modify startle amplitude; however, in conformity with previously published data [499], its highest dose (5 mg/kg, s.c.) produced a marked reduction of PPI in comparison with saline (data not shown) [$F(2,24) = 8.71$, $P < 0.05$]. To determine the specificity of this deficit we pretreated the rats (n = 10 per group) with the D1 receptor antagonist SCH 23390. SCH 23390 produced a significant reduction in startle amplitude [main effect: $F(1,36) = 5.28$, $P < 0.05$]; conversely, this parameter was not affected by either SKF 82958 (5 mg/kg, s.c.) treatment or its interaction with SCH 23390 (Fig. 8.1A). In the same strain, SKF 82958 significantly reduced PPI [main effect: $F(1,36) = 35.30$, $P < 0.05$]; however, in confirmation of previous data [477], this effect was not countered by the D1 receptor antagonist (Fig. 8.1B), confirming that, in SD rats, the PPI-disrupting effects of SKF 82958 are not mediated by D1 receptors.

In contrast to the albino SD rats, the lowest dose of SKF 82958 (1mg/kg, s.c.) was sufficient to reduce PPI in LE rats (data not shown; n = 9 per group) [$F(1,16) = 29.38$, $P < 0.05$}, without altering startle amplitude. Furthermore, SCH 23390 pretreatment in LE rats (n = 8-10 per group)

produced a significant enhancement in startle amplitude [main effect: $F(1,34) = 10.75$, $P < 0.05$]; conversely, ANOVA failed to detect a significant main effects for SKF 82958 or interactions between the two treatments (Fig. 8.1C). The analysis of PPI confirmed that SKF 82958 significantly reduced this index [$F(1,34) = 26.84$, $P < 0.05$]; however, in contrast with the SD rats, this effect was prevented by SCH 23390 [pretreatment × treatment interaction: $F(1,34) = 6.76$, $P < 0.05$], suggesting that the PPI-disrupting effects of SKF 82958 were mediated by D1 receptors in this strain (Fig. 8.1 D).

We then examined whether the PPI deficits induced by SKF 82958 may be countered by the selective D2 receptor antagonist L 741626. The combination of L 741626 (1 mg/kg, s.c.) and SKF 82958 (5 mg/kg, s.c.) failed to induce significant alterations in startle magnitude in both SD (Fig. 8.2A; n = 10 per group) and LE rats (Fig. 8.2C; n = 8 per group). Conversely, the PPI deficits

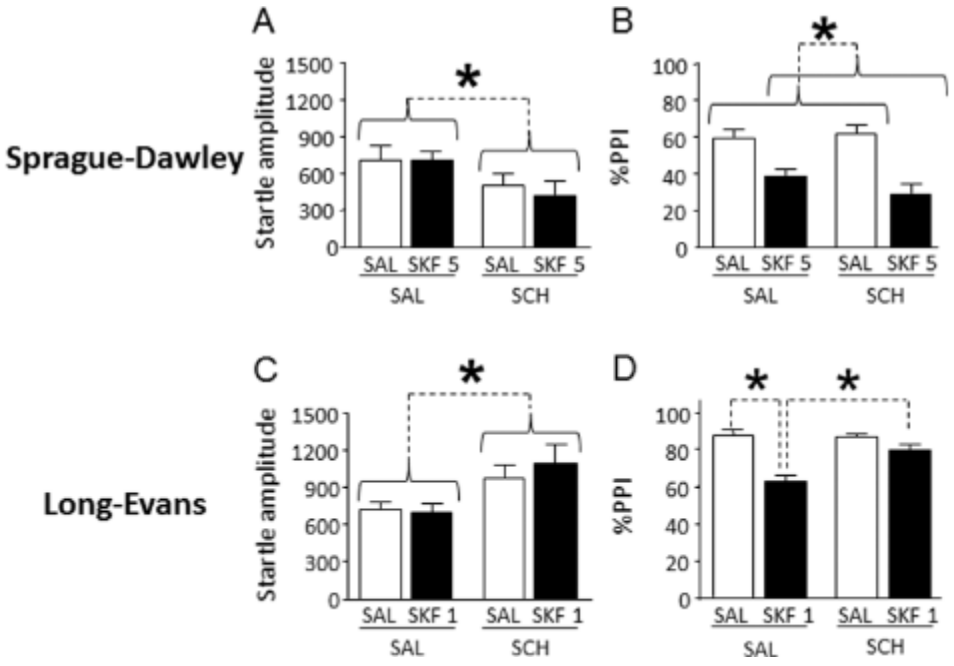

Figure 8.1 Different responses of SD and LE rats to D1 receptor agonists in PPI. SKF82958 (SKF; 5 mg/kg, s.c.) in SD rats and LE rats (1 mg/kg, s.c.) was tested in combination with the selective D1 receptor antagonist SCH23390 (SCH; 0.1 mg/kg, IP). Values represent mean ± SEM for each experimental group. Doses of SKF are indicated in mg/kg. *$P < 0.05$, significantly different as indicated. For more details, see text.

induced by SKF 82958 were significantly prevented by L 741626 in SD [$F(1,36) = 17.29$, $P < 0.05$] (Fig. 8.2B), but not LE rats (Fig 8.2D).

Assessment of effects of D2 receptor activation in SD and LE rats. In SD rats (n=10 per group), quinpirole treatment decreased the mean startle amplitude [$F(1,36) = 48.51$, $P < 0.05$], but this effect was not modified by the D2 antagonist L 741626 (Fig. 8.3A). Both L 741626 [main effect; $F(1,36) = 10.12$, $P < 0.05$] and quinpirole [main effect; $F(1,36) = 14.88$, $P < 0.05$] significantly modified PPI, but no significant interaction of their effects was found (Figure 8.3B). In LE rats (n = 8 per group), startle analyses showed a significant interaction between quinpirole and L 741626 [$F(1,28) = 6.8$, $P < 0.05$]; post hoc analyses revealed that L 741626 increased startle response, while quinpirole significantly decreased it both in the vehicle for L 741626 and L 741626-pretreated animals (Figure 8.3C). The analysis of PPI in LE rats detected a significant

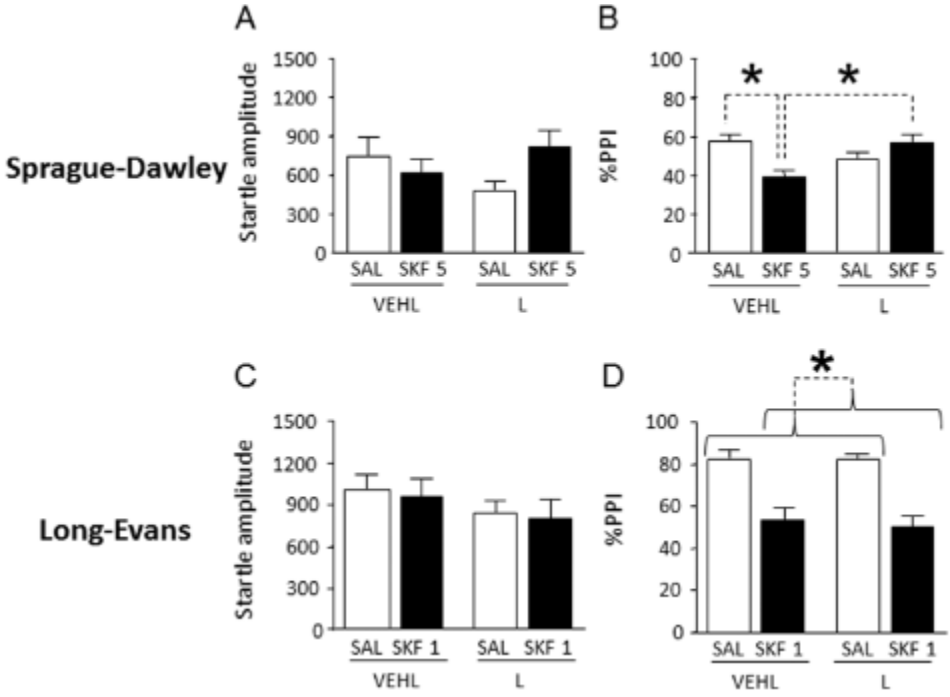

Figure 8.2 The D2 receptor mediates the effects of SKF 82958 in SD rats. The involvement of D2 receptors in the effects of SKF82958 (SKF) on the regulation of startle reflex and PPI in different rat strains, as tested through the combined treatment with the selective D_2 receptor antagonist L741,626 (L; 1 mg/kg, s.c.). Values represent mean ± SEM for each experimental group. PPI values are represented as the means of all prepulse-loudness values. Doses of SKF are indicated in mg/kg. VEHL, vehicle for L 741626; *$P < 0.05$, significantly different as indicated. For more details, see text.

interaction between quinpirole and L 741626 [F(1,28) = 8.07, $P < 0.05$]. Post hoc analyses revealed that quinpirole led to a significant PPI deficit, which was fully countered by L 741626 (Figure 6D).

Assessment of effects of apomorphine in SD and LE rats. In SD rats (n = 10 per group), apomorphine failed to affect startle magnitude (data not shown), but reduced PPI [F(2,27) = 5.09, $P < 0.05$]. Post hoc analyses showed that the reduction in PPI was produced by the dose of 0.25 mg/kg (data not shown). In LE rats (n = 10-12 per group), apomorphine did not significantly affect startle amplitude, but produced a robust PPI disruption [F(2,32) = 13.27, $P < 0.05$]. Significant differences were found for both the doses of 0.25 and 0.5 in comparison with the vehicle for apomorphine (data not shown).

In LE rats, finasteride counters the PPI deficits induced by D1-like, but not D2-like receptor agonists. We investigated the effects of finasteride on startle response and PPI in LE

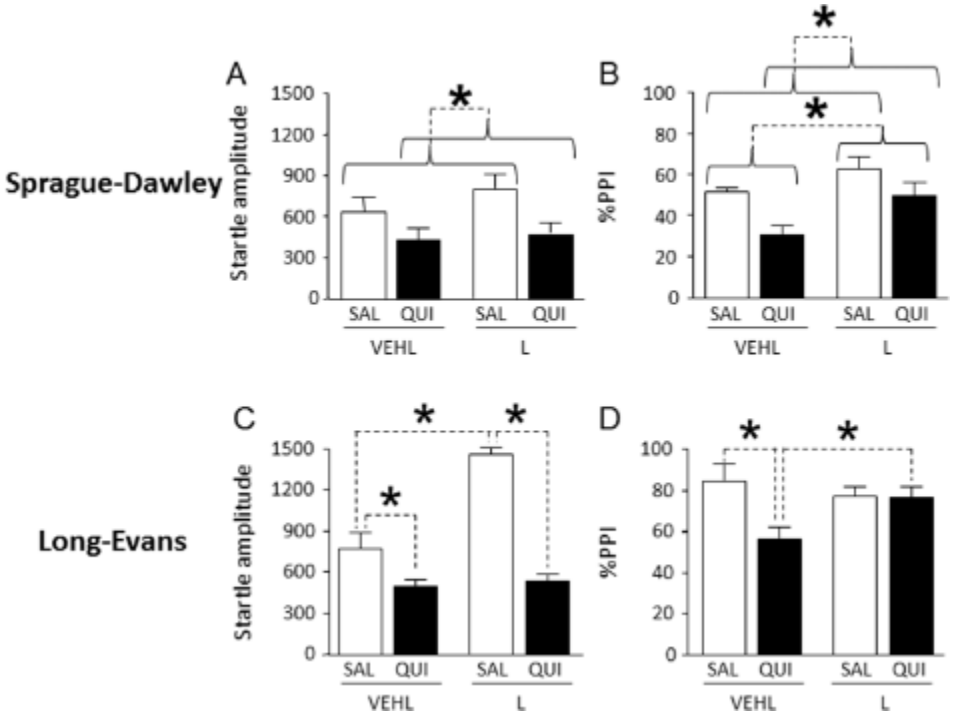

Figure 8.3 Quinpirole induces PPI deficits in SD but not LE rats. Involvement of D_2 receptors in the effects of quinpirole (QUI) on the regulation of startle reflex and PPI in different rat strains, as tested through the combined treatment with the selective D_2 receptor antagonist L741,626 (L; 1 mg/kg, s.c.). Values represent mean ± SEM for each experimental group. PPI values are represented as the means of all prepulse-loudness values. VEHL, vehicle for L741,626; *$P < 0.05$, significantly different as indicated. For more details, see text.

rats. Our first experiment was aimed at testing whether finasteride may counter apomorphine-induced PPI deficits (n=8– 9/treatment group) (Figs. 8.4A–B). In agreement with our previous results ([320]; summarized above), apomorphine (0.5 mg/kg, s.c.) reduced startle amplitude [(Main effect: $F(1, 29)=4.63$, $P < 0.05$]. Conversely, finasteride (100 mg/kg, i.p.) did not significantly affect this parameter [Main effect: $F(1,29)=1.83$, NS]. Furthermore, we found no significant interactions between the two drugs [$F(1, 29)=0.79$, NS] (Fig. 8.4A). %PPI analyses indicated that, while apomorphine significantly reduced %PPI in LE rats [Main effect: $F(1, 29)=48.47$, $P < 0.001$], finasteride surprisingly failed to counter this effect [Interaction: $F(1, 29)=0.08$, NS] (Fig. 8.4B). We then examined the effects of finasteride on the disruption of PPI induced by the full D1-like receptor agonist SKF 82958 (1 mg/kg, s.c.; n=8/treatment group), which we recently documented in LE rats ([320]; summarized above). Neither SKF 82958 nor finasteride produced significant effects on startle amplitude [Main SKF 82958 effect: $F(1, 28)=1.08$, NS; Main finasteride effect: $F(1, 28)=0.03$, NS]. Furthermore, no significant interaction between the two factors was detected [$F(1, 28)=1.0$, NS] (Fig. 8.4C). The analysis of %PPI in LE rats revealed that SKF 82958 significantly reduced %PPI [Main effect: $F(1, 28)=52.01$, $P < 0.001$], but this effect was significantly prevented by finasteride [$F(1, 28)=15.39$, $P < 0.001$; Ps<0.001 for comparisons between vehicle-saline vs vehicle-SKF 82958 and vehicle-SKF 82958 and Finasteride-SKF 82958]. (Fig. 8.4D).

Finally, we tested whether finasteride may oppose the effects of the D2-like receptor agonist quinpirole (0.6 mg/kg, s.c.) on PPI (n=9–10/treatment group). As shown in Fig. 8.4E, quinpirole markedly reduced startle amplitude [Main effect: $F(1,33)=11.27$; $P < 0.01$], while finasteride did not change this parameter [Main effect: $F(1,33)=1.71$; NS] and failed to reverse the effects of quinpirole [Interaction: $F(1,33)=0.01$; NS] (Fig. 8.4E). Notably, quinpirole disrupted

%PPI [Main effect: F(1,33)=48.47; $P < 0.001$]; conversely, finasteride failed to either affect %PPI [Main effect: F(1,33)=0.34; NS] or counter the effect of quinpirole [Interaction: F(1,33)=0.90; NS] (Fig. 8.4F).

In SD rats, finasteride counters the PPI deficits induced by apomorphine, but not D2 receptor agonists. In line with our prior results in SD rats [422], startle magnitude was significantly reduced by finasteride [Main effect: F(1, 35)=32.85, $P < 0.001$], and increased by

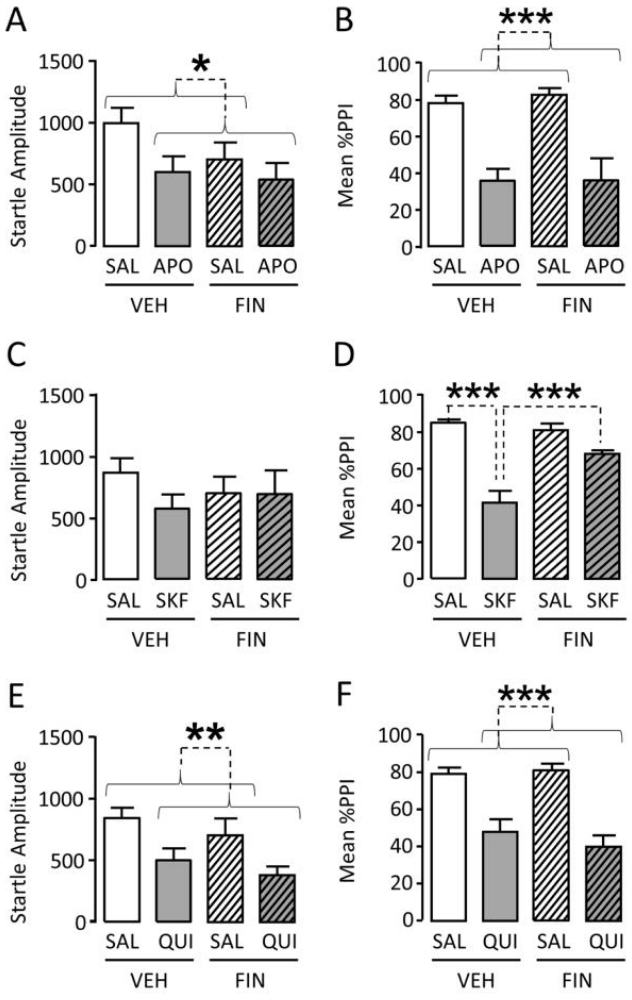

Figure 8.4 Finasteride counters the effects of D1 receptor agonists in PPI. Effects of finasteride (FIN, 100 mg/kg, IP) on the changes in acoustic startle and prepulse inhibition (PPI) induced by (A–B) the non-selective D1–D2 receptor agonist apomorphine (APO, 0.5 mg/kg, SC), (C–D) the D1 receptor agonist SKF 82958 (SKF, 1 mg/kg, SC), (E–F) the D2-like receptor agonist quinpirole (QUI, 0.6 mg/kg, SC) in male Long-Evans rats. Values represent mean ± SEM for each experimental group. N = 8–10/group. SAL, saline; VEH, vehicle of finasteride; *, P<0.05; **, P<0.01; ***, P<0.001 for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. For more details, see text.

apomorphine [Main effect: F(1, 35)=6.91, $P < 0.05$] (n=9–10/treatment group); however, ANOVA failed to identify any significant interaction between these two effects [Interaction: F(1, 35)=1.03, NS] (Fig. 8.5A). %PPI analyses revealed significant main effects for both finasteride pretreatment [F(1, 35)=10.93, $P < 0.01$] and apomorphine treatment [F(1, 35)=15.62, $P < 0.001$]. In addition, a significant interaction between these effects was found [F(1, 35)=4.33, $P < 0.05$]; post-hoc analyses revealed that, while apomorphine significantly reduced %PPI ($P < 0.05$), this effect was significantly prevented by finasteride pre-treatment ($P < 0.05$) (Fig. 8.5B). The same effects were detected through the analysis of corresponding ΔPPI values [finasteride x apomorphine interaction: F(1,31)=4.55, P<0.05; Ps<0.05 for post-hoc comparisons between vehicle + saline and vehicle + apomorphine as well as vehicle + apomorphine and finasteride + apomorphine] (data not shown).

Next, we tested whether the PPI-disrupting effect of the D2-like receptor agonist quinpirole was reversed by finasteride (n=10–11/treatment group) (Fig. 8.5C–D). Both quinpirole and finasteride significantly reduced startle amplitude [Main quinpirole effect: F(1,37)=46.68, $P < 0.001$; Main finasteride effect: F(1,37)=13.50, $P < 0.001$], yet no significant interaction between the two drugs was found [F(1,37)=2.80, NS]. %PPI analysis detected that this parameter was significantly decreased by quinpirole [F(1,37)=16.76; $P < 0.001$], and increased by finasteride [F(1,37)=8.64; $P < 0.01$]; however, no significant finasteride x quinpirole interaction was detected [F(1,37)=0.81; NS] (Fig. 8.5D).

Since both D2 and D3 receptor agonists reduce PPI in SD rats, we verified whether the specific contribution of each receptor may be countered by finasteride. Thus, we tested whether finasteride may counter the effect of the selective D2 receptor agonist sumanirole (3 mg/kg, s.c.) (n=8–9/treatment group). In contrast with quinpirole, sumanirole did not affect startle magnitude

[F(1,31)=1.16; NS] (Fig. 8.5E), while finasteride significantly reduced this response [F(1,31)=20.50; $P < 0.001$]; however, no significant interaction between these two drugs was detected [F(1,31)=0.81; NS]. Analyses of PPI showed that sumanirole disrupted PPI [F(1,31)=8.64; $P < 0.001$], but finasteride pretreatment failed to prevent this effect [F(1,31)=0.09; NS]. (Fig. 8.5F).

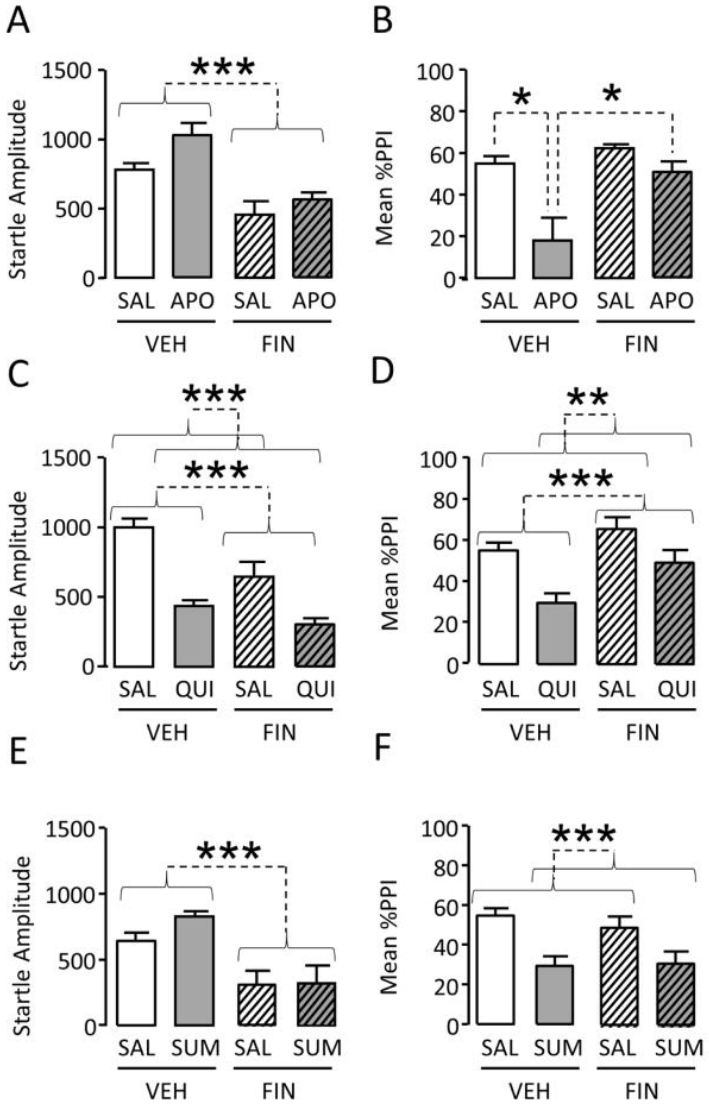

Figure 8.5 Finasteride ablates the effects of apomorphine in SD rats. Effects of finasteride (FIN, 100 mg/kg, IP) on the changes in acoustic startle and prepulse inhibition (PPI) induced by (A–B) the non-selective D1–D2 receptor agonist apomorphine (APO, 0.25 mg/kg, SC), (C–D) the D2-like receptor agonist quinpirole (QUI, 0.6 mg/kg, SC), (E–F) the D2 selective agonist sumanirole (SUM, 3 mg/kg, SC) in male Sprague-Dawley rats. Values represent mean ± SEM for each experimental group. N = 8–10/group. SAL, saline; VEH, vehicle of finasteride; *, P<0.05; **, P<0.01; ***, P<0.001 for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. For more details, see text.

In SD rats, finasteride counters the %PPI deficits induced by D3 receptor activation. We then tested the effects of finasteride on the %PPI reduction induced by D3 receptor agonist PD 128907 (n=10–12/treatment group). While finasteride reduced acoustic startle amplitude [F(1,40)=19.35; $P < 0.001$], PD 128907(0.1 mg/kg, i.p.) [F(1,40)=1.83; NS] failed to affect this parameter. In addition, a significant interaction of these two treatments was found [F(1,40)=7.57; $P < 0.01$]; post-hoc analyses revealed that the group treated with vehicle and saline exhibited a significantly higher startle magnitude than those treated with vehicle and PD 128907 (P<0.05) as well as finasteride and saline ($P < 0.001$) (Fig. 8.6A). Two-way ANOVA analyses of %PPI parameters showed significant main effects of finasteride [F(1,40)=15.86; $P < 0.001$] and PD 128907 [F(1,40)=4.38; $P < 0.05$]. Interestingly, finasteride countered the reduction in %PPI induced by PD 128907 [Interaction:F(1,40)=4.38; $P < 0.001$; *P*s<0.001 for comparisons between vehicle + saline and vehicle + PD 128907 as well as vehicle + PD 128907 and finasteride + PD 128907] (Fig. 8.6B). In contrast with these findings, the analysis of ΔPPI values indicated a significant interaction between finasteride and PD 128907 [F(1,40)=12.92; $P < 0.001$]; however, post-hoc comparisons found a significant difference between vehicle + saline and vehicle + PD 128907 ($P < 0.001$) as well as between vehicle + saline and finasteride + saline ($P < 0.01$), but not between vehicle + PD 128907 and finasteride + PD 128907 (Fig. 8.6C).

To confirm that the observed effects by PD 128907 were mediated by D3 receptors, we tested the effects of the D3 receptor antagonist GR 103691 (n=8–9/treatment group). GR 103691 (0.2 mg/kg, s.c.) countered both the reduction of startle amplitude [Interaction: F(1,30)=4.24, $P < 0.05$; *P*s < 0.05 for comparisons between vehicle + saline and vehicle + PD 128907 as well as vehicle + PD 128907 and GR 103691 + PD 128907] (Fig. 8.6A) and %PPI caused by PD 128907 [Interaction: F(1,30)=6.23; P<0.05; Ps<0.001 for comparisons between vehicle + saline and

vehicle + PD 128907 as well as vehicle + PD 128907 and GR 103691+PD 128907] (Fig. 8.6B). These results were fully confirmed by ΔPPI analyses, which found main effects for both [GR 103691 F(1,28)=7.66; $P < 0.01$] and PD 128907 [F(1,28)=9.51; $P < 0.01$]. Furthermore, a significant interaction between the two treatments [F(1,28)=6.87; $P < 0.05$] was found to reflect significant differences between vehicle + saline and vehicle + PD 128907 ($P < 0.01$) as well as between vehicle + PD 128907 and GR 103691 + PD 128907 (P<0.01)] (Fig. 8.6C).

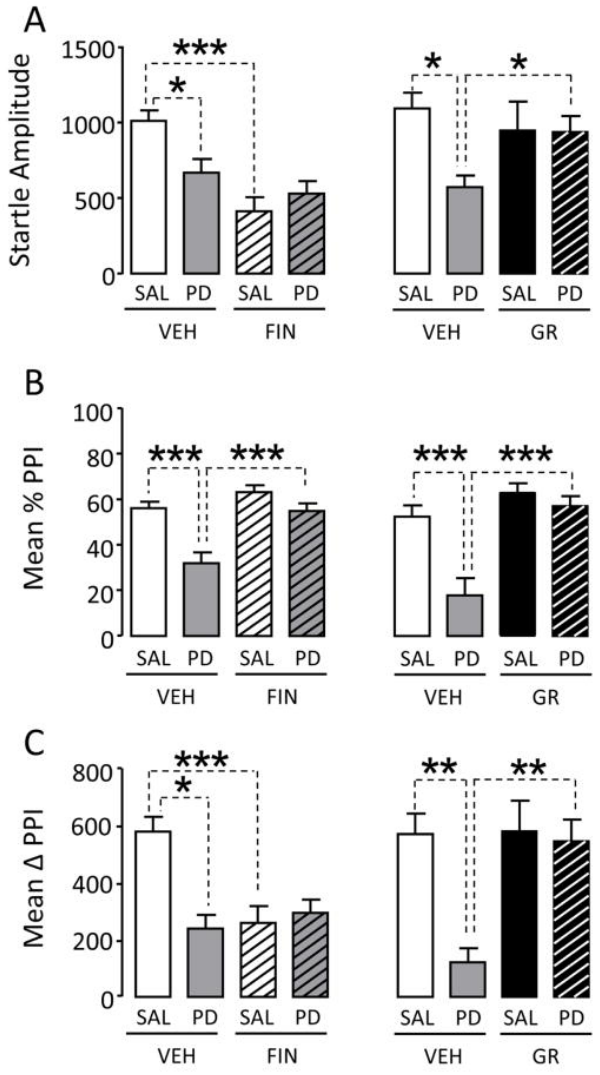

Figure 8.6 Finasteride ameliorates the PPI deficits induced by D3 agonists in SD rats. Effects of finasteride (FIN, 100 mg/kg, IP) and the D3 receptor antagonist GR103691 (0.2 mg/kg, SC) on the changes in (A) acoustic startle and (B) prepulse inhibition (PPI) induced by the D3 receptor agonist PD 128907 (PD, 0.1 mg/kg, IP) in male Sprague-Dawley rats. Values represent mean ± SEM for each experimental group. N = 8–10/group. SAL, saline; VEH, vehicle; *, P<0.05; **, P<0.01; ***, P<0.001 for comparisons indicated by dotted lines. For more details, see text.

Given that our results qualified the %PPI-ameliorating properties of finasteride in relation to the mechanisms of D1 and D3 receptors, we further tested whether apomorphine-induced PPI disruption in SD rats could be countered by D1 and D3 receptor antagonists (Fig. 8.7). Both the D1 receptor antagonist SCH 23390 (0.1 mg/kg, s.c.) [$F(1,36)=6.61$; $P < 0.05$] and apomorphine [$F(1,36)=12.30$; $P < 0.01$] reduced acoustic startle amplitude (Fig. 8.7A) (n=10/treatment group). However, no significant interaction between these two treatments was found. PPI analyses revealed a significant interaction between SCH 23390 and apomorphine [$F(1,36)=5.96$; $P < 0.05$]. Post-hoc analyses revealed that, while apomorphine caused a significant PPI disruption ($P < 0.05$ for comparison between vehicle + saline and vehicle + apomorphine), SCH 23390 reversed this phenomenon ($P < 0.05$ for comparison between vehicle + apomorphine vs vehicle + SCH 23390)

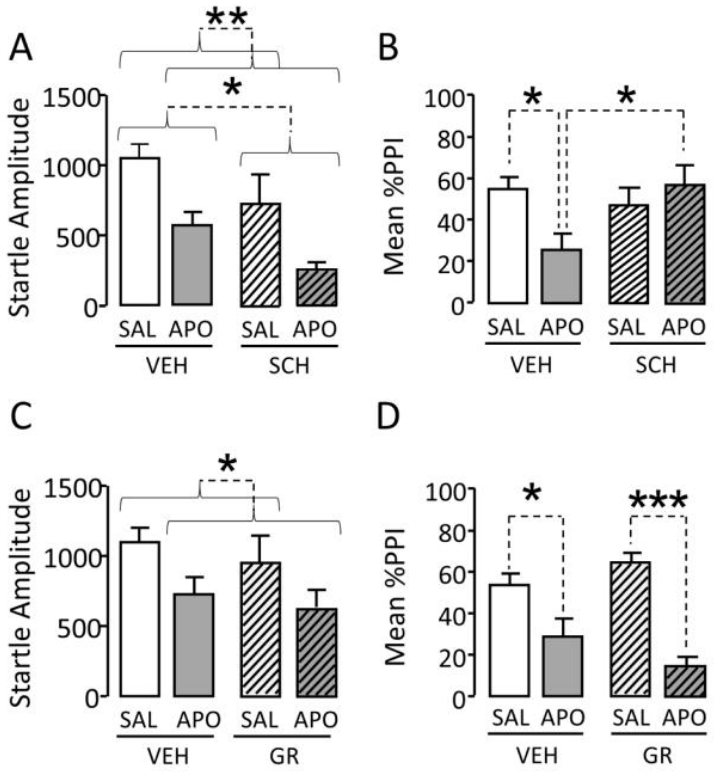

Figure 8.7 D1 but not D3 antagonists counter apomorphine induced PPI deficits in SD rats. Effects of the D1 receptor antagonist SCH 23390 (SCH, 0.1 mg/kg, SC) and the D3 receptor antagonist GR103691 (GR, 0.2 mg/kg, SC) on the changes in (A–C) acoustic startle and (B–D) prepulse inhibition (PPI) induced by the D1–D2 receptor agonist apomorphine (APO, 0.25 mg/kg, SC) in male Sprague-Dawley rats. Values represent mean ± SEM for each experimental group. N = 8–10/group. SAL, saline; VEH, vehicle; *, P<0.05; **, P<0.01; ***, P<0.001 for comparisons indicated by dotted lines. Curvy brackets are used to indicate main effects. For more details, see text.

(Fig. 8.7B), in agreement with previous findings [499]. We then tested the effects of the D3 receptor antagonist GR 103691 on the changes in startle and PPI produced by apomorphine (Fig. 8.7C– D) (n=8/treatment group). The analysis of acoustic startle response revealed a main effect for apomorphine (Fig. 8.7C) [$F(1,28) = 4.20$; $P = 0.05$], but not for GR103691 [$F(1,28) = 2.14$; NS]; furthermore, no significant interaction between these two treatments was found [$F(1,28)=0.16$; NS]. Finally, PPI analyses confirmed that apomorphine disrupted PPI [$F(1,28)=42.86$; $P<0.0001$; Main effect for apomorphine] (Fig. 8.7D), while GR 103691 did not affect PPI [$F(1,28)=0.11$; NS; Main effect for GR 103691]. ANOVA also detected a significant interaction between GR 103691 and apomorpine [$F(1,28)= 4.72$; $P<0.05$]; post-hoc comparisons revealed significant differences between vehicle + saline and vehicle + apomorphine ($P<0.05$) and between GR 103691+saline and GR 103691+apomorphine ($P<0.001$).

Finasteride counters the changes in dopamine levels in Nucleus Accumbens shell induced by D3 receptor activation. To verify whether the effects of finasteride on PPI may be reflective of changes in extracellular dopamine levels in the nucleus accumbens, we tested the effects of selective dopaminergic agonist on levels of the dopamine and DOPAC levels in the nucleus accumbens shell by means of microdialysis in freely moving SD rats (n=7–8/group). Extracellular basal values (mean ± SEM) were: dopamine=2.7±0.2 pg, DOPAC = 1.7±0.1 ng per sample (20 μl dialysate). Confirming our previous study [423], finasteride (100 mg/kg, i.p.) significantly increased extracellular dopamine [$F(6,42)=6.05$; $P<0.0001$] and DOPAC [$F(6,42)=2.53$; $P<0.05$] above the baseline, starting at 60 and 80 min after finasteride injection (100 mg/kg, i.p.), respectively. Vehicle plus saline administration did not affect dopamine and DOPAC levels (Fig. 8.8). Dopamine levels were significantly increased by the selective D1 agonist SKF 82958 (1 mg/kg, s.c.) [$F(6,18)=4.09$, $P<0.01$]; however, no interactions between finasteride

and SKF 82958 were detected [F(1,19)= 0.78, NS; 2-way ANOVA]. The D2 agonist sumanirole (3 mg/kg, s.c.) significantly decreased extracellular dopamine levels [F(6,24)= 11.9, $P< 0.0001$; however, this effect also failed to significantly interact with the effects of finasteride on PPI [F(1,24)= 0.38, NS). Conversely, the D3 receptor agonist PD 128907 (0.1 mg/kg, i.p.) significantly reduced extracellular dopamine levels [F(6,42)=6.05, $P< 0.001$], but this effect was significantly reversed by finasteride [F(1,20)=11.0, $P<0.01$] (Fig. 8.8). Temporal analysis showed that this effect was significant at 80 min after PD 128907 injection (Fig. 8.8E).

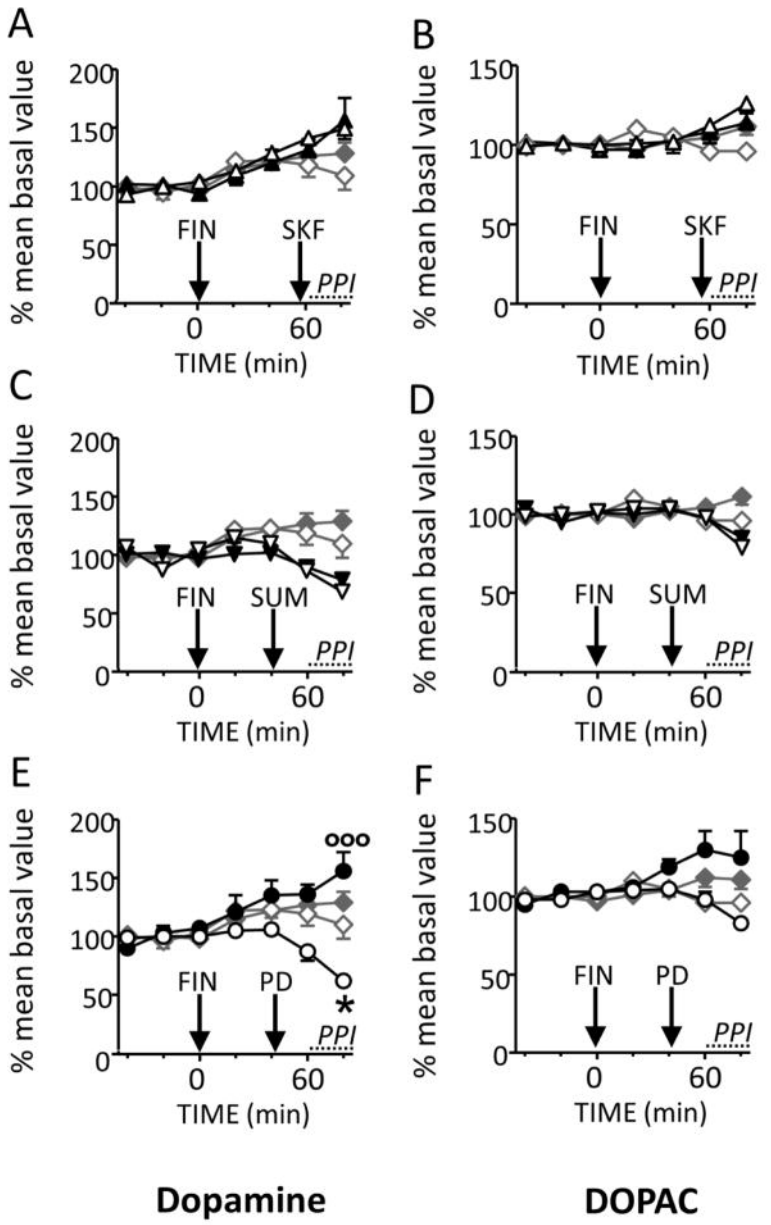

Figure 8.8 Finasteride counters the changes in dopamine levels in Nucleus Accumbens shell induced by D3 receptor activation. Time-related effects of systemic finasteride (FIN, 100 mg/kg, IP) on extracellular concentrations of dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in the nucleus accumbens shell of Sprague-Dawley rats. FIN (shaded symbols) or vehicle (white symbols) was tested in combination with (A–B) D1 agonist SKF 82958 (1 mg/kg, SC, triangles); (C–D) D2 agonist sumanirole (SUM, 3 mg/kg, SC, upside down triangles); (E–F) D3 agonist PD 128907 (0.1 mg/kg, IP, circles) or saline (diamonds). Arrows represent injection time of FIN or its vehicle (VEH) and the dopaminergic agonist or saline (SAL). The interval corresponding to PPI testing is indicated by a dotted bar alongside the time axis. Values are expressed as mean percent of the baseline (average values of the three first samples) ± S.E.M for each time point. N = 7–8/group. *, $P<0.05$ compared with baseline values; °°°, $P<0.001$ in comparison with VEH+PD 128907. Significant within-group (time-dependent) effects are not indicated. For further details, see text.

Discussion

In the present study, we first demonstrated that in contrast to SD albino rats, hooded LE rats display a significant impairment in sensorimotor gating in response to selective, full stimulation of D1 dopamine receptors. Specifically, under our experimental settings, the full D1 agonist SKF 82958, but not the partial D1 agonist SKF 38393 produced a significant PPI reduction, which was not paralleled by variations in startle amplitude, and was countered by D1, but not D2 receptor antagonism.

Following the establishment of dopamine receptor responses in SD versus LE rats we showed that the 5αR inhibitor finasteride prevented the PPI deficits induced by the activation of D1-like, but not D2 receptor agonists, across these different rat strains (Table 8.1). Specifically, in LE rats, finasteride effectively countered the PPI impairment induced by the potent D1 receptor agonist SKF 82958, but failed to significantly prevent the deficits mediated by the D2-like receptor agonist quinpirole or the non-selective D1–D2 receptor agonist apomorphine. Conversely, in SD rats, finasteride countered the PPI-disrupting effects of apomorphine, but not the D2 receptor

Effects of finasteride (FIN; 100 mg/kg, IP) in PPI	
Apomorphine; D1-D2 receptor agonist	FIN opposes % PPI and ΔPPI deficits in SD rats FIN does not oppose %PPI deficits in LE rats
SKF 82958; D1 receptor agonist	FIN opposes %PPI and ΔPPI deficits in LE rats
Quinpirole; D2–D3 receptor agonist	FIN does not oppose %PPI deficits in SD rats FIN does not oppose %PPI deficits in LE rats
Sumanirole; D2 receptor agonist	FIN does not oppose %PPI deficits in SD rats
PD 128907; D3 receptor agonist	FIN opposes %PPI, but not ΔPPI, deficits in SD rats

Table 8.1 Synoptic table of the combined effects of finasteride and dopaminergic agonists on the prepulse inhibition (PPI) of the startle in Long-Evans (LE) and Sprague-Dawley (SD) rats.

agonists quinpirole and sumanirole. Furthermore, our analyses revealed that, in SD rats, finasteride opposed the reduction of %PPI, but not ΔPPI, values induced by the D3 receptor agonist PD 128907. In parallel with these effects on sensorimotor gating, finasteride reversed the reduction in extracellular dopamine levels caused by D3, but not D2 receptor activation in the nucleus accumbens of SD rats. The identification of the selective involvement of D1 receptors in finasteride's effects across different rat strains extends and complements our previous reports on the antipsychotic-like properties of 5αR inhibitors [277, 422, 423, 504], and points to a specific mechanism of action for the emerging therapeutic potential of 5αR inhibitors in neuropsychiatric disorders [374].

The implication of D1-like receptors in finasteride-induced PPI amelioration was documented both directly in LE rats and indirectly in SD rats. The latter strain does not exhibit PPI impairments in response to administration of D1-like receptor agonists [499, 505]; however, finasteride countered the gating deficits induced by the D1–D2 receptor agonist apomorphine, but not the D2 activators quinpirole and sumanirole; furthermore, the actions of finasteride mirrored those of the D1 receptor antagonist SCH 23390. The implication of D1 receptor in the antipsychotic-like mechanisms of finasteride is in agreement with our previous results on C57BL/6 mice [277]. Although these animals do not exhibit PPI deficits in response to D2-like receptor agonists [300], finasteride fully prevented the PPI deficits induced by SKF 82958 and paradoxically led to PPI deficits following treatment with the D2 receptor agonist quinpirole [277]. Interestingly, key neurosteroids, such as AP and DHEAS, modulate the behavioral effects of D1 receptor activation [506, 507]; furthermore, progesterone and AP affect the phosphorylation of DARPP-32 [508, 509], a key molecule in D1 receptor signaling cascade [510].

We previously documented that the mechanism of action of finasteride in reversing apomorphine-induced gating deficits is likely reflective of changes in neurosteroid profiles in the nucleus accumbens [423]. Interestingly, the intra-accumbal effects of finasteride were not accompanied by any significant variations in dopamine extracellular levels, suggesting that the effects are not mediated by changes in dopamine release [423]. Those results, together with the lack of significant interactions between finasteride and SKF 82958 on dopamine levels in the nucleus accumbens documented in this study, strongly suggest that presynaptic D1 receptors are not directly involved in the modulatory role of 5αR on gating. Accordingly, the effects of apomorphine on PPI are regarded as primarily due to the activation of postsynaptic receptors [184]. Striatal D1 receptors are predominantly located in extrasynaptic locations of GABAergic medium-spiny neurons [511]. This particular localization is posited to enable D1 receptors to be preferentially activated by transient elevations of dopamine levels due to phasic bursts of dopaminergic neuron activity [512, 513]. Building on this perspective, the modulation of neurosteroidogenesis in the nucleus accumbens may affect sensorimotor gating by altering the response to different dynamics of dopamine neurotransmission. Notably, neurosteroids influence tonic and phasic GABA activity [514], whose cross-talk plays a fundamental role in PPI regulation [515]. Further studies are necessary to understand whether changes in tonic and phasic activity in GABAergic activity may be directly related to dynamic alterations in accumbal dopamine activity.

A second potentially important finding of this study was that finasteride countered the %PPI deficits induced by the D3 receptor agonist PD 128907. However, these results were not validated by parallel ΔPPI analyses, raising the possibility that the observed effects may be due to computational artefacts. Nevertheless, it is worth mentioning that the effects of finasteride on PPI were time-locked with a reversal of the PD 128907-mediated reduction in extracellular dopamine

levels, which has been linked to the stimulation of presynaptic D3 autoreceptors [516]. The results of these microdialysis studies (and, to a more limited extent, those on %PPI values) suggest that 5αR regulates D3 receptor signaling. Given that finasteride does not bind to D3 receptors (S. Ruiu, personal communication), our data suggest that 5αR may regulate their signaling by interfering with the function of one of their downstream effectors, which include Gi /Go proteins and inward rectifying potassium channels [517, 518]. Irrespective of the mechanisms, further studies with different testing protocols will be necessary to verify whether the interaction between 5αR and D3 receptors is actually relevant to PPI regulation.

Although finasteride opposed the %PPI disruption induced by both apomorphine and PD 128907, the lack of effects of the D3 receptor antagonist GR 103691 on apomorphine-mediated effects suggest that these two effects were likely underpinned by distinct processes, namely the actions of finasteride on D1-like and D3 receptors. This difference is in line with the strikingly different properties of these two receptor subtypes: on one hand, D1-like receptors are conducive to excitatory effects, through the concatenated activation of Gαs and Gαolf proteins and their downstream effectors [519]; on the other hand, D3 receptor function appears to be primarily inhibitory [520, 521]. Despite this phenomenological dichotomy, some of the actions of 5αR on D1 and D3 receptor signaling may affect common intracellular substrates. Indeed, these receptor types are highly colocalized in extrasynaptic compartments of the nucleus accumbens [522], and have been found to interact at multiple levels [523], including the formation of heteromers [524]. With respect to this issue, it should be noted that the implication of D1 receptors in the formation of dimers often requires σ1 receptors, which are targeted by several neurosteroids [525]. Future studies will be needed to verify the implication of σ1 receptors in the actions of finasteride.

In keeping with previous evidence [422], we found that the dose of finasteride used in these studies (100 mg/kg, i.p.) reduced startle amplitude in SD rats; however, this drug had surprisingly no such effect on LE rats. The difference in the effects of finasteride on startle amplitude across these two strains may reflect the diverse properties of this drug with respect to locomotor activity: in fact, the same dose of finasteride used in this study produced a generalized decrease in locomotor activity in SD rats [422]; conversely, ongoing studies in our lab are indicating that LE rats exhibit a greater resistance to the locomotor depression induced by high doses of finasteride (data not shown). Future studies are warranted to elucidate the neurobiological bases of the different reactivity of SD and LE rats to finasteride with respect to startle and locomotor activity.

The marked differences between the effects of finasteride in SD and LE rats are in line with previous evidence on the distinct PPI responses in these two strains [526]. Although the molecular underpinnings of these differences remain unclear, our data suggest a potential role of neurosteroids in these changes. Previous studies have documented that LE rats exhibit higher dopamine turnover in comparison with SD rats [527], possibly due to changes in the dopamine-metabolic enzyme catechol-O-methyl transferase (COMT) [528]. Notably, this enzyme has been shown to be affected by neuroactive steroids; for example, COMT expression is enhanced by testosterone and dihydrotestosterone (DHT) [529] and reduced by estrogens [530]. These premises suggest that differences in 5αR or other neurosteroids may contribute to the differences in gating regulation between SD and LE rats.

Several limitations of the present study should be acknowledged. First, our analyses did not include analyses of neurosteroid profiles to evaluate the mechanisms underpinning the observed interstrain differences with respect to the role of finasteride on the effects of dopaminergic agonists in both strains. Nevertheless, in preliminary studies, we have verified that

the analysis of neurosteroid profile in the nucleus accumbens alone is not currently feasible, given the limited size of this region and the detection limits of available systems.

Secondly, our data cannot rule out that the observed effects of finasteride may be partially mediated by peripheral effects; in particular, finasteride inhibits the conversion of testosterone into the potent androgen hormone DHT [374]. However, this possibility is tempered by our prior finding that the effects of finasteride on sensorimotor gating are not affected by gonadectomy [423]; in addition, the involvement of testosterone in these effects is unlikely, given that, in separate studies performed on the neurosteroid profile of the combination of striatum and nucleus accumbens, the same dose of finasteride used in this study (100 mg/kg, i.p.) failed to modify the levels of this steroid [504].

Thirdly, given the broad scope of our studies, behavioral and microdialysis experiments were restricted to the analysis of the effects of optimal doses of finasteride and dopaminergic agonists, based on our prior research and other relevant scientific literature. The lack of dose-response curves, however, limits a comprehensive assessment of the interstrain differences in the dopaminergic regulation of rat PPI, and leaves open the possibility that the actions of different finasteride concentrations may result in different effects on PPI regulation in combination with different dopaminergic drugs. In a similar way, PPI was consistently tested with 100-ms interstimulus intervals, as this particular setting allowed us to reveal PPI disrupting effects of D1 and D2 receptor agonists in LE rats [320], as well as D2 and D3 receptor agonists in SD rats. Different testing conditions and protocols, however, may reveal different effects of finasteride with respect to the dopaminergic modulation of PPI.

Finally, although our experiments were performed on equivalent experimental protocols and apparatuses, it is worth noting that the experiments were performed in two different

laboratories (SD at the University of Cagliari, and LE at the University of Kansas). Thus, we cannot completely exclude divergences in the colonies from the suppliers. Indeed, differences in PPI can reflect sub-strain variations based on the specific location of the supplier [501]. Nevertheless, these potential concerns are tempered by the similarity of results obtained in both laboratories on the effects of finasteride in modifying PPI preventing, in co-treatment with both APO and QUI in SD rats (data not shown).

These limitations notwithstanding, our results highlight a neurobiological link between 5αR, neurosteroids and dopamine receptors, which may be particularly important in the pathophysiology of neuropsychiatric disorders characterized by gating deficits, including schizophrenia and TS. In preliminary clinical observations, we documented that finasteride elicits potential therapeutic effects in these disorders [416, 417, 531, 532]. Furthermore, emerging data indicate the potential of D1 and D3 receptor blockers in the treatment of TS and schizophrenia, respectively [56, 533]. The selective action of finasteride on these receptors, rather than D2, may help explain the lack of extrapyramidal symptoms associated with 5αR inhibitors [422].

Whereas further research is needed to address these limitations, our findings highlight the critical role of 5αR in the pathophysiology of gating deficits, and point to an important functional link between neurosteroids and D1 and D3 receptors, which may be implicated in the pathophysiology of schizophrenia, TS and other related disorders.

Conflict of interest

The authors declare no conflict of interest.

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Authors’ contributions

R.F. and L.M. monitored data collection, analyzed behavioral data and performed statistical analyses. VB, performed behavioral tests and performed statistical analyses. L.M., G.P., R.P., P.S., S.F. performed the behavioral tests. P.D. designed the experiments, analyzed data and discussed the paper. MB designed the experiments, supervised the experimental execution, monitored data collection, performed statistical analyses, wrote and revised the manuscript.

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9. 5α-reductase type 1 mediates the PPI ameliorating effects of finasteride against dopamine D1 receptor agonists

Introduction

The research presented up to this point in this dissertation has emphasized the role of neurosteroids in the mediation of stress-induced fluctuations of TS-related symptoms and highlighted a therapeutic option for blocking steroidogenesis with the 5αR inhibitor finasteride. However, as presented in chapter 5, there are side effects of finasteride that make it a less desirable treatment option, which include demasculinizing effects for children. To address this issue, we have further explored the contributions of the two isoforms of 5αR that finasteride inhibits. As discussed previously, 5αR2 is the primary mediator of androgen synthesis; therefore, targeting 5αR1 specifically would result in a safer option for children if 5αR1 proves to be a therapeutic option for TS. To explore this possibility we used mice deficient of either 5αR1 or 5αR2 and studied the response of these mice to dopamine receptor agonists in the PPI paradigm.

Materials and Methods

Animals. The experiments included in this study were performed on adult (3-5-month old), experimentally naïve male 5αR1KO and 5αR2KO mice along with the HZ and WT littermates (strain: C57BL/6), obtained from breeding colonies at the Universities of Kansas and Utah. All mice were generated from HZ x HZ crosses. Progenitors were obtained by Dr. Mala Mahendroo (Southwestern University). Unless stated otherwise for specific experimental purposes, all mice were housed in groups of 4-5/cage, with at least 1 mouse/genotype, and had ad libitum access to food and water. Housing facilities were maintained at 22ºC with a reverse light/dark cycle (lights off at 08:00 AM hours and on at 08:00 PM). Whenever the same mice were used for multiple behavioral paradigms, the order of animals in each test was counterbalanced throughout the study.

Tests were arranged from least to most stressful (separated by at least one week) to minimize carry-over stress. Experimental manipulations were carried out in the animals' dark cycle between 10:00 AM and 6:00 PM. All handling and experimental procedures were performed in compliance with the National Institute of Health guidelines and approved by the local Institutional Animal Care and Use Committees.

Genotyping. Mouse genotyping was performed by PCR as described in chapter 7.

Locomotor Activity. Locomotor behaviors were measured in a square force-plate actometer as previously described in chapter 3. Mice (n=8/genotype) were placed in the center and their behavior was monitored for 20 min for the baseline locomotor activity studies. In a subsequent study 5αR1 KO, HZ, and WT mice (n=8/genotype) were placed in the center and their behavior was monitored for 60 min before they were removed, treated with SKF 82958, quinpirole or vehicle and placed back into the actometer for an additional 120 min.

Acoustic Startle Reflex and Prepulse Inhibition (PPI) of the Startle. Acoustic startle reflex and PPI were tested as previously described in chapter 3.

Western blot. Prefrontal cortices and ventral nucleus accumbens were harvested and homogenized on ice in homogenization buffer containing 1% SDS, protease and phosphatase inhibitor cocktail. Small aliquots of the homogenate were used for protein determination by a modified Lowry protein assay method (DC protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Immunoblotting was performed as previously described with slight modifications [534, 535]. Equal amounts of proteins were separated on a 4–15% Criterion TGX stain free Precast Gel (Bio-Rad Laboratories, Hercules, CA, USA) by electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were then blocked with 3% bovine serum albumin in TRIS-buffered saline supplemented with 0.1% Tween 20 for 2 h at room temperature and then incubated

overnight with primary antibodies at 4 °C. Primary antibodies used in this study include the following: ERK (Cell-Signaling, Danvers, MA, USA); Phospho-ERK (Cell-Signaling, Danvers, MA, USA); DARPP-32 (Cell-Signaling, Danvers, MA, USA); Phospho-THR75-DARPP-32 (PhosphoSolutions, Aurora, CO, USA); Phospho-THR34-DARPP-32 (PhosphoSolutions, Aurora, CO, USA) antibodies. After washing, membranes were incubated with secondary horseradish peroxidase antibodies and developed with Clarity Western chemiluminescence blotting substrate (Bio-Rad Laboratories, Hercules, CA, USA). Stain-free gels were analyzed on a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and bands were normalized to total protein per lane [536].Samples containing the same amount of total proteins from mice in each experimental group were run on the same immunoblots.

Statistical Analyses. Data were tested for normality and homoscedasticity by the Kolmogorov-Smirnov and Bartlett's test. Based on these results, parametric and non-parametric statistical analyses were performed by a one-way ANOVA and Kruskal-Wallis test, followed by Tukey's or Nemenyi's tests for post-hoc comparisons, respectively. Significance was set at $P = 0.05$.

Results

Sensorimotor gating and locomotor activity in mice deficient of 5αR type 1 or type 2. We first analyzed the sensorimotor gating capabilities and locomotor activity in mice with a genetic knockout of 5αR1 or 5αR2. As described in figure 9.1, 5αR1 KO mice did not display differences for WT or HZ littermates in mean startle amplitude (Fig 9.1A), average % PPI (Fig 9.1C), or total distance moved in the force plate actometer (Fig 9.1C). Similarly, 5αR2 KO mice also did exhibit any marked alterations in these paradigms (Fig 9.1D-E).

D1 agonists do not induce PPI deficits in 5αR1 knockout mice. We next performed experiments to determine the responses of 5αR1 KO mice to D1 and D2 receptor agonists. We found that the D1 agonist, SKF 82958, did not alter mean startle amplitude in 5αR1 KO mice or the WT and HZ littermates (Fig 9.2A), although there was a trend toward a significant decrease in startle in the 5αR1 KO mice (main effect of genotype: $F(2,42) = 5.57$, $P = 0.07$). However, we did find genotype differences in the PPI response following SKF 82958 treatment (Fig 9.2B; significant interaction: $F(2,42) = 4.38$, $P < 0.05$). Post hoc analysis revealed that the WT mice displayed the expected PPI deficit induced by SKF 82958 (WT: vehicle vs SKF 82958: $P < 0.01$).

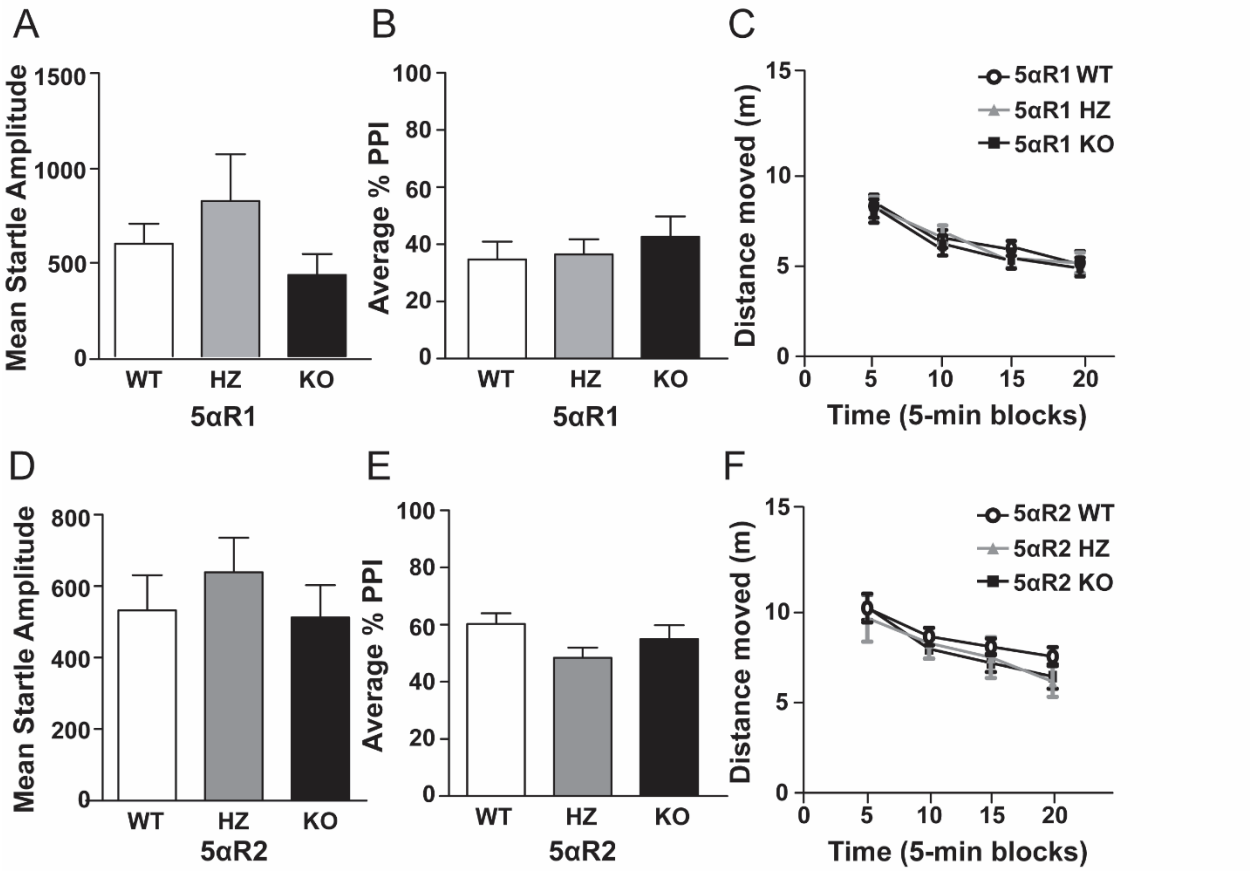

Figure 9.1 Mice deficient of either 5αR1 or 5αR2 do not display alterations in sensorimotor gating capabilities or locomotor behavior. The mean startle response recorded during the prepulse inhibition (PPI) of the startle response was analyzed in 5αR1 KO (A) or 5αR2 KO (D) mice along with their WT and HZ littermates. The average inhibition of the startle by a 3, 6, or 12 dB prepulse was calculated in 5αR1 KO (B) or 5αR2 KO (E) mice along with control littermates. Locomotor activity was tested in the force plate actometer. The distance moved (5 min bins) is represented for 5αR1 KO (C) and 5αR2 KO (D) mice. Data are shown as mean ± SEM. N=8-9/genotype. WT, wildtype; HZ, heterozygous; KO, knockout, 5αR1 5α-Reductase type 1; 5αR2 5α-Reductase type 2

However, SKF 82958 did not induce a significant decrease in PPI in either HZ or KO mice; furthermore, both HZ and KO mice had higher PPI compared to WT treated with SKF 82958 (all treated with SKF: WT vs HZ, p<0.05; WT vs KO, $P < 0.01$). In a separate cohort of mice, we investigated the response to the D2 agonist, quinpirole in PPI. In this experiment there a significant effect of genotype on startle responses ($F(2,53) = 37.57$, $P < 0.001$) and a trend toward an effect from quinpirole treatment ($F(1,53) = 3.52$, $P = 0.08$). In addition, we also found a significant effect of both genotype ($F(2,53) = 10.29$, $P<0.05$) and quinpirole on PPI responses ($F(1,53) = 7.50$, $P<0.05$). These data mirror our previous findings of WT mice treated with finasteride [277], in

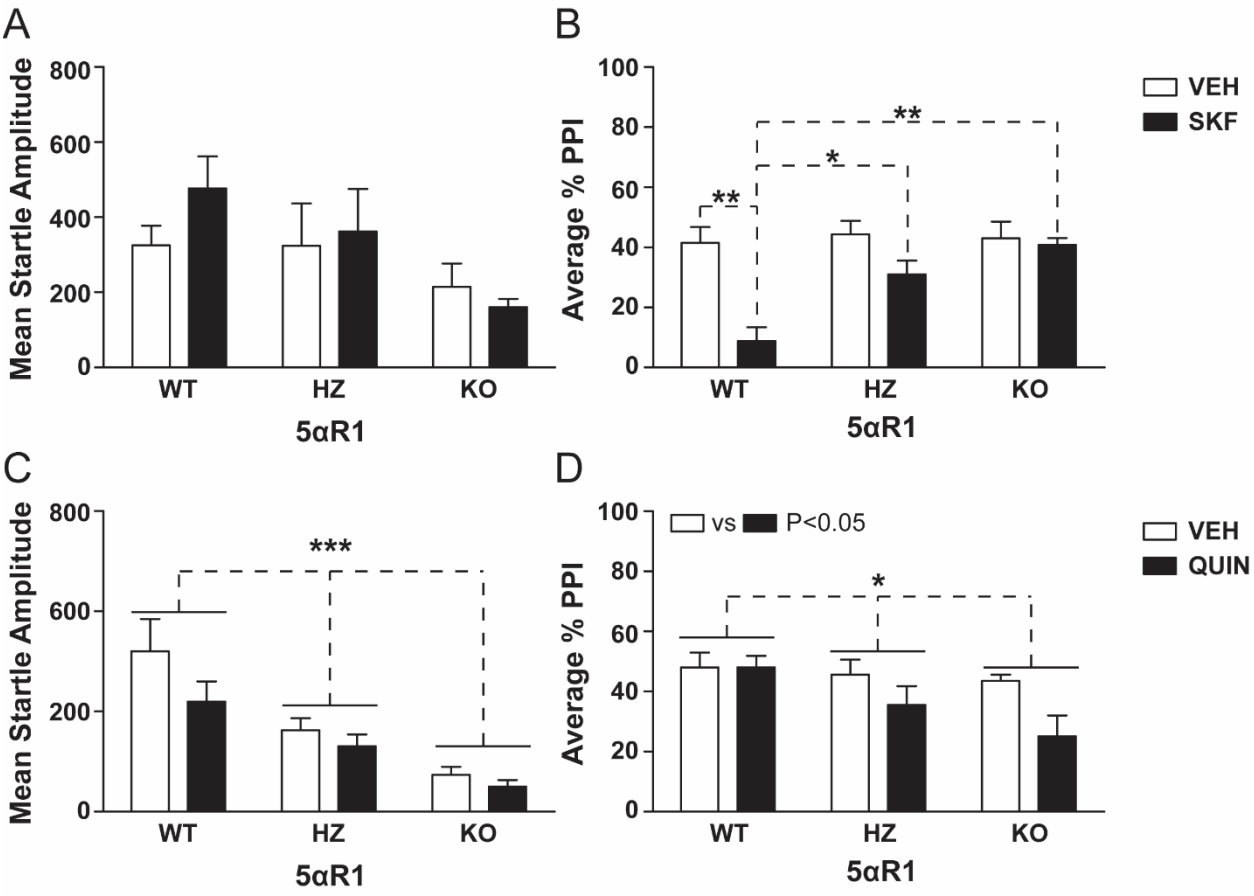

Figure 9.2 5αR1 KO mice are insensitive to PPI disrupting effects from D1 receptor agonists. Mice were treated with SKF 82958 (0.3m/kg, IP) and tested in the PPI paradigm. The mean startle amplitude (A) and the average % PPI (B) were recorded. A second cohort of mice was treated with Quinpirole (0.5mg/kg, IP) and also tested in the PPI paradigm. The mean startle amplitude (C) and average % PPI (D) are reported. Data are shown as mean ± SEM. N=8/genotype.*P<0.05, **P<0.01, ***P<0.001 for comparisons indicated. Main effects are described in graph. WT, wildtype; HZ, heterozygous; KO, knockout, 5αR1 5α-Reductase type 1; VEH, vehicle; SKF, SKF 82958; QUIN, quinpirole.

which we demonstrated that finasteride ablated the effect of SKF 82958 to induce PPI deficits but synergized with quinpirole to induce deficits.

The effects of D1 and D2 agonists on PPI in 5αR2 knockout mice. To determine if 5αR1 and 5αR2 serve the same function in PPI regulation we next tested 5αR2 KO in the PPI following treatment with either the D1 agonist SKF 82958 or the D2 agonist quinpirole. Similar to the studies in 5αR1 KO mice we did not observe any alteration in mean startle amplitude as a result of SKF 82958 treatment (Fig 9.3A). However, unlike 5αR1 KO and HZ littermates, the 5αR2 KO and HZ mice were sensitive to the PPI disrupting effects of SKF 82958 (Fig 9.3B; main effect of treatment: $F(1,41) = 38.19$, $P < 0.001$). We also analyzed the PPI response of the 5αR2 KO mice to the D2 receptor agonist, quinpirole. We found that quinpirole significantly decreased the mean startle

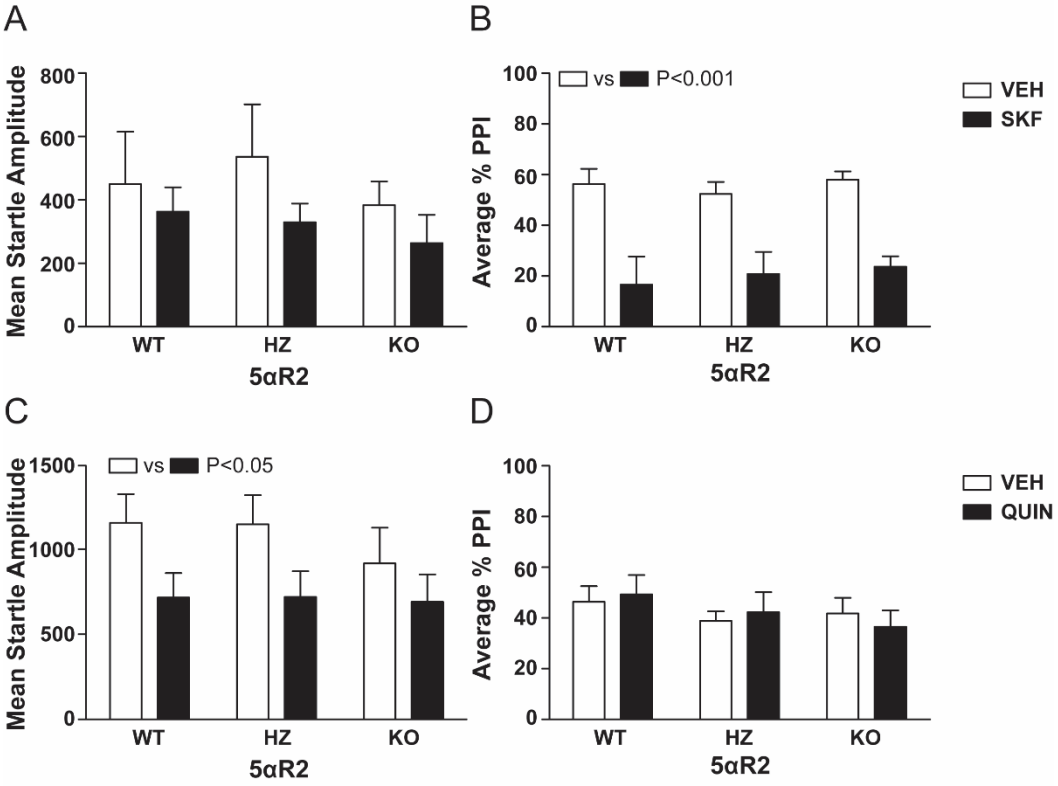

Figure 9.3 5αR2 KO mice do not display altered PPI responses to dopamine agonists. Mice (n=7-9/genotype) were treated with SKF 82958 (0.3m/kg, IP) and tested in the PPI paradigm. The mean startle amplitude (A) and the average % PPI (B) were recorded. A second cohort of mice (n=8/genotype) was treated with Quinpirole (0.5mg/kg, IP) and also tested in the PPI paradigm. The mean startle amplitude (C) and average % PPI (D) are reported. Data are shown as mean ± SEM. Significant main effects are described in graph. Abbreviations: WT, wildtype; HZ, heterozygous; KO, knockout, 5αR1 5α-Reductase type 2; VEH, vehicle; SKF, SKF 82958; QUIN, quinpirole

amplitude in all genotypes (Fig 9.3C; main effect of treatment $F(1,42) = 6.97$, $P < 0.05$). In addition, quinpirole did not elicit any significant alterations in average % PPI (Fig 9.3D). Due to the decrease in startle we also analyzed the delta PPI to account for an exaggerated PPI response; however, analysis of delta PPI did not reveal any deficits induced by quinpirole (data not shown).

Finasteride does not counter D1 agonist SKF 82958 in 5αR2 knockout mice. We have previously described [277] that the 5αR inhibitor finasteride attenuated the PPI disrupting effects of SKF 82958 in WT C57 mice. To verify if 5αR2 KO mice also responded to finasteride in this manner we pretreated the mice with finasteride prior to SKF 82958 treatment. Since the HZ mice did not display any alterations compared to WT or KO littermates we limited these studies to WT mice and KO littermates. In WT mice we found significant alterations in the startle response due to SKF 82958 treatment (Fig 9.4A; $F(1,36) = 6.93$, $P < 0.05$). We also confirmed the data previously published in WT mice the finasteride treatment counted the PPI disrupting effects of SKF 82958 (Fig 9.4B; pretreatment*treatment interaction $F(1,36) = 5.2$, $P < 0.05$; relevant post hoc comparisons: vehicle + vehicle vs vehicle + SKF 82958, $P < 0.05$; vehicle + SKF 82958 vs finasteride + SKF 82958, $P < 0.01$). In the 5αR2 KO mice we found a significant interaction for the mean startle amplitude analysis (Fig 9.4C; $F(1,44) = 5.58$, $P < 0.05$); however, no significant comparisons were revealed in post hoc analysis. Interestingly, we did not find an interaction in the PPI responses of the 5αR2 KO mice (Fig 9.4D). Instead we observed only a main effect of SKF 82958 treatment ($F(1,44) = 33.69$, $P < 0.001$), indicating that finasteride does not ablate SKF 82958 induced deficits in the 5αR2 KO mice and that 5αR2 may be required for the therapeutic effect of finasteride.

The effects of D1 and D2 agonists on locomotor activity in 5αR1 knockout mice. Due to the alterations found in PPI regulation from D1 and D2 receptors in the 5αR1 KO mice, we next performed experiments on the effects of the agonists on locomotor activity. We did not include HZ littermates since there were no significant alterations between the HZ mice and their littermates. We found that 5αR1 KO mice behaved equivalently to WT littermates following treatment with SKF 82958 (Fig 9.5A) and quinpirole (Fig 9.5B). As expected, SKF 82958 induced a significant hyperlocomotion in the mice following the 60 minute habituation to the arena (Fig 9.5A) in both WT and KO mice (significant time*treatment interaction ($F(17,594) = 12.9$, $P <$

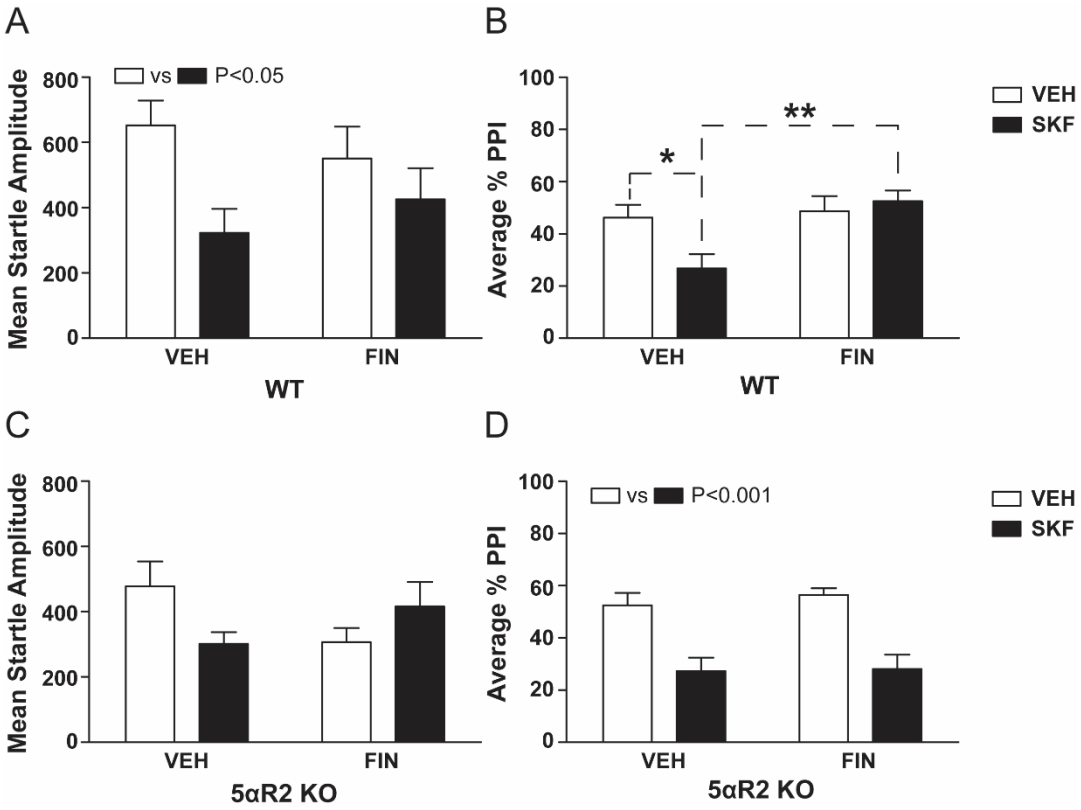

Figure 9.4 Finasteride does not counter PPI deficits induced by SKF 82958 in 5αR2 KO mice. Mice (10/WT group, 12/KO group) were pretreated with finasteride (50 mg/kg, IP) 30 minutes prior to treatment with SKF 82958 (0.3mg/kg, IP). The mean startle amplitude was recorded for both WT (A) and KO (C) mice. The average % PPI was also analyzed for WT (B) and KO (D) mice. Data are shown as mean ± SEM. *P<0.05, **P<0.01 for comparisons indicated. Main effects are described in graph. Abbreviations: WT, wildtype; KO, knockout, 5αR2 5α-Reductase type 2; VEH, vehicle; SKF, SKF 82958; FIN, finasteride

0.001; significant points compared to the 60 minute baseline are marked in graph). In addition, quinpirole induced hypolocomotion in the mice (Fig 9.5B) however post hoc analysis only identified significant points in the WT mice (time*treatment interaction: $F(17,522) = 2.8$; $P < 0.001$; significant points compared to the 60 minute baseline are marked in graph).

Allopregnanolone restores the PPI disrupting effect of D1 agonists. Given the results concerning AP in other animal models of TS, we designed experiments to determine if low levels of endogenous AP is protective against PPI deficits induced by D1 agonists. To this end, we

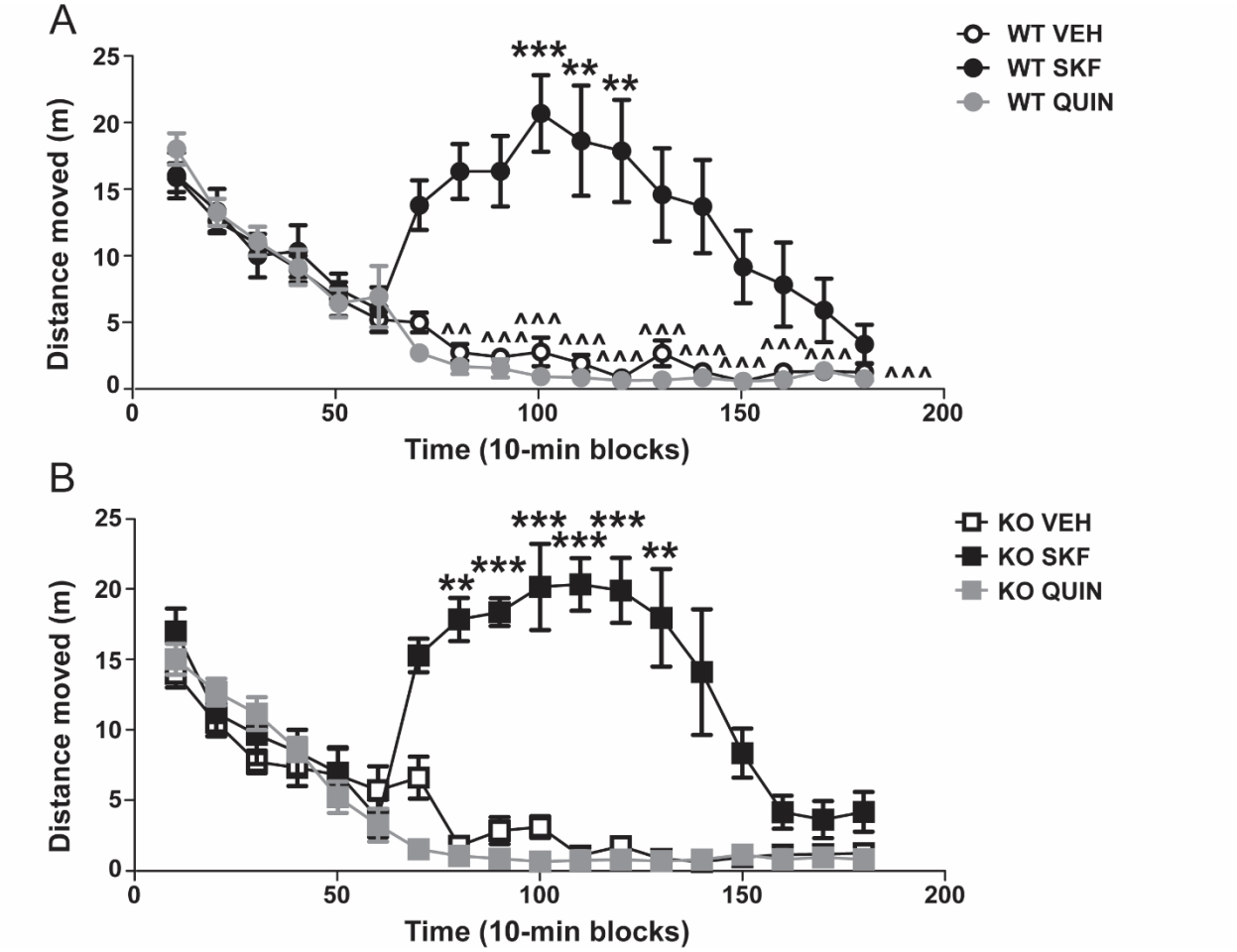

Figure 9.5 Locomotor responses to dopamine D1 and D2 agonists in 5αR1 KO mice. Mice (7-10/group) were allowed to habituate to the force plate actometer for 60 minutes. At 60 minutes the mice were briefly removed and injected with VEH, SKF 82958 (0.3mg/kg, IP) or Quinpirole (0.5mg/kg, IP) and returned to the actometer. The responses of WT mice (A) and 5αR1 KO littermates (B) are described. Data are shown as mean ± SEM. **P<0.01, ***P<0.001 for comparisons between baseline values at 60 minutes and SKF treatment at time indicated; ^^P<0.01, ^^^P<0.001 for comparisons between baseline values at 60 minutes and QUIN treatment at time indicated. Abbreviations: WT, wildtype; KO, knockout, 5αR1 5α-Reductase type 1; VEH, vehicle; SKF, SKF 82958; QUIN, quinpirole

pretreated 5αR1 KO mice with a low dose of AP prior to SKF 82958 treatment. We observed a trend toward an effect of AP on mean startle amplitude (Fig 9.6A; $F(1,27) = 3.67$, $P = 0.07$). We also found a significant interaction on average %PPI (Fig 9.6B; $F(1,27) = 4.90$, $P < 0.05$). This interaction revealed that AP pretreatment synergized with SKF 82958 to induce PPI deficits (vehicle + SKF 82958 vs AP + SKF 82958: $P < 0.01$; AP + vehicle vs AP + SKF 82958: $P < 0.01$).

Allopregnanolone in the PFC reveals PPI deficits following treatment with D1 agonists in SD rats. To further characterize the actions of AP and pinpoint a region of action we performed AP infusions into the PFC of SD rats prior to SKF 82958 treatment. As presented in

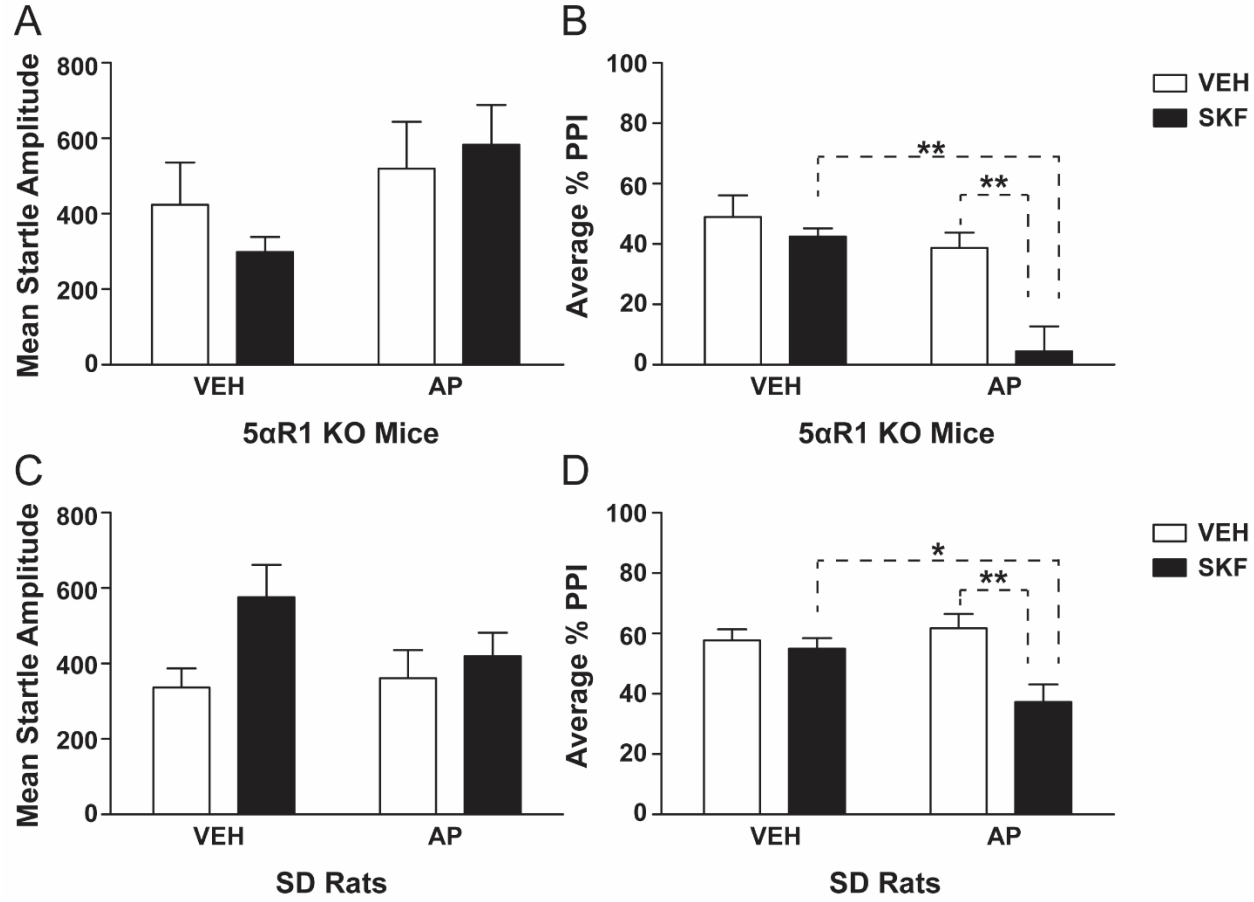

Figure 9.6 Allopregnanolone synergizes with SKF 82958 to induce PPI deficits in the 5αR1 KO mice and SD rats. 5αR1 KO mice were pretreated with AP (3mg/kg, IP) shortly before SKF 82958 (0.3mg/kg, IP) treatment and then tested in the PPI paradigm. (A) The mean startle response and (B) the average % PPI of the 5αR1 KO mice were recorded. AP (1ug/0.5ul/side) was infused directly into the PFC of SD rats shortly before SKF 82958 systemic injection (0.05mg/kg, IP). (C) The mean startle amplitude and (D) average % PPI were subsequently recorded. Data are shown as mean ± SEM. **P<0.01 for comparisons indicated. Abbreviations: KO, knockout, 5αR1 5α-Reductase type 1; VEH, vehicle; SKF, SKF 82958; AP, allopregnanolone; SD, Sprague-Dawley

chapter 8, SD rats are not inherently sensitive to SKF 82958, much like the 5αR1 KO mice. In this analysis we found a main effect of SKF 82958 to increase the startle of the rats (Fig 9.6C; $F(1,36)=4.58$, $P < 0.05$) but no interaction between AP and SKF 82958. However, we did find an interaction between AP infusion into the PFC and SKF 82958 treatment on PPI in SD rats (Fig 9.6D; $F(1,36) = 5.70$, $P < 0.05$). Specifically, these results showed that the combination of AP + SKF 82958 induced PPI deficits that were significantly lower than the vehicle + vehicle ($P < 0.05$), vehicle + SKF 82958 ($P < 0.05$), or the AP + vehicle ($P < 0.01$) treatment groups.

Dopamine signaling in 5αR1 knockout mice. To determine if dopamine signaling was altered in the 5αR1 KO mice we treated mice with SKF 82958 prior to dissecting out the brain regions of importance to PPI regulation. We started our analysis in the PFC since that was the brain region identified in the rat studies to be involved in the synergism between D1 and AP. However, we did not reveal any effect from genotype or SKF 82958 on ERK phosphorylation (Fig. 9.7A). Therefore, we extended our analysis to the ventral striatum, which has previously been demonstrated to be a key region for the effects of finasteride on PPI [423]. We found that in the ventral striatum there was a significant increase in the phosphorylation of the dopamine signaling molecule ERK1/2 (Fig 9.7B; main effect of treatment $F(1,19) = 0.23$, $P < 0.05$). Additionally, there was a trend toward an increase in the phosphorylation of DARPP-32 at THR75 (Fig 9.7C; main effect of treatment $F(1,20) = 13.3$, $P = 0.095$). Analysis of the phosphorylation of DARPP-32 at THR34 revealed a significant main effect of SKF 82958 treatment (Fig 9.7D; main effect of treatment $F(1,21) = 5.24$, $P < 0.05$). There was also a trend toward a main effect of genotype

(F(1,21) = 3.09, *P* = 0.09) and a trend toward a genotype x treatment interaction (F(1,21) = 3.15, *P* = 0.09).

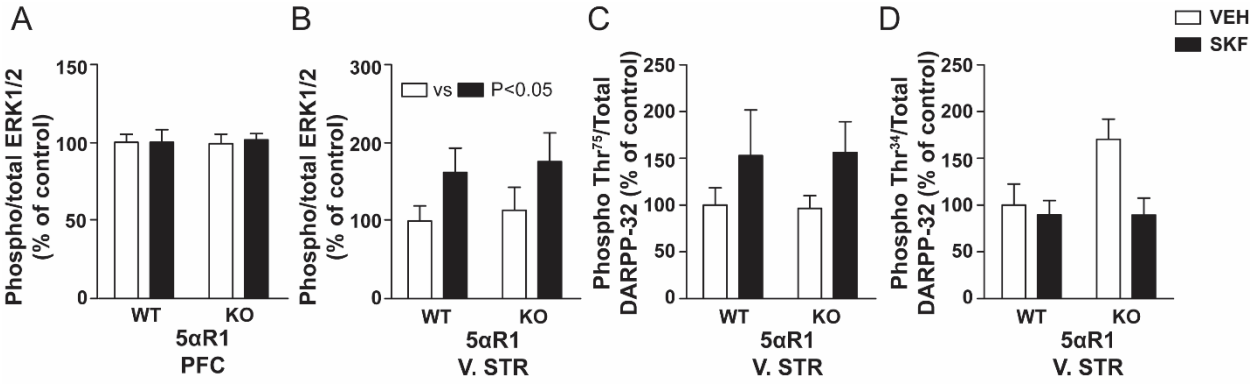

Figure 9.7 Dopamine D1 receptor signaling in 5αR1 KO mice. The PFC and ventral striatum were extracted and analyzed for the D1 signaling molecules P-ERK (A, D), Phospho Thr75 DARPP-32 (B) and Phospho Thr34 (C) following treatment with SKF 82958 (0.3mg/kg, IP). Data are shown as mean ± SEM. Main effects are indicated in graphs. Abbreviations: PFC, prefrontal cortex; V. STR, ventral striatum; WT, wildtype; KO, knockout, 5αR1 5α-Reductase type 1; VEH, vehicle; SKF, SKF 82958

Discussion

The results presented in this chapter demonstrate that AP synergizes with D1 receptor agonists to induce PPI deficits in both mouse and rat models. This was presented through several different animal models and pharmacological manipulations.

First we demonstrated that despite similar baseline responses (Fig 9.1) 5αR1 KO mice were not sensitive to the PPI disrupting effects of SKF 82958 in the same manner as their WT littermates (Fig 9.2B). In contrast, the 5αR2 KO mice responded in an identical manner to their WT littermates following treatment (Fig 9.3B). Several possible conclusions can be drawn from this data. First, the results from the 5αR1 KO mice mirror the results obtained in WT C57Bl/6 mice where finasteride ablated the PPI deficits induced by SKF 82958 [277]. Hence, it could be assumed that the primary mechanism of finasteride in this ameliorating SKF 82958 induced PPI deficits is through the inhibition of 5αR1. This is confirmed by the 5αR2 KO data which demonstrates that these mice retain the sensitivity to SKF 82958.

Alternatively, these data could point to the importance of homeostatic AP (and other neurosteroid) levels. The characteristics of 5αR1 suggest that this isoenzyme regulates tonic neurosteroid levels: it is widespread throughout the brain, the pH optima is at a neutral pH of 6-8.5, and it has a relatively low substrate affinity [383, 385-391]. These characteristics point to a looser regulation of 5αR1 activity which would allow it to function in normal physiological environments. In contrast, 5αR2 is more heterogeneously expressed in the brain, has a narrow and more acidic pH optima of 5-5.5 and has a higher affinity for substrates than 5αR1 [372, 374, 392, 394]. Hence, a tighter regulation on 5αR2 activity and high substrate affinity suggest a more specific function that must be turned on and off quickly. Therefore, due to the tonic nature of 5αR1, the levels of the 5α-reduced steroids would be decreased in the mice lacking 5αR1; whereas, loss

of 5αR2 would not as drastically effect tonic steroid levels since the characteristics of this enzyme suggest a more phasic nature. Hence, it is possible that the 5αR1 KO mice are resistant to SKF 82958 due to low levels of AP. This hypothesis is supported by two key experiments performed in the 5αR1 KO and 5αR2 KO mice. First, we found that finasteride does not attenuate PPI deficits induced by D1 receptor stimulation in the 5αR2 KO mice (Fig 9.4). This surprising finding indicates that while there are sufficient levels of neurosteroids to allow SKF 82958 to produce PPI deficits, finasteride requires 5αR2 to be effective. Secondly, we found that low doses of AP synergized with SKF 82958 treatment to induce PPI deficits in both 5αR1 KO mice and SD rats, which are inherently resistant to D1 receptor-induced PPI deficits (Fig 9.6). Taken together these data indicate that the primary mechanism of resistance in 5αR1 KO mice and SD rats is likely due to low levels of AP; however, further experiments are needed to determine the molecular underpinnings of these observations. In addition, the finding that the 5αR2 KO mice do not respond to the therapeutic effects of finasteride indicates that the primary mechanism of action of finasteride in through inhibition of 5αR2 not 5αR1.

Finasteride is primarily an inhibitor of 5αR2 in humans, although due to the chronic nature of therapy it is likely that 5αR1 is inhibited to a significant extent as well. However, the inhibitor sensitivity of the isoenzymes differs between rodents and humans. Indeed, it has been reported that in rats, finasteride displays a 4-13 selectivity for 5αR2 over 5αR1, while in humans there is >100-fold selectivity [537]. The results from the studies presented here and clinical data on finasteride indicate that in the case of finasteride's effects against D1 agonists in PPI, 5αR2 is the primary enzyme to focus on. Yet, further research must be conducted on specific 5αR1 inhibitors before this conclusion is drawn. For one, finasteride appears to inhibit rodent 5αR1 differently from rodent 5αR2 or human 5αR1 and 2. Inhibition studies have indicated that while the reaction to

inhibit rodent 5αR2, human 5αR1, and human 5αR2 produces an enzyme-bound NADP-dihydrofinasteride adduct [397], the dissociation constant of this adduct for 5αR results in a very slow turnover of the complex, creating a practically irreversible inhibition [383]. However, this is not the case for rat 5αR1. The inhibition of 5αR1 by finasteride in rats is not time-dependent, like it is for rodent 5αR2 and human 5αR1 and 2, and this inhibition of rat 5αR1 is fully reversible by washout. In addition, no detection of dihydrofinasteride is found in inhibition studies of rat 5αR1 but is in the study of the other enzymes [537]. Mouse 5αR1 might differ from rat 5αR1; however, these data indicate that we cannot yet determine that 5αR1 is not a viable option for countering D1 agonist induced deficits, especially since the mice deficient of this enzyme are not responsive to SKF 82958. Due to the differences between rodent 5αR1 and human 5αR1, the specific 5αR1 inhibitors that have been manufactured for human studies are not valid options for preclinical studies. However, future studies employing humanized 5αR1 mice may shed light on the potential of targeting 5αR1 therapeutically.

We have previously shown that finasteride treatment is an effective therapy for adult male patients with refractory TS [416, 417]. However, since finasteride cannot be used in children due to the high inhibition of 5αR2, determining the mechanism of action of finasteride will be crucial to developing novel therapies for TS patients. If 5αR1 inhibition proves to be a beneficial therapy this would bypass the issue of demasculinzation.

Another interesting finding of this research was the response of 5αR1 KO and 5αR2 KO mice to the D2 receptor agonist quinpirole. The PPI response of C57Bl/6 is not altered by quinpirole [300]. However, we previously reported that pretreatment with finasteride combined with quinpirole, induced PPI deficits in these mice [277], indicating that the steroidal milieu may be modulating the contributions of different dopamine receptors in the modulation of sensorimotor

gating mechanisms. In line with these findings we found that quinpirole induced PPI deficits in 5αR1 KO mice, but not 5αR2 KO mice (Fig 9.2D and Fig 9.3D). To determine if this is truly due to lower levels of AP in the 5αR1 KO mice compared to the 5αR2 KO mice, further studies must be completed. First, determining if finasteride pretreatment allows for quinpirole induced PPI deficits in the 5αR2 KO mice will provide insight on the necessity of 5αR2 in the mechanism of finasteride in this pathway. Second, experiments on the 5αR1 KO, in which AP is administered prior to quinpirole, would determine if AP could abolish this effect of quinpirole. This would be instrumental in determining both if low levels of AP is allowing the D2 receptor agonist to induce PPI deficit, as well as demonstrate if AP is essential in orchestrating the switch between D1 and D2 mechanisms of PPI regulation.

The molecular pathways through which 5α-reduced steroids alter PPI mechanisms is unknown and will be the topic for the next chapter. However, several hypotheses are viable. The 3α, 5α-steroids such as AP are potent positive allosteric regulators of the $GABA_A$ receptor. Research on the effects of $GABA_A$ receptor agonists or positive allosteric regulators on PPI is scant; however, altering the balance between GABA and glutamate in any fashion could produce an imbalance between brain regions (such as the prefrontal cortex and nucleus accumbens) that would lead to PPI deficits. For example, extensive research has found that inhibitors of the $GABA_A$ receptor, such as picrotoxin, reduce PPI. Interestingly, this has been observed when picrotoxin was infused directly in the medial prefrontal cortex, and pretreatment with systemic haloperidol ablated these effects [538]. On the other hand, dizocilpin, an NMDA antagonist has been demonstrated to induce PPI deficits when infused into the amygdala or dorsal hippocampus but not the nucleus accumbens, ventral hippocampus, or dorsomedial thalamus [539]. A trend toward a significant reduction was also seen when dizocilpin was infused into the medial prefrontal cortex in the same

study and further research has demonstrated that excitotoxic lesions of the medial prefrontal cortex prevent the PPI deficit induced from systemic administration of dizocilpin [540]. In contrast, dopamine agonists primarily induce PPI deficits via subcortical brain regions [184]. The results from our studies also suggest that alterations in the 5αR1 KO mice are not affecting dopamine neurotransmission in the dorsal striatum since SKF 82958 is still capable of producing hyperlocomotion in these mice (Fig 5B). Although quinpirole does not induce hypolocomotion in these mice, it is possible that this is due to floor effect after the long habituation. However, should AP and finasteride be inducing these effects in the medial prefrontal cortex, as is suggested by the results of chapter 6, or through the nucleus accumbens, as demonstrated in previously published data on rats [423] there are several possibilities to be considered.

First, AP could be inducing these deficits through modulation of the $GABA_A$ receptor. An increased inhibitory tone from $GABA_A$ in the prefrontal cortex would produce excessive inhibition of glutamatergic neurons and lead to activation of the subcortical brain regions that regulate PPI. Alternatively, the effects of AP could be mediated by AP sulfate, which has been demonstrated to negatively modulate the NMDA receptor [492]. Alternatively pregnenolone sulfate potentiates the NMDA receptor containing GluN1/GluN2A, B while at the same time inhibits AMPA, kainite, NMDA receptors containing GluN1/Gul N2C, D and $GABA_A$ receptors [541-543]. Finasteride treatment would increase levels of the precursors to AP and hence would increase pregnenolone sulfate. An increase in the positive modulation of NMDA would decrease dopamine release in the nucleus accumbens which could reduce the effectiveness of SKF 82958. The possibility of an involvement of NMDA in very interesting in light of previous publications from our lab. We found that the partial D1 agonist SKF 38393 potentiated the PPI disrupting effects of the NMDA antagonist dizocilpine in SD rats [505]. These data indicate that there are D1-NMDA interactions

in the mediation of PPI mechanisms. Furthermore, there is research that NMDA activation might alter D2 signaling mechanism in the PFC from inhibitory G_i signaling pathways to excitatory G_s signaling pathways to enhance the excitability of the prefrontal neurons [544].

Other possible mechanisms involve lesser well known and studied receptors for AP, PXR and P2X4R. While both of these receptors have been studied in the periphery, relatively little is known about how they may mediate the effects of AP in the brain, although both receptors have been demonstrated to be involved in dopamine mediated behaviors [453, 454, 456-460, 468, 469, 473]. Further research into these roles is warranted to determine if they are involved in the regulation of PPI processes as well.

Besides the additional experiments outlined above, there are several limitations to this study. First, we did not test female mice. Preliminary studies indicate that the alterations in behavior observed in 5αR1 are not present in the female mice; however, these studies need to be expanded on. Still, these findings are most relevant to disorders characterized by a pronounced male predominance, so the results presented here are the most salient. In addition, we did not find any alterations in dopamine signaling due to 5αR1 knockout. We found the expected increase in the phosphorylation of ERK in the ventral striatum but there was no effect from genotype. No alterations were seen in the phosphorylation of DARPP-32, a dopamine signaling molecular shown to be differentially regulated by D1 and D2 receptors. Finally, we also did not see any differences in the prefrontal cortex. These results suggest that the actions of AP are likely not mediated by dopamine D1 receptors; however, more extensive analyses need to be completed. Despite these limitations, the results of these studies strongly support a role for AP in the mediation of dopamine induced PPI deficits and raise important questions for future research on the balance between D1 and D2 receptors in the regulation of sensorimotor gating mechanisms.

10. Allopregnanolone mediates stress-induce PPI deficits

Introduction

The results of the previous chapters highlight a key role for the steroid AP. This neurosteroid has numerous actions in the brain, including mediating dopamine motivated behaviors (such as mating) [545-547] and as a negative modulator of the stress response through the $GABA_A$ receptor [349, 437]. To further research the role of AP in stress-exacerbation of TS symptoms we performed a series of experiments in WT mice to determine if AP alone could induce PPI deficits and further explore the molecular pathways that induce these alterations. We demonstrated in chapter 6 that AP is sufficient to induce an increase in tics and PPI deficits in the TS animal model, the D1CT-7 mouse line; however, we chose to translate these findings into another animal model to strengthen the findings from the D1CT-7 experiments.

Based on the research up to this point, we hypothesize that AP is sufficient to induce TS-like symptoms (PPI deficits) in WT mice; however, research completed in the D1CT7 mouse line suggest that these responses are not solely due to the $GABA_A$ receptor. Therefore, we also utilized knockout mice of other receptor targets of AP, PXR and P2X4 to investigate other mechanisms of action. Finally, we further investigated the hypothesis that stress induced TS-like symptoms are contributed to by increased AP synthesis through experiments exposing WT mice to stress paradigms prior to PPI testing.

Methods

Animals. The experiments included in this study were performed on adult (3-5-month old), experimentally naïve male WT C57BL/6 mice obtained from Jackson Laboratories (Bar Harbor, ME). Experiments were performed on PXR KO mice along with their WT littermates obtained from breeding colonies at the Universities of Kansas and Utah. All mice were generated from HZ

x HZ crosses. Progenitors were obtained by Dr. Jeffrey Staudinger (University of Kansas). P2X4 KO mice were obtained from breeding colonies at the University of Southern California (USC) [548]. All mice were housed in groups of 4-5 and had ad libitum access to food and water. Housing facilities were maintained at 22ºC with on 12 hour light/dark cycle (06:00 AM hours and on at 06:00 PM). Experimental manipulations were carried out in the animals' light cycle between 8:00 AM and 4:00 PM. All handling and experimental procedures were performed in compliance with the National Institute of Health guidelines and approved by the local Institutional Animal Care and Use Committees.

Genotyping. Animal genotyping was performed by PCR. Samples of genomic DNA were extracted from tail biopsies acquired from mice at weaning (postnatal day 21). For the P2X4R KO mice, primers were used to identify LacZ (5′-GCGAACGCGAATGGTGCAGC-3′) and P2X4R (5′-TCGCTCTCTGGGTCTGGGGC-3′). Reaction conditions were 5 min at 95 °C, followed by 32 cycles of 15 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. For the PXR KO mice, the following primers were used: 1) CTG GTC ATC ACT GTT GCT GTA CCA; 2) GCA GCA TAG GAC AAG TTA TTC TAG AG; 3) CTA AAG CGC ATG CTC CAG ACT GC. Reaction conditions were 3 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 30 s at 60 °C, and 60 s at 72 °C, and then a final elongation of 6 min at 72 °C.

Experimental Compounds. The following drugs were used in this study: SCH 23390 (1mg/kg), haloperidol (0.3 mg/kg), ganaxolone (15 mg/kg), obtained from Tocris Bioscience (Bristol, UK) and Sigma-Aldrich (Saint Louis, MO); AP (1-15mg/kg, synthesized by the laboratory of Jeff Aubé at the University of Kansas) and isopregnanolone (15mg/kg, Steraloids, Newport RI). SCH 23390 was dissolved in distilled water. Haloperidol was dissolved in 10%

acetic acid buffered with sodium hydroxide and diluted with saline. Ganaxolone, AP and isopregnanolone were dissolved in 5% Tween 80 and diluted with saline.

Acoustic Startle Reflex and Prepulse Inhibition (PPI) of the Startle. Acoustic startle reflex and PPI were tested as previously described in chapter 3.

Statistical Analyses. Parametric and non-parametric statistical analyses on behavioral parameters were performed by a one-way ANOVA or Kruskal-Wallis test, followed by Tukey's HSD or Nemenyi's test for post-hoc comparisons, respectively. Normality and homoscedasticity of data were verified by the Kolmogorov-Smirnov and Bartlett's test. Significance was set at $P = 0.05$.

Results

AP induces PPI deficits in WT mice. The first experiment was conducted to determine the effects of AP on sensorimotor gating. WT C57/BL mice were treated with AP (1-15mg/kg). AP did not significantly alter the mean startle amplitude (Fig 10.1A) but did induce PPI deficits at the highest dose tested (Fig 10.1B; $F(1,43) = 5.13$, $P < 0.001$; post hoc comparisons: vehicle vs 15mg/kg AP, $P < 0.01$).

Dopamine D1 or D2 receptors do not mediate the effects of AP. We next tested the effects of dopamine receptor antagonists on the PPI disrupting effect of 15mg/kg AP. Dopamine has been well documented to mediate sensorimotor processes. Indeed, dopamine antagonists are used clinically to treat patients with TS and dopamine agonists are commonly used to model TS in animals. We first tested the dopamine D1 receptor antagonist SCH 23390. In this experiment we found that AP increased the startle amplitude of the test mice (Fig 10.1C; main effect of AP: $F(1,32) = 7.24$, $P < 0.05$), but there was no effect of SCH 23390 on the startle amplitude. In addition, while AP induced PPI deficits in the test mice, SCH 23390 did not restore PPI levels (Fig

10.1D; main effect of AP: $F(1,32) = 8.56$, $P < 0.05$). We also tested the D2 receptor antagonist haloperidol. There was no effect of either drug on mean startle amplitude (Fig 10.1E) in this experiment, but AP treatment did result in PPI deficits that were not ablated by haloperidol treatment (Fig 10.1F; main effect of AP: $F(1,28) = 14.49$, $P < 0.001$).

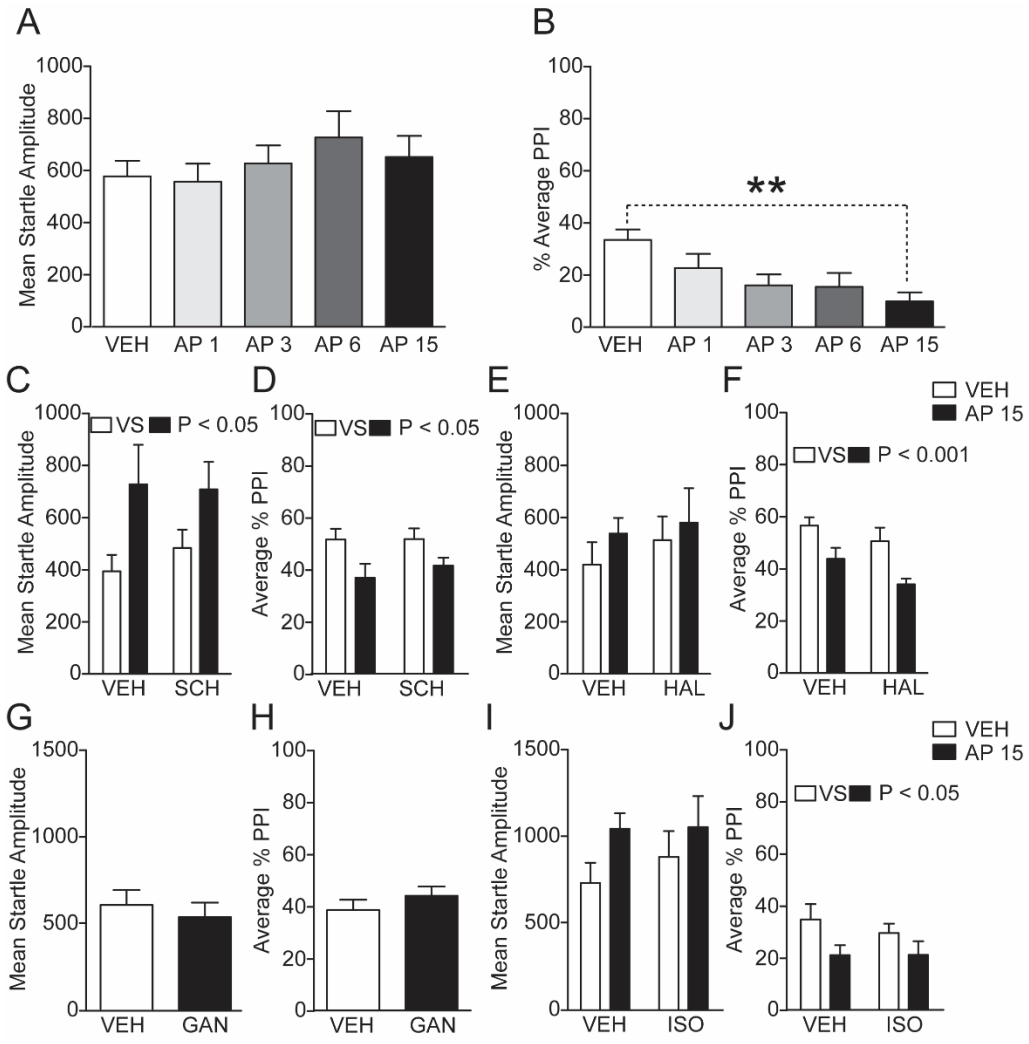

Figure 10.1 Allopregnanolone induces PPI deficits in WT C57 mice. Mice were pretreated with AP (1-15 mg/kg) prior to PPI testing. (A) Mean startle amplitude and (B) % Average PPI were recorded. (C-D) The D1 antagonist SCH 23390 (1mg/kg) was tested against AP in PPI. (E-F) The D2 antagonist haloperidol (0.3mg/kg) was tested against AP in PPI. (G-H) The selective $GABA_A$ positive modulator ganoxolone (15mg/kg) was tested in PPI. (I-J) The negative $GABA_A$ receptor modulator isopregnanolone (15 mg/kg) was tested against AP in PPI. Data are shown as mean ± SEM. **P<0.01 compared to VEH treated mice. All main effects are represented in the corresponding graphs. Abbreviations: VEH, vehicle; AP, allopregnanolone; SCH, SCH 23390; HAL, haloperidol; GAN, ganaxolone; ISO, isopregnanolone.

GABAA receptors are not involved in the mechanism of AP. To determine if AP was inducing PPI deficits by acting at the GABAA receptor we next treated with the GABAA receptor specific synthetic analog of AP, ganaxolone. We did not find any effect of ganaxolone on startle amplitude or PPI levels in WT mice (Fig 10.1G). In addition, we pretreated mice with isopreganolone, which has been shown to be a negative modulator of the GABAA receptor at the same site of action as AP [549]. In confirmation of the ganaxolone results, isopregnanolone did not alter startle amplitude (Fig 10.1I) or attenuate the PPI deficit from AP (Fig 10.1J; main effect of AP: $F(1,28) = 5.39$, $P < 0.05$).

Alternative receptors: PXR and P2X4. Research has shown that besides the $GABA_A$ receptor, AP can also exert effects through the purinergic receptor, P2X4R, and the PXR, both of which have been demonstrated to be expressed in the brain. To study the contributions of these receptors we performed experiments in mice deficient of either P2X4R or PXR. We found that the P2X4R KO mice were resistant to the effect of AP on PPI (performed by Sheraz Khoja and Dr. Daryl Davies, University of Southern California; data not included). In addition, AP also did not

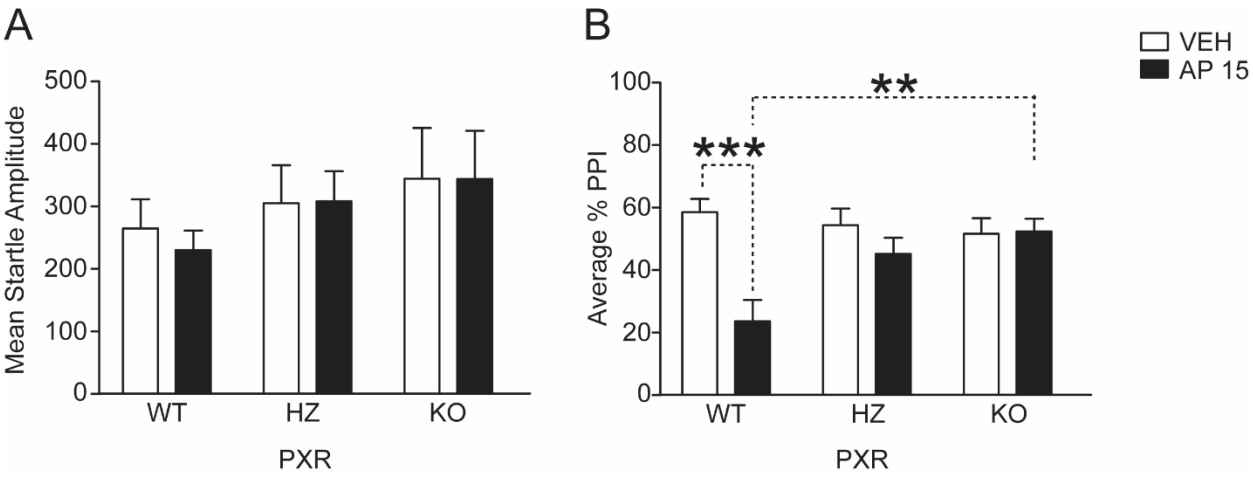

Figure 10.2 Pregnane xenobiotic receptor (PXR) is involved in AP induced PPI deficits. PXR KO mice were treated with AP (15 mg/kg,) and tested in the startle reflex paradigm. (A) Mean startle amplitude and (B) average % PPI were recorded. Data are shown as mean ± SEM. **P<0.01 and ***P<0.001 for comparisons indicated. Abbreviations: VEH, vehicle; AP, allopregnanolone.

induce PPI deficits in the PXR KO mice (Fig 2B, genotype x treatment interaction: $F(2,54) = 6.30$, $P < 0.01$; post hoc comparisons: WT + vehicle vs WT + AP, $P < 0.001$; WT + AP vs KO + AP, $P < 0.01$). These data indicate that both the P2X4R and PXR contribute to the role of AP in sensorimotor gating regulation.

Restraint stress induces PPI deficits. To translate these findings to a physiological situation we exposed mice to restraint stress to induce an increase in stress hormones, such as AP. We found that similar to AP, restraint stress did not affect mean startle amplitude (Fig 3A) but did induce PPI deficits in a time dependent fashion (Fig 3B; $F(1,44) = 3.39$, $P < 0.017$; post hoc

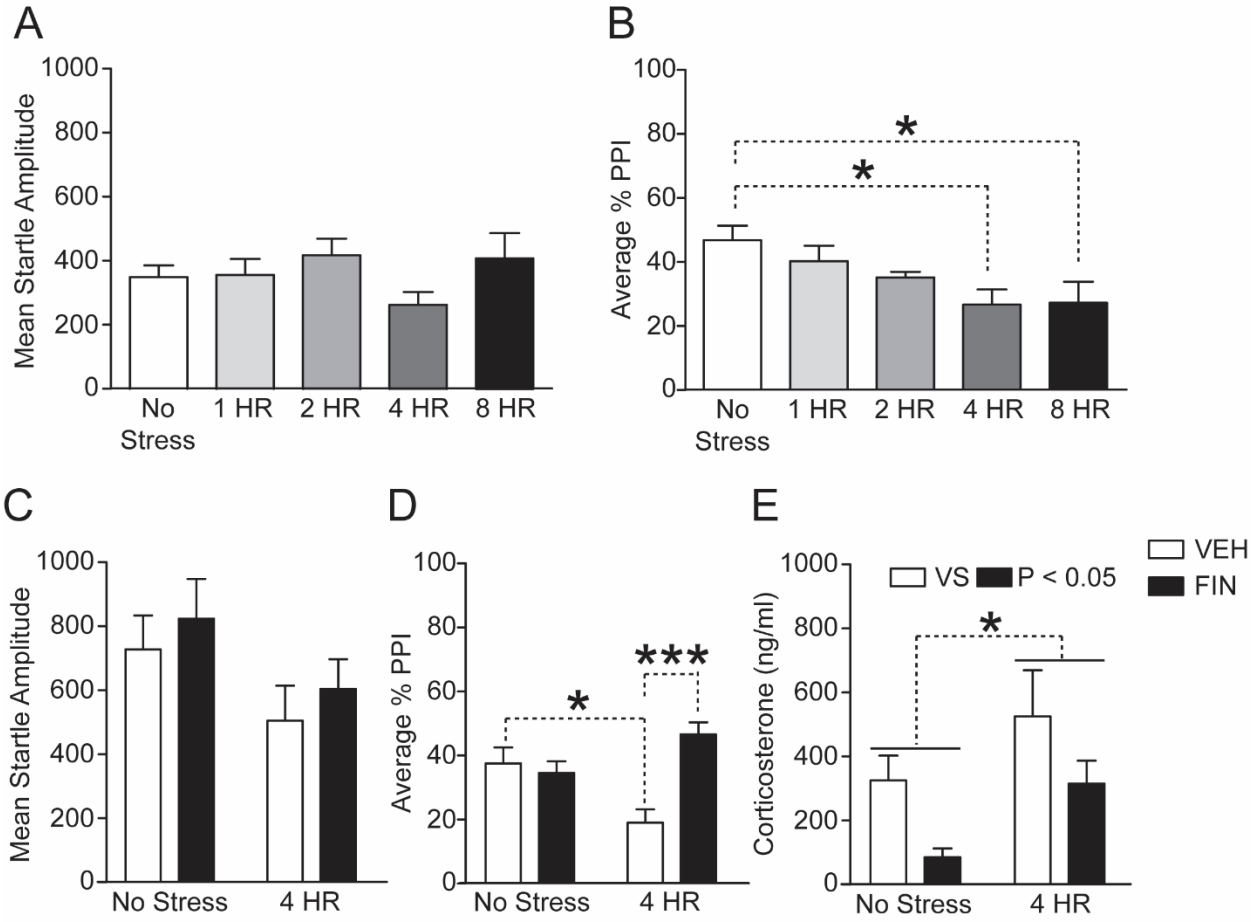

Figure 10.3 Restraint stress induces PPI deficits that are ablated by finasteride. (A-B) WT C57 mice were exposed to restraint stress (0-8hr) and testing for sensorimotor responses, mean startle amplitude and average % PPI. In a subsequent experiment, mice were exposed to 4hr restraint stress and treated with finasteride (50mg/kg) prior to PPI testing. (C) Mean startle amplitude, (D) average % PPI, and (E) plasma corticosterone levels were measured in this second cohort of mice. Data are shown as mean ± SEM. *P<0.05, ***P<0.001 for the comparisons indicated. Main effects are indicated in the corresponding graph. Abbreviations: HR, hour; FIN, finasteride.

comparisons: no stress vs 4hr restraint stress, $P < 0.05$; no stress vs 8hr restraint stress, $P < 0.05$). To confirm that these observations were due to an increase in AP we blocked the synthesis of AP with the 5α-reductase inhibitor finasteride. We found that a main effect of the 4hr restraint stress on mean startle amplitude (Fig 3C; $F(1,36) = 4.14$, $P < 0.05$). In addition, finasteride treatment before PPI testing ablated the effect of restraint stress on PPI (Fig 3D; condition x treatment interaction: $F(1,36) = 13.57$, $P < 0.001$; post hoc comparisons: no stress + vehicle vs 4hr restraint + vehicle, $P < 0.05$; 4hr restraint + vehicle vs 4hr restraint + finasteride, $P < 0.001$). We also analyzed corticosterone levels following restrain stress. We found a significant main effect of both stress to increase corticosterone (Fig 3E; $F(1,18) = 17.42$, $P < 0.05$) and of finasteride treatment to decrease corticosterone levels ($F(1,18) = 19.09$, $P < 0.05$).

Discussion

The results of these studies confirm previously published data from our group that demonstrates that the neurosteroid AP mediates important behaviors related to TS symptom fluctuations. Here we find that AP is sufficient to induce PPI deficits in WT C57BL/6 mice through the receptors P2X4 and PXR. Furthermore we report that stress induced PPI deficits in the WT C57BL/6 mice are ablated by treatment with the 5αR inhibitor finasteride.

Our initial studies expanded on previously published data that highlighted a key role for AP in the exacerbation of TS-like symptoms in the D1CT-7 mouse model ([550], chapter 6). We repeated aspects of this study in the WT C57BL/6 mice and found that systemic AP was sufficient to induce PPI deficits in a dose dependent fashion (Fig 10.1). This is in contrast to the WT Balb/c mice tested in our previous publication. In those studies the same dose of AP did not induce PPI deficits in the WT BALB/c littermates of the D1CT-7 mice but did in the D1CT-7 mice. These opposing findings are likely the result of strain differences. It has been published repeatedly by

our group and others that albino strains display altered PPI responses to dopamine agonists in comparison to non-albino strains ([267, 320, 489] also see chapter 8); therefore, the albino Balb/c strain may not be as susceptible to the AP induced PPI deficits without the D1 mutation, as observed in the D1CT-7 mutants.

We next investigated the contributions of various receptors to the effects of AP. We found that neither antagonists for the dopamine D1 receptor, not the dopamine D2 receptor blocked the effects of AP on PPI (Fig 10.1). This suggests that there are either two converging mechanisms from AP and dopamine or AP is exerting its effects downstream of the dopamine receptors. We previously reported that AP did synergize with D1 agonists to induce PPI deficits (see chapter 9), indicating that the two pathways do interact. However, in the same studies (chapter 9) we also observed no alteration in dopamine signaling molecules 5αR1 KO mice indicating that AP may not interact with dopamine at the level of receptor or signaling cascade.

Following these studies we focused on the $GABA_A$ receptor, which is the most studied receptor for AP (Fig 10.1). AP acts as a positive allosteric modulator of $GABA_A$ so we attempted to replicate the results of AP treatment using a synthetic analog of AP, ganaxolone. Ganaxolone has been demonstrated to be specific for the $GABA_A$ receptor and induce effects within the same time course and dose as AP [551]. However, ganaxolone was not sufficient to induce PPI deficits in the WT C57Bl/6 mice. We further confirmed that there was not a primary mechanism involving the $GABA_A$ receptor by pretreating mice with the 3β, 5α- isomer of AP, isopregnanolone. Contrary to AP, isopregnanolone acts as a negative modulator of the $GABA_A$ receptor and has been demonstrated to block the $GABA_A$-potentiating effect of AP [549]. In this study isopregnanolone was not effective at blocking the results of AP on PPI. Together these results suggest that the influence from $GABA_A$ receptors is not a primary mechanism through which AP induces PPI

deficits. This does not rule out the possibility that $GABA_A$ is working in concert with other mechanisms in this pathway such as the dopamine receptors or P2X4R.

We next turned to other possible receptors for AP. The first we investigated was the PXR receptor [455]. We found that the PXR KO mice were resistant to PPI deficits induced by AP (Fig 10.2). PXR has been primarily studied for its role in drug clearance in the liver [442]. However, research surrounding the role of PXR in the brain has recently become more prominent. PXR has been found in several brain regions [452-455, 552]. In addition, researchers have demonstrated that PXR knockdown results in a decreased level of AP in the midbrain, striatum and hippocampus. These same studies also identified PXR as a key mediator of dopamine motivated behaviors such as mating; however these results are likely due to the observed decrease in AP levels [453, 454, 456-460]. One possible explanation for these results is that PXR is both a receptor for AP and is also involved in the transcriptional regulation of steroidogenic enzymes to act as part of a feedforward mechanism to upregulate AP. The finding that PXR knockdown decreases AP levels implies that the PXR KO mice are resistant to PPI disrupting effects of AP because the critical level of AP in the brain has not been reached. Furthermore, since PXR is a nuclear receptor that mediates transcription it is not feasible for AP to exert the effects seen on PPI in the time frame observed, which further supports the hypothesis that PXR mice are resistant to AP induced PPI deficits due to low baseline levels of the neurosteroid.

The other receptor we investigated was the P2X4R, which has also been demonstrated to bind to AP [461]. We found that these mice were also protected from PPI deficits induced by AP. Since this receptor is a membrane bound cation channel, the possibility that this receptor directly mediates the effects of AP in PPI is greater than for PXR. The research on P2X4R in the brain has focused largely on the role of this receptor in inflammation and microglia. This is especially

interesting in the context of TS because multiple lines of study have suggested inflammation or infection may predispose individuals to developing TS and clinical studies indicate that TS patients have altered immune responses [102, 553, 554]. While further research is needed to determine the connection between P2X4R, inflammation/immune responses and TS, this could prove to be a fruitful line of inquiry. Interestingly P2X4Rs modulate N-methyl-D-aspartate (NMDA) glutamate receptors [470] which could provide a direct mechanism through which AP affects sensorimotor processes. NMDA receptor antagonists, such as MK-801, have been shown to induce PPI deficits in rodent models [505, 539, 540]. P2X4R has been implicated in PPI regulation previously. Ivermectin, which potentiates P2X receptors, and genetic knockout of P2X4R both induce PPI deficits [468, 469]. Of note, P2X4Rs have also been shown to regulate striatal dopamine homeostasis which would also contribute to sensorimotor gating functions [473]. Taken together the data presented here and the previous research conducted indicate that P2X4 may directly mediate the effects of AP in PPI processes through modulation of the NMDA receptor, likely in the prefrontal cortex, or dopamine neurotransmission in the striatum.

Finally we performed experiments to induce an increase in AP physiologically. This was accomplished through restraint stress of up to eight hours. We found that restraint stress between 4-8 hours induced significant PPI deficits in mice, which were ablated by treatment with the 5αR inhibitor finasteride (Fig 10.3). These data indicate that the PPI deficits induced by stress are mediated by an increase in 5α-reduced neurosteroids. In addition, we confirmed the stressfulness of the paradigm by measuring corticosterone levels in mice following a 4 hour restraint with or without finasteride treatment. As expected, stress increased corticosterone levels in the restrained mice. Surprisingly, finasteride decreased corticosterone levels both in mice not exposed to stress and those that underwent restraint stress. This was unexpected because corticosterone is a substrate

of 5αR so inhibition of this enzyme should increase, not decrease corticosterone levels. This suggests that finasteride is inhibiting the cascade at a level before corticosterone synthesis is triggered in the adrenal gland. These data are in partial agreement with published data demonstrating that restraint stress induces PPI deficits; however, the restraint stress required to induce a significant PPI deficit was markedly shorter than we found in our study [555]. Interestingly, the researchers also found that D-serine, an agonist of NMDA receptors ablated these stress induced deficits.

In conclusion, the results presented here further characterize the mechanism through which AP exacerbates TS-like behaviors in rodents. Of note, these studies are limited by the exclusion of females and the lack of molecular data. Even though TS is far more prevalent in males the contribution of AP to stress-exacerbated behaviors is highly relevant to females as well, and so should be the topic of future research. Furthermore, exploration of signaling pathways altered by AP will be valuable to determine the mechanisms involved. Finally, we did not pinpoint a region through which AP is exerting these effects. Previous data strongly suggests the prefrontal cortex is the key region involved but this has not been confirmed here. In spite of these limitations, this research extends the current knowledge concerning the role of AP in the brain and provides novel targets for future research and therapies.

11. Finasteride inhibits 5α-Reductase Type 2 to induce depressive-like behaviors

Introduction

There are two critical issues that prevent finasteride from being an ideal candidate for TS therapy. The first, the demasculinizing side effects may be circumvented by employing 5αR1 specific inhibitors [556], since 5αR2 rather than 5αR1 is the primary mediator of androgen synthesis and puberty. The second issue, however, also warrants significant research and consideration. As mentioned above, a subset of patients prescribed finasteride to treat alopecia have developed a severe, untreatable depression [403-405]. Therefore, before committing to a course of suggesting 5αR1 as a suitable treatment option for TS patients, the mechanisms underpinning this side effect must be evaluated.

To investigate the roles of 5αR1 and 5αR2 in the development of depressive behaviors we tested WT mice and rats treated with finasteride in the forced swim test, a well-validated measure of depressive behaviors. To parse out the contributions of 5αR1 versus 5αR2 we studied the knockout mice for each enzyme in the forced swim test to determine if finasteride was exerting its actions through one of them.

Materials and Methods

Animals. The experiments included in this study were performed on adult (3-5 month old), WT C57 mice (Jackson Laboratories, Bar Harbor, ME, USA), LE rats (Charles River Laboratories, Raleigh, NC, USA) and experimentally naïve male 5αR1KO and 5αR2KO mice along with the HZ and WT littermates (strain: C57BL/6), obtained from breeding colonies at the Universities of Kansas and Utah. All mutant mice were generated from HZ x HZ crosses. Progenitors were obtained by Dr. Mala Mahendroo (Southwestern University). Unless stated otherwise for specific experimental purposes, all mice were housed in groups of 4-5/cage, with at least 1

mouse/genotype, and had ad libitum access to food and water. Housing facilities were maintained at 22ºC with a reverse light/dark cycle (lights off at 08:00 AM hours and on at 08:00 PM). Whenever the same mice were used for multiple behavioral paradigms, the order of animals in each test was counterbalanced throughout the study. Tests were arranged from least to most stressful (separated by at least one week) to minimize carry-over stress. Experimental manipulations were carried out in the animals' dark cycle between 10:00 AM and 6:00 PM. All handling and experimental procedures were performed in compliance with the National Institute of Health guidelines and approved by the local Institutional Animal Care and Use Committees.

Genotyping. Mouse genotyping was performed by PCR as described in chapter 9.

Locomotor Activity. Locomotor behaviors were measured in a square force-plate actometer as previously described in chapter 3 for mice. Rats were placed in a larger actometer (side: 42 cm; height 30 cm) [557] and the data analyzed as described in chapter 3. 5αR1KO and 5αR2KO mice were tested in an open field for analysis of locomotor activity following finasteride treatment. The open field consisted of a square arena (40 cm x 40 cm) surrounded by black Plexiglas walls. The locomotor activity in the open field was analyzed using Ethovision (Noldus, Leesburg, VA).

Forced Swim Test. Mice were placed in a clear Plexiglas cylinder (10 cm in diameter × 30 cm in height) filled to 15 cm with water (25°C) for 10 minutes before removed. The first five minutes were scored for immobility. Rats were similarly tested in a larger cylinder (29.21 cm in diameter x 44.45 cm in height) [558].

Statistical Analyses. Data distributed binomially (such as the results of the tube test) were analyzed by a binomial test. Continuously distributed data were tested for normality and homoscedasticity by the Kolmogorov-Smirnov and Bartlett's test. Based on these results,

parametric and non-parametric statistical analyses were performed by a one-way ANOVA and Kruskal-Wallis test, followed by Tukey's or Nemenyi's tests for post-hoc comparisons, respectively. Significance was set at $P = 0.05$.

Results

Finasteride increases immobility in the forced swim test. We tested both Long-Evans rats and C57bl/6 mice in the force swim test following treatment with finasteride. In rats we found that a dose of 50 mg/kg significantly increased immobility in this test, while the other doses tested did not exert a significant effect ($F(3,32) = 4.46$, $P < 0.05$; post hoc comparisons: vehicle vs finasteride 50, $P < 0.01$). The same was observed in the latency to immobility ($F(3,32) = 5.113$, $P < 0.01$; post hoc comparisons: vehicle vs finasteride 50, $P < 0.05$; finasteride 10 vs finasteride 50, $P < 0.05$) In mice we observed a similar behavioral response to finasteride treatment; however, the mice were more sensitive to lower doses of finasteride ($F(3,28) = 12.83$, $P < 0.001$; post hoc comparisons: vehicle vs finasteride 25, $P < 0.01$; vehicle vs finasteride 50, $P < 0.001$; finasteride 12.5 vs finasteride 50 $P < 0.01$). In addition, the latency to immobility was decreased due to finasteride treatment ($H(3) = 16.15$, $P < 0.01$; post hoc comparisons: vehicle vs finasteride 50 $P < 0.01$, finasteride 12.5 vs finasteride 50 $P < 0.05$). Since drugs that induce hypolocomotion or sedation can produce increases in immobility in the forced swim test that do not correlate with a depressed mental state we also tested the animals in the open field actometer to confirm that finasteride did not decrease locomotion. In rats we found that the range of doses tested in the forced swim test did not alter total distance moved. In contrast, the mice treated with finasteride exhibited a severe decrease in total distance moved at the highest dose of 50 mg/kg compared to all other doses ($F(3,27) = 6.66$, $P < 0.01$; post hoc comparisons: vehicle vs finasteride 50, $P < 0.01$; finasteride 10 vs finasteride 50, $P < 0.01$; finasteride 25 vs finasteride 50, $P < 0.05$). Importantly,

the finasteride treatment of 25 mg/kg did not induce hypolocomotion but did produce a significant increase in immobility in the forced swim test.

Finasteride induces depressive-like behaviors by blocking 5αR type 2. To determine which isoform of 5αR contributes to the depressive behaviors observed following finasteride treatment we conducted tests on mice deficient of either 5αR1 or 5αR2. Previous observations have indicated no overt behavioral alterations of these mice in the forced swim test compared to wildtype littermates; therefore, we only conducted analyses on the knockout mice to reduce the number of animals needed. In the 5αR1 KO mice we found that finasteride treatment at 25mg/kg and 50mg/kg induced a decrease in the latency to immobility ($F(2,21) = 23.20$, $P < 0.001$; post hoc comparisons: vehicle vs finasteride 25, $p < 0.001$; vehicle vs finasteride 50, $p < 0.001$). However, only the highest dose at 50mg/kg increased immobility ($F(2,21) = 6.17$, $p < 0.01$; post

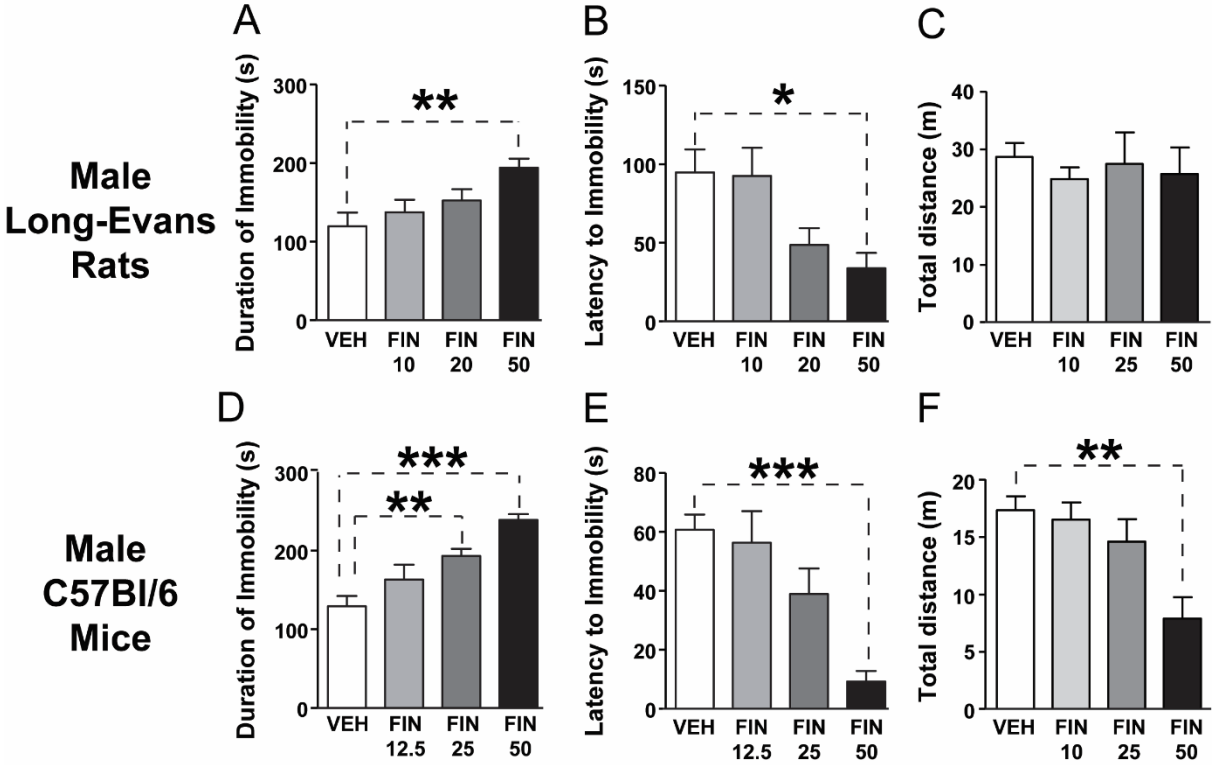

Figure 11.1 Finasteride induces increased immobility in the forced swim test. Rats (A-C) and mice (D-F) were treated with finasteride (0-50 mg/kg) and tested in the forced swim test and open field. The duration of immobility (A,D) and latency to immobility (B,E) was analyzed in the forced swim test. Total distance moved (C,F) was recorded in the open field paradigm. Data are shown as mean ± SEM. *P<0.05, **P<0.01; ***P<0.001 for the comparisons indicated. Abbreviations: VEH, vehicle FIN, finasteride.

hoc comparisons: vehicle vs finasteride 50, $P < 0.01$). Finasteride treatment also induced a significant hypolocomotion in the 5αR1 KO mice at 50mg/kg ($P < 0.05$). In contrast the 5αR2 KO mice were not affected by finasteride at either dose in the latency to immobility or duration of immobility. Finasteride at 50mg/kg induced a significant decrease in locomotion ($P < 0.001$).

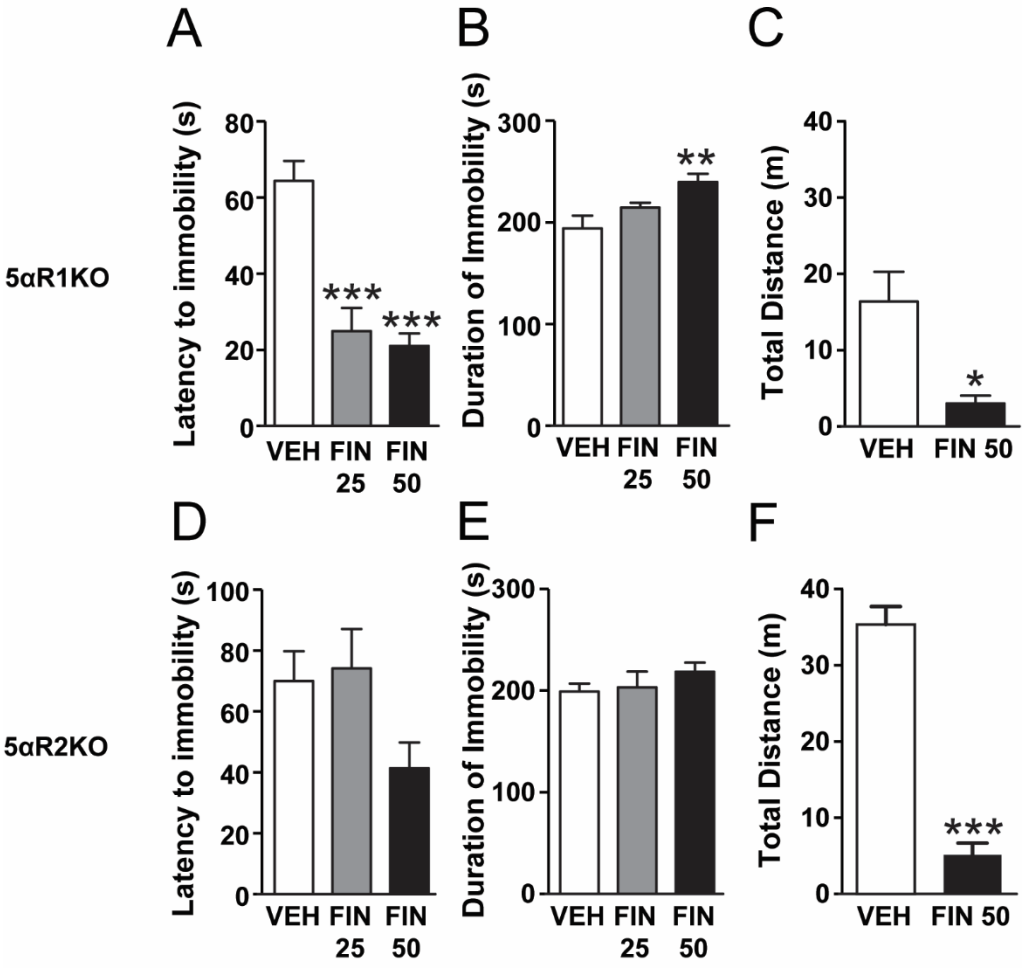

Figure 11.2 5αR2 KO mice are resistant to FIN-induced depressive-like behaviors. 5αR1 and 5αR2 KO mice were tested in the forced swim test following treatment with finasteride (0-50mg/kg) and in the open field following treatment with finasteride at 50 mg/kg. (A, D) The latency to immobility in the forced swim test. (B,E) The total duration of immobility during the forced swim test. (C,F) the total distance moved during the open field test. Data are shown as mean ± SEM. *P<0.05, ***P<0.001 for the comparisons indicated. Abbreviations: VEH, vehicle FIN, finasteride; 5αR1KO, 5α-Reductase type 1 knockout; 5αR2KO, 5α-Reductase type 2 knockout.

Discussion

The results of these studies demonstrate that finasteride induces depressive-like behaviors in both mice and rats. Importantly, we also demonstrate that these behaviors are mediated by 5αR2 rather than 5αR1.

Our initial characterization of finasteride in a well validated paradigm aimed at measuring depressive-like behaviors, the forced swim test, found that acute doses of finasteride produced a robust increase in immobility. These findings are in agreement with clinical data indicating that finasteride can induce depression in patients [403-406]. While not a common side effect of finasteride, this is nevertheless an important consideration since the depression appears to be untreatable and does not remit with termination of finasteride treatment.

To determine if the depressive-like behaviors induced by finasteride were mediated by the 5αR1 or 5αR2 we conducted similar experiments in mice with genetic inactivation of these isoenzymes. We found that the immobility of the 5αR1 KO mice was not altered by the intermediate dose of finasteride but highest dose did induce an increase in immobility. In contrast the 5αR2 KO mice were not affected by either dose of finasteride.

Several limitations to these studies exist. This preliminary analysis of the effects of finasteride on depressive-like behaviors is far from complete. First, these studies only focus on one behavior related to depression. Ongoing research is analyzing the effects of finasteride on anhedonia and risk taking behaviors before definitive conclusions are drawn. In addition, it will be important to investigate the effect finasteride has on molecules in the stress signaling cascade such as CRH, ACTH and corticosterone. Preliminary data indicates that finasteride is drastically inhibiting CRH in the PVN and inducing a mild reduction on ACTH; however, further analyses are warranted to determine the effect of finasteride on corticosterone in this paradigm. Finally, the

inclusion of females in this study would be a valuable addition. Depression greatly affects women so even though post finasteride syndrome does not directly relate to women, insights into the contributions of neurosteroids to depression would be valuable for studies on women as well.

Despite these limitations, these studies indicate that 5αR1 inhibition could a safe alternative to 5αR2 inhibition for the treatment of TS. However, further studies on 5αR1 inhibitors will be necessary before this is concluded and clinical trials are initiated. As mentioned above, rodent 5αR1 is not inhibited by finasteride in the same manner as rodent 5αR2 so these results may be influenced by these differences. Further research on humanized 5αR1 mice treated with specific 5αR1 inhibitors will be valuable to determine the true translational and therapeutic potential of 5αR1 inhibition.

12. Final Discussion and Future Directions

The critical findings of this dissertation

The research presented in this dissertation extends the currently available range of animal models for the study of TS and provides valuable insights into the mechanisms of stress-induced fluctuations of TS symptoms. Furthermore, the findings of these studies can greatly inform the direction of future clinical studies and determine the direction for pursuing novel therapeutic options for TS patients.

Stress exacerbates tics in mouse models through AP. Stress has a very clear exacerbating effect on symptoms in TS patients; however, the mechanism of this exacerbation has remained unclear. To provide further insight into these mechanism, the studies outlined in this dissertation have been designed to delve into the role of stress and stress neurosteroids on TS symptoms. We started our research using the D1CT-7 mouse model of TS. This model is extremely useful in the study of TS-like behaviors and is the only model currently available to display spontaneous tics (face validity), PPI deficits (construct validity) and responds to common TS therapeutics (predictive validity). Our studies confirmed the validity of this model (Chapter 3) and further explored the impact of stress on these behaviors. Importantly, we found that all the TS-like behaviors observed in these mice were exacerbated by acute stress. In addition, we have taken the critical next step in this line of research by demonstrating that this exacerbation of TS-like symptoms are induced by an increase in the neurosteroid AP (Chapter 6). While the role of AP in stress regulation has been well characterized (Chapter 5), this is the first report of AP mediating TS symptoms. These results also provide valuable insight into the mechanism of action of finasteride in TS patients. Based on these studies, the reduction of AP induced by finasteride's

inhibition of 5αR is a key mechanism through which this compound exerts its therapeutic effects in clinical patients.

We further confirmed these findings in WT C57BL/6 mice. First, we studied eye blinks in these mice to study a behavior in these animals that mimics a common tic in TS patients. In these studies we found that both D1 receptor activation and stress (restraint) increased eye blink frequency (Chapter 4). We also demonstrated predictive validity by demonstrating that common TS therapies reduced the eye blink responses that were increased following D1 receptor activation or stress. To determine the role of neurosteroids in this model we pretreated the mice with finasteride before either D1 agonist treatment or restraint stress. Surprisingly, we found that finasteride ablated stress- but not the D1 agonist-induced enhancement of eye blink frequency. This was surprising considering the anti-dopaminergic effects of finasteride that was observed in the D1CT-7 mice and other models of this dissertation, as well as previously published data from our lab [277, 422, 423, 489]. However, the anti-stress effects of finasteride were in agreement with our other presented data. We also showed that these effects are contributed by both isoenzymes of 5αR indicating that an overall reduction in neurosteroids, regardless of the isoenzyme targeted, is sufficient to attenuate stress-induced eye blink responses.

AP and dopamine interactions in the regulation of PPI. We next moved our studies to focus on perceptual alterations associated with TS, specifically PPI. Previous data from our lab and the results of the D1CT-7 series of experiments indicates that neurosteroids support dopaminergic mechanisms of PPI regulation [277, 422, 423, 489]. However, the translational impact of these findings was previously challenged due to strain differences observed between rats and mice; PPI deficits were only induced by D1 agonists in mice but D2 agonists in rats. This was a concerning state of the field because it raised questions on the validity of animal research to

study PPI deficits in a translational manner. Further research by Ralph and Caine [267] demonstrated that these assumptions were not entirely correct in mice but rather the result of the strain of mouse used. We expanded on this research by demonstrating that the same is true for rat strains. While this is still concerning and should be taken into account when translating findings from animal studies to the clinic these findings expand the number of appropriate animal models and highlight that vast heterogeneity that can occur within species.

Following the identification of appropriate and varied models to use in these studies we confirmed previously published data from our lab [277] indicating that finasteride ablates PPI deficits induced by D1, but not D2 agonists, and actually exacerbates D2 receptor deficits in mice. These data on D1 mechanisms were repeated in rats and expanded on in mice to determine the role of the 5αR isoenzymes (Chapters 8 and 9). Interestingly, we found that 5αR1 but not 5αR2 genetic knockout provided protection from the PPI disrupting effects of D1 agonists. Furthermore, 5αR1 but not 5αR2 KO mice were sensitive to PPI deficits induced by D2 agonists. These data indicate that low levels of neurosteroids alter the dopaminergic regulation from a system that is disrupted by D1 agonists to one that is disrupted by D2 agonists. Surprisingly, our data also indicates that finasteride requires 5αR2 to exert its anti-dopaminergic effect in PPI. These data indicate that low tonic levels of neurosteroids is protective; however, targeting 5αR2 acutely to inhibit phasic increases in neurosteroids has therapeutic potential as well and is the primary mechanism of finasteride. We further identified that AP was mediating these observations by demonstrating that AP synergized with D1 agonists to induce PPI deficits in both 5αR1 KO mice and albino SD rats, which belong to a strain of rat not susceptible to D1 agonist-induced PPI deficits.

Molecular mechanisms of AP. The mechanism through which AP mediates these behaviors was our next avenue of study (Chapter 10). Surprisingly we found that AP was not

working directly through the $GABA_A$ receptor but through either the PXR or P2X4R. Based on the specific characteristics of each receptor, either might contribute to TS pathogenesis. PXR is a nuclear receptor and regulates the transcription of various genes. This is unlikely to be involved in acute effects of AP but it has been demonstrated that PXR is involved in the regulation of AP synthesis [454]; therefore, increased activation of this receptor in TS could lead to a higher production of AP and the deleterious effects observed in this research. On the other hand, the P2X4R is a cation channel that would exert effects quickly, on the timescale through which AP is observed to begin inducing tic-like behaviors and PPI deficits (10 minutes). P2X4R is on neurons and glia cells throughout the brain, but of special interest to TS has been found on GABAergic interneurons and spiny neurons in the striatum [559]. Furthermore Jo et al [560] demonstrated that cross-talk between P2X4R and $GABA_A$ receptors exists. Of note these studies showed that P2X4 expression reduces GABAergic currents in the same neurons and provided evidence for a physical coupling between P2X4R and $GABA_A$. P2X4R has also been found co-localized with NMDA receptor in excitatory synapses and is involved in the ATP-mediated down-regulation of NMDA receptors [561]. Therefore, P2X4R is posed to act as a key regulator in the GABA-glutamate balance in signaling pathways of the brain. NMDA receptors have been demonstrated to interact with D1 receptors to produce PPI deficits and have also been shown to regulate D2 signaling [505, 544]. These data indicate that NMDA antagonists, such as dizocilpine, induce PPI deficits through the same pathway as D1 agonists: increased dopamine release in the nucleus accumbens, triggered either indirectly via NMDA antagonists in the PFC or increased dopamine receptor activation directly from the D1 agonist in the nucleus accumbens. Alternatively, D2 receptor stimulation in the PFC leads to decreased dopamine release in the nucleus accumbens; however, when NMDA is stimulated directly before the D2 receptor it has been demonstrated that this is capable of

inducing a switch in D2 to an excitatory signaling pathway. This could explain the ability of quinpirole to induce PPI deficits in mice pretreated with finasteride or in the 5αR1 KO mice. Further research is required to determine the validity of this hypothesis but the following scenarios are hypothesized to mediate these effects

1) AP has been demonstrated to act in the PFC to exacerbate D1-mediated TS-like symptoms as shown indirectly in the D1CT-7 mice (chapter 6) and directly in the SD rats (chapter 9). Therefore we hypothesize that AP is modulating the P2X4R to potentiate the ATP-mediated down regulation of NMDA receptors which in turn would increase dopamine release in the nucleus accumbens and exacerbate the TS symptoms. Finasteride would reduce the down regulation of NMDA receptors and therefore ablate this response.
2) Following finasteride treatment or genetic knockout of 5αR1 there is a decrease in AP but not in the precursors. An increase in pregnenolone sulfate would potentiate the NMDA receptors [562] and could facilitate a switch from an inhibitory D2 receptor to an excitatory pathway. This could lead to an increase in dopamine release in the nucleus accumbens when quinpirole in administered. Alternatively, if a similar mechanism is observed in the nucleus accumbens (so far NMDA mediated alterations in D2 signaling has only been observed in the prefrontal cortex) quinpirole could be acting directly there to induce PPI deficits in a similar manner as the D1 agonist.

5αR isoenzymes in depression. Finally, we investigated the role of 5αR in the mediation of depression-like behaviors. This line of inquiry resulted from the rare side effect of finasteride treatment to induce post finasteride syndrome, a state of anhedonia and depression [403-406].

Furthermore, there have been reports of decreased 5αR1 in depressed patients [563] which we have confirmed for both 5αR1 and 5αR2 in unpublished data. Here we have identified 5αR2 to be the primary regulator of post finasteride syndrome in preclinical animal models. This is important for the research on TS because it identifies 5αR2 as the isoenzyme to be avoided in developing novel therapeutics due its role in masculinization and post finasteride syndrome. However, before 5αR1 inhibition is the chosen as the therapy of choice more research must be conducted. Targeting an enzyme that regulates such broad physiological processes that are central to regulating homeostasis and behavior, such as 5αR1, should not be undertaken lightly and so further research to determine the level of inhibition that is therapeutic and/or more specific targets would be beneficial.

Future studies and limitation

D1 vs D2 induced PPI deficits. First, further research on the molecular mechanisms of neurosteroid regulation of dopamine receptor signaling will be instrumental in understanding the complete picture of TS. Our research has only just initiated these studies and further investigation into the pathways that mediate the role of both dopamine D1 and D2 receptors in the regulation of sensorimotor gating will be valuable. This can be accomplished first by performing additional experiments on the 5αR1 and 5αR2 KO mice. We have demonstrated that 5αR1 KO are protected from PPI deficits induced by D1 agonists but these mechanisms also induce susceptibility to D2 agonist initiated PPI deficits (Chapter 9). However, we do not know if this entirely mediated by AP. Therefore, conducting experiments in which the 5αR1 KO mice are pretreated with AP prior to D2 agonist treatment would answer these question. In addition, to determine if 5αR2 is involved in acute processes of D1 and D2 sensorimotor regulation, the experiment conducted in Frau et al., 2013 [277] should be replicated in the 5αR2 KO mice (finasteride + the D2 agonist quinpirole) to

parallel our finding that finasteride does not ablate D1 induced PPI deficits in the 5αR2 KO mice. In this experiment, the 5αR2 KO mice would be pretreated with finasteride before D2 agonist treatment and PPI testing. The results of these experiments would support the hypothesis that 5αR2 inhibition is the primary mechanism of finasteride in mice and that this inhibition drives acute alterations in neurosteroid levels to induce the switch between D1 induced PPI deficits and D2 induced deficits. Determining the neurosteroid that is driving this switch is also an important future direction. We have highlighted a key role for AP; however, the role of the other 3α, 5α-neurosteroids and the precursors to AP should be investigated as well. It is possible that finasteride is exerting its therapeutic effects through increasing the levels of precursors, such as pregnenolone sulfate, so further research should focus on determining if this neurosteroid is beneficial in our TS animal models. Since sulfated compounds cannot cross the blood brain barrier these studies would have to conducted through ICV infusions or directly into the brain regions of interest, such as the PFC.

Our research strongly suggests that the neurosteroid regulation of NMDA or $GABA_A$ in the PFC is critical for regulation of subcortical brain regions such as the nucleus accumbens. Therefore, further experiments verifying that AP is directly acting in the PFC will be critical to understanding these pathways in more detail. This can be accomplished through direct infusion of AP into the PFC. Alternatively, finasteride infusion into the PFC, to determine if decreasing AP there would ablate D1 agonist induced PPI deficits, would indirectly support this hypothesis. We have previously demonstrated that finasteride was attenuating PPI deficits through actions in the nucleus accumbens; however, these experiments were completed in SD rats rather than mice or LE rats (D2 driven sensorimotor processes versus D1 driven). Therefore, the regulation from the PFC might be different in C57Bl/6 mice or LE rats. Once this is confirmed it will be interesting to

explore the contribution of the P2X4R in the mediation of these processes. As stated above, the P2X4R is well situated to be involved in modulating the balance between GABAergic neurotransmission vs glutamatergic neurotransmission.

5αR1 and 5αR2 in the regulation of mood and emotion. We have recently published that 5αR2 KO mice display alterations in dominance behaviors ([564], full text in appendix). While outside the direct scope of this dissertation, this paper highlights the role 5αR2 plays in mediating dominance and impulse-control disorders but did not reveal any overt alteration in depression-like behaviors or anxiety. However, the preliminary research presented in chapter 11 indicates that finasteride is inducing depression-like behaviors through inhibition of 5αR2. The fact that we did not see any such behaviors in the 5αR2 mice might indicate that there are compensatory effects taking place in the constitutive KO mice. Therefore, it will be important to also study the effect of condition KO in adulthood, rather than chronic deficiency. Finally, the results presented in chapter 11 do not completely rule out 5αR1 in the production of depression-like behaviors. As stated above, finasteride does not inhibit 5αR1 in rodents in the same manner as 5αR2 [537]. Furthermore, 5αR1 specific inhibitors developed for use in humans are not effective on the rodent 5αR1 [565]. Experiments using humanized 5αR1 mice, which are currently being developed in our lab, will provide insight into the therapeutic potential of these specific 5αR1 inhibitors and possible adverse side effects associated with this inhibition.

In conclusion, this research will be instrumental in developing novel therapeutics for TS patients. It also points to actions of AP and neurosteroid-AP interactions that were previously unknown. Despite the limitations of not also testing female rats and the experiments outlined above that would add further depth to this presentation, this research will be highly valuable in furthering the field of TS research and the physiological response to stress.

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Appendix

A.1 List of Abbreviations

Abbreviation	Full Name
ACTH	Adrenocorticotropic hormone
ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analysis of Variance
AP	Allopregnanolone
APO	Apomorphine
CLON	Clonidine
CRH	Corticotrophin-releasing hormone
CSTC	Cortico-Striato-Thalamo-Cortio
DAT	Dopamine Transporter
DHP	Dihydroprogesterone
FIN	Finasteride
HAL	Haloperidol
HPA	Hypothalamic-Pituitary-Adrenal Axis
HZ	Heterozygous
i.p.	Interperitoneal
KO	Knockout
LE	Long Evans
OCD	Obsessive Compulsive Disorder
PFC	Prefrontal Cortex
PPI	Prepulse Inhibition
PROG	Progesterone
QUI(N)	Quinpirole
SCH	SCH 23390
SC	Space Confinement
s.c.	Subcutaneous
SD	Sprague Dawley
SKF	SKF 82958
TS	Tourette Syndrome
WT	Wildtype

Table A.1 List of abbreviations used in this dissertation

A.2 List of Publications

The following publications were included in this dissertation:

Mosher, L.J., Godar, S.C., Nelson, M., Fowler, S.C., Pinna, G., Bortolato, M., *Allopregnanolone mediates the exacerbation of Tourette-like responses by acute stress in mouse models.* Sci Rep, 2017. **7**(1): p. 3348.

Frau, R., **Mosher, L.J.,** Bini, V., Pillolla, G., Pes, R., Saba, P., Fanni, S., Devoto, P., Bortolato, M., *The neurosteroid enzyme 5α-reductase modulates the role of D1 dopamine receptors in rat sensorimotor gating.* Psychoneuroendocrinology, 2016. **63**: p. 37-44.

Godar, S.C., **Mosher, L.J.,** Strathman, H.J., Gochi, A.M., Jones, C.M., Fower, S.C., Bortolato, M., *The D1CT-7 mouse model of Tourette syndrome displays sensorimotor gating deficits in response to spatial confinement.* Br J Pharmacol, 2016. **173**(13): p. 2111-21.

Mosher, L.J., Frau, R., Pardu, A., Pes, R., Devoto, P., Bortolato, M., *Selective activation of D1 dopamine receptors impairs sensorimotor gating in Long-Evans rats.* Br J Pharmacol, 2016. **173**(13): p. 2122-34.

Godar, S.C., **Mosher, L.J.,** Di Giovanni, G., Bortolato, M., *Animal models of tic disorders: a translational perspective.* J Neurosci Methods. 2014. **238**: p. 54-69.

Bortolato, M., Frau, R., Godar, S.C., **Mosher, L.J.,** Paba, S., Marrosu, F., Devoto, P., *The implication of neuroactive steroids in Tourette's Syndrome Pathogenesis: a role for 5α-reductase?* J Neuroendocrinol, 2013. **25**(11): p. 1196-208.

The following papers were published during Laura's graduate studies but were not included in the main text of the dissertation:

Mosher, L.J., Godar, S.C., Morissette, M., McFarlin, K.M., Scheggi, S., Gambarana C., Fowler S.C., Di Paulo, T., Bortolato M., *Steroid 5alpha-reductase 2 deficiency leads to reduced dominance-related and impulse-control behaviors.* Psychoneuroendocrinology, 2018. **91**: p. 95-104.

Santangelo, A., Bortolato, M., **Mosher, L.J.,** Crescimanno, G., Di Giovani, G., Cassioli, E., Ricca, V., Casarrubea, M., *Behavioral fragmentation in the D1CT-7 mouse model of Tourette's syndrome.* CNS neuroscience & therapeutics, 2018.

Fowler, S.C., **Mosher, L.J.,** Godar, S.C., Bortolato, M., *Assessment of gait and sensorimotor deficits in the D1CT-7 mouse model of Tourette syndrome.* J Neurosci Methods, 2017. **292**:p. 37-44.

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A.4 Selective Activation of D1 Dopamine Receptors Impairs Sensorimotor Gating in Long-Evans Rats

Mosher, L.J., Frau, R., Pardu, A., Pes, R., Devoto, P., Bortolato, M., *Selective activation of D1 dopamine receptors impairs sensorimotor gating in Long-Evans rats.* Br J Pharmacol, 2016. **173**(13): p. 2122-34.

Introduction

The enactment of adaptive behavioural responses to salient environmental cues is contingent on the ability to filter out irrelevant or redundant sensory information [1]. Deficits in this function, termed sensorimotor gating, have been documented in numerous neuropsychiatric disorders characterized by information-processing deficits, including schizophrenia and Tourette syndrome [2].

One of the best-validated operational indices to measure gating integrity is the prepulse inhibition (PPI) of the acoustic startle reflex. This endophenotype consists of the reduction of startle response triggered by a dim pre-stimulus immediately preceding the response-eliciting burst [3]. Over the past two decades, PPI has attracted substantial interest in neuroscience and psychiatric research, in view of its well-consolidated relevance to psychopathology as well as a number of operational advantages, including its validity as a cross-species testing paradigm for humans and experimental animals, which makes it particularly appealing in the context of translational studies [4, 5].

In line with the pivotal function of dopamine in information-processing functions, several studies have shown that this neurotransmitter plays a major role in the orchestration of PPI in humans [6], as well as rodents [7-10] and other vertebrates [11]. In rats and mice, agonists of dopamine receptors have been shown to produce robust PPI deficits [7, 8, 12]. These impairments

have been likened to the sensorimotor gating deficits observed in neuropsychiatric patients, by virtue of their sensitivity to antipsychotic agents [8, 12-14].

Numerous studies have shown that the role of dopamine in PPI is mediated by both D1- and D2-like receptors; nevertheless, the specific contributions of these receptors to sensorimotor gating vary across different rodent models. While PPI deficits are elicited by D2 dopamine receptor agonists in Sprague-Dawley (SD) and Wistar (WIS) albino outbred rats [15, 16], these drugs fail to disrupt PPI in most mouse strains commonly used in behavioural research [17, 18]. Conversely, D1-like receptor agonists produce robust PPI deficits in most mouse strains, but are inherently unable to reduce PPI in SD rats. However, these drugs potentiate the effects of D2 receptor agonists and other key PPI disruptors, such as NMDA glutamate receptor antagonists [19, 20]. It should be noted that high doses of the full D1 receptor agonist SKF82958 were shown to induce PPI deficits in SD rats but these changes were found to be mediated by D2, rather than D1 receptors [20].

The dichotomy between mice and rats with respect to their different sensitivity to dopamine receptor agonists was originally posited to reflect interspecies differences. More recently this interpretation has been challenged by Ralph and Caine [21], who identified PPI deficits also in response to D2 receptor agonists in less commonly used mouse strains. Although few studies have documented the occurrence of PPI deficits in rats following administration of D1 receptor agonists [17], the lack of concomitant experiments on D1 receptor antagonists in those studies leaves the question open as to whether some rat strains may

exhibit independent D1-mediated PPI deficits in a fashion similar to that observed in most mouse strains.

To address this issue, we have studied the effects of different D1 receptor agonists and antagonists in SD, WIS and Long–Evans (LE) hooded rats. Our data indicate that, under specific

protocol settings, LE rats (but not albino strains) showed a specific reduction of PPI in response to a moderate, D1-selective dose of SKF82958 and that this effect was sensitive to D1 receptor antagonism.

Materials and Methods.

Animals. All animal care and experimental procedures were in compliance with the National Institute of Health guidelines and approved by the Institutional Animal Use Committees of the University of Kansas and Cagliari. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [22, 23]. The present study was conducted on 207 male SD, 106 WIS (Harlan, Italy) and 174 LE rats (Charles River Laboratories, Raleigh, NC, USA). Rats (3–4 months old; 300–350 g of body weight) were housed 3–4 per cage in rooms maintained at a temperature of 22 ± 2°C and a humidity of 60%. Animals were given ad libitum access to food and water and held under an artificial 12/12 h light/dark cycle, with lights off from 10:00 a.m. to 10:00 p.m. In order to reduce stress during the experiment, each rat was handled gently for 5 min each day of the week preceding the behavioural testing. Care was taken in ascertaining the uniformity of all husbandry conditions across the two facilities where the experiments were performed (University of Kansas and University of Cagliari, Italy).

Apparatus and Experimental Procedure. Startle and PPI were tested as previously described [24]. The apparatus used for detection of startle reflexes (Med Associates, St Albans, VT, USA) consisted of six standard cages placed in sound-attenuated chambers with fan ventilation. Each cage consisted of a Plexiglas cylinder of 9 cm diameter, mounted on a piezoelectric accelerometric platform connected to an analogue-digital converter. Two separate speakers conveyed background noise and acoustic bursts, each one properly placed so as to produce a variation of sound within 1 dB across the startle cage. Both speakers and startle cages

were connected to a main PC, which detected and analysed all chamber variables with specific software. Before each testing session, acoustic stimuli and mechanical responses were calibrated via specific devices supplied by Med Associates.

Rats were first subjected to a pre-test session, during which they were exposed to a sequence of seventeen trials, consisting of 40 ms, 115 dB burst, with a 70 dB background white noise. Experimental groups were defined based on the average startle amplitude of the rats, so as to maintain comparable values of average startle response across all groups.

Three days after the pre-test session, rats were treated and were exposed to a test session (Figure A4.1). This session featured a 5 min acclimatization period, with a 70 dB background white noise, which continued for the remainder of the session. The acclimatization period was followed by three blocks, each consisting of a sequence of trials: the first and the third block consisted of five pulse-alone trials of 115 dB (identical to those used in the pre-test session). The second block consisted of a pseudorandom sequence of 50 trials, including 12 pulse-alone trials, 30 trials of pulse preceded by 74, 78 or 82 dB pre-pulses (10 for each level of pre-pulse loudness), and eight no-pulse trials, where only the background noise was delivered. Inter-trial intervals (i.e. the time between two consecutive trials) were selected randomly between 10 and 15 s.

The % PPI was calculated only on the values relative to the second period, as well, using the following formula:

$$100 - \frac{mean\ startle\ amplitude\ for\ prepulse\ pulse\ trials}{mean\ startle\ amplitude\ for\ pulse\ alone\ trials} \times 100$$

For both the pre-test and the test session, the interstimulus interval (i.e. the duration between the prepulse and the pulse in each trial) was kept at 100 ms. The selection of this interstimulus interval was based on pilot data and previously published experiments from our group [19], which showed

this parameter to be optimally suited to reveal PPI deficits in response to indirect and direct DA receptor agonists in rats under our experimental settings.

Experimental Procedure. This study encompassed six experiments, each involving 8–13 rats per group. The first experiment was aimed at assessing what doses of the full D1 receptor agonist SKF82958 (1–5 mg/ml, s.c., in comparison with saline, 10 min before behavioural testing) may produce alterations in PPI in SD, WIS and LE rats under our experimental conditions.

Although SKF82958 is one of the most potent D1 receptor agonists, its D1:D2 selectivity ratio (10:1) has been shown to be relatively modest in comparison with other benzazepine D1 receptor agonists [25]. Indeed, previous reports showed that, in SD rats, its PPI-disrupting effects were primarily mediated by D2, rather than D1 receptors [20]. To assess whether the effects of this agent on other rat strains may be ascribed to similar phenomena, in the second experiment, we tested whether the PPI disrupting effects of SKF82958 across different strains may be prevented by the selective D1 receptor antagonist SCH23390 (0.1 mg/ml, s.c.). Rats from each strain were therefore pretreated with either saline or the potent D1 receptor antagonist, SCH23390; 10 min later, rats were injected with either saline or a dose of SKF82958 that induced PPI deficits (based on the results of the first experiment). Testing occurred 10 min after SKF82958 injection. The third experiment mirrored the design of the second, and assessed the highly selective D2 receptor antagonist L741626 (1 mg/ml, s.c.) in SD and LE rats. Rats were pretreated with either the D2 receptor antagonist L741626 or VEHL; 20 min later, animals were injected with either saline or SKF82958. Testing occurred 10 min after SKF82958 administration.

The fourth experiment was conducted to determine the effects of the partial D1 receptor agonist SKF38393 (5–10 mg/ml, s.c.) on PPI in SD, WIS and LE rats. Animals were treated with SKF38393 or saline, 10 min prior to being placed in the startle apparatuses for testing.

In the fifth experiment, we evaluated the effects of the prototypical D2 receptor agonist quinpirole (0.6 mg/ml, s.c., 5 min prior to testing) in SD and LE rats. Furthermore, to assess the specificity of this effect, we assessed whether the PPI deficits induced by quinpirole may be prevented by L741626 (1 mg/ml, s.c., 25 min prior to quinpirole injection).

In the sixth and final experiment, we tested the effects of the D1/D2 receptor agonist apomorphine (0.25–0.5 mg/ml, s.c.) on sensorimotor gating in SD, WIS and LE rats. Apomorphine was injected immediately before placing the animals in the startle apparatuses for testing.

Materials. The following drugs were used: SKF 82958 hydrobromide, SKF 38393, SCH23390, L741626, apomorphine hydrochloride and quinpirole (Sigma Aldrich, St. Louis, MO, USA). SKF 82958, SKF 38393, SCH 23390 and quinpirole were dissolved in 0.9% saline solution. L741626 was dissolved in a vehicle (VEHL) of 1 mg/ml lactic acid and 0.9% saline. Apomorphine was dissolved in a vehicle (VEHA) of 0.9% saline and 1 mg/ml ascorbic acid to prevent oxidation. All drugs were administered via either s.c. or i.p injection, in 1 mL·kg−1 injection volume.

Data analysis. Normality and homoscedasticity of data were verified by Kolmogorov–Smirnov and Bartlett's tests. Data were compared across groups by one-way or two-way ANOVAs, as appropriate. As no interaction between prepulse levels and treatment were found in the statistical analysis, %PPI values were collapsed across prepulse intensity to represent average %PPI. Post hoc analyses were performed using Tukey's test with Spjøtvoll Stoline correction. Significance threshold was set at 0.05.

Results

Assessment of effects of D1 receptor agonists in SD, WIS and LE rats. In the first series of experiments (Figure A4.2), we tested the effects of the full D1 receptor agonist SKF82958 (1–5 mg/ml, SC) on the startle responses and PPI of SD, WIS and LE rats. In SD rats (Figure A4.2A–

B; n = 9 per group), this drug did not significantly modify startle amplitude; however, in conformity with previously published data [20], its highest dose (5 mg/ml, s.c.) produced a marked reduction of PPI in comparison with saline [$F(2,24) = 8.71$, $P < 0.05$]. Conversely, the dose of 1 mg/ml of SKF82958 was sufficient to reduce PPI in both WIS (Figure A4.2C–D) and LE rats (Figure A4.2E–F; n = 9 per group for each strain) (WIS: [$F(1,16) = 4.69$]; LE: [$F(1,16) = 29.38$], $Ps < 0.05$) , without altering startle amplitude.

In SD rats (n = 10 per group), SCH23390 produced a significant reduction in startle amplitude [main effect: $F(1,36) = 5.28$, $P < 0.05$]; conversely, this parameter was not affected by either SKF82958 (5 mg/ml, s.c.) treatment or its interaction with SCH23390 (Figure A4.3A). In the same strain, SKF82958 significantly reduced PPI [main effect: $F(1,36) = 35.30$, $P < 0.05$]; however, in confirmation of previous data [20], this effect was not countered by the D1 receptor (Figure A4.3B), confirming that, in SD rats, the PPI-disrupting effects of SKF82958 are not mediated by D1 receptors. These results were mirrored by our findings in WIS rats (n = 9 per group). Indeed, in this strain, SCH23390 produced a significant reduction of startle amplitude [main effect: $F(1,32) = 19.09$, $P < 0.05$] (Figure A4.3C); furthermore, while SKF82958 (1 mg/ml, s.c.) reduced PPI levels in this strain [$F(1,32) = 15.82$, $P < 0.05$], this effect was not prevented by SCH23390 (Figure A4.3D).

In contrast with albino strains, in LE rats (n = 8–10 rats per group), SCH23390 pretreatment produced a significant enhancement in startle amplitude [main effect: $F(1,34) = 10.75$, $P < 0.05$]; conversely, ANOVA failed to detect a significant main effects for SKF82958 or interactions between the two treatments (Figure A4.3E). The analysis of PPI confirmed that SKF82958 significantly reduced this index [$F(1,34) = 26.84$, $P < 0.05$]; however, in contrast with the other rat strains, this effect was prevented by SCH23390 [pretreatment × treatment interaction: $F(1,34) =$

6.76, $P < 0.05$], suggesting that the PPI-disrupting effects of SKF82958 were mediated by D1 receptors in this strain (Figure A4.3F).

We then examined whether the PPI deficits induced by SKF82958 may be countered by the selective D2 receptor antagonist L741626. The combination of L741,626 (1 mg/ml, s.c.) and SKF82958 (5 mg/ml, s.c.) failed to induce significant alterations in startle magnitude in both SD (n = 10 per group; Figure A4.4A) and LE rats (n = 8 per group; Figure A4.4C). Conversely, the PPI deficits induced by SKF82958 were significantly prevented by L741,626 in SD [$F(1,36) = 17.29$, $P < 0.05$] (Figure A4.4B), but not LE rats (Figure A4.4D).

Finally, we studied the effects of the partial D1 receptor agonist SKF38393 (5–10 mg/ml, s.c.) on the startle reflex and PPI of SD, WIS and LE rats. Notably, this drug failed to affect either parameter in any strain (Figure A4.5).

Assessment of effects of D2 receptor activation in SD and LE rats. In SD rats, quinpirole treatment decreased the mean startle amplitude [$F(1,36) = 48.51$, $P < 0.05$], but this effect was not modified by the D2 antagonist L741626 (Figure A4.6A). Both L741626 [main effect; $F(1,36) = 10.12$, $P < 0.05$] and quinpirole [main effect; $F(1,36) = 14.88$, $P < 0.05$] significantly modified PPI, but no significant interaction of their effects was found (Figure A4.6B). In LE rats, startle analyses showed a significant interaction between quinpirole and L741626 [$F(1,28) = 6.8$, $P < 0.05$]; post hoc analyses revealed that L741626 increased startle response, while quinpirole significantly decreased it both in VEHL- and L741626-pretreated animals (Figure A4.6C). The analysis of PPI in LE rats detected a significant interaction between quinpirole and L741626 [$F(1,28) = 8.07$, $P < 0.05$]. Post hoc analyses revealed that quinpirole led to a significant PPI deficit, which was fully countered by L741626 (Figure A4.6D).

Assessment of effects of apomorphine in SD, WIS and LE rats. In SD rats (n = 10 per group), apomorphine failed to affect startle magnitude (Figure A4.7A), but reduced PPI [$F(2,27) = 5.09$, $P < 0.05$]. Post hoc analyses showed that the reduction in PPI was produced by the dose of 0.25 mg/ml (Figure A4.7B). In WIS rats (n = 10 per group), apomorphine did not reduce startle amplitude (Figure A4.7C); the higher dose of apomorphine significantly decreased PPI [$F(2,27) = 4.25$, $P < 0.05$]. In LE rats (n = 10–12 per group), apomorphine did not significantly affect startle amplitude, but produced a robust PPI disruption [$F(2,32) = 13.27$, $P < 0.05$]. Significant differences were found for both the doses of 0.25 and 0.5 in comparison with VEHA.

Discussion

The main result of this study show that, in contrast with SD and WIS albino rats, hooded LE animals display a significant impairment in sensorimotor gating in response to selective, full stimulation of D1 dopamine receptors. Specifically, under our experimental settings, the full D1 agonist SKF82958, but not the partial D1 agonist SKF38393 produced a significant PPI reduction, which was not paralleled by variations in startle amplitude, and was countered by D1, but not D2 receptor antagonism.

To the best of our knowledge, this is one of the first reports demonstrating PPI deficits following the selective and independent activation of D1 receptors in rats. Numerous rat studies have shown the implication of both D1 and D2 receptors in the PPI-disrupting properties of non-selective dopaminergic agonists in SD, WIS and LE rats [12, 20, 26, 27]. The general consensus, however, has pointed to an ancillary role of D1 dopamine receptors in the regulation of sensorimotor gating in rats. This assumption has been largely based on numerous experimental results on albino rat strains, which showed that D1 receptor agonists, albeit able to potentiate the PPI-disrupting properties of D2 receptor agonists or NMDA glutamate receptor blockers, failed to

intrinsically reduce PPI in a selective fashion [19, 20]. For example, while SD rats display PPI deficits in response to SKF82958 or related agents (such as R-6-Br-APB [R(+)-6-bromo-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine]; [9, 17, 20]), these impairments were found to reflect the activation of D2, rather than D1 receptors [20]. Accordingly, the present results showed that, in albino rats, the PPI disruption caused by SKF82958 was prevented by administration of L741626, but not SCH23390. In this perspective, our findings highlight an unequivocal link between D1 receptor activation and sensorimotor gating deficits, and provide an experimental model to elucidate the role of these receptors in the regulation of rat PPI.

The mechanisms underpinning the role of D1 or D2 receptors in PPI regulation are incompletely understood. While several studies have identified that the PPI-disrupting properties of non-selective dopaminergic agonists are primarily contributed by the nucleus accumbens [28], the specific localization of each receptor subtype is not well understood. In particular, recent data have shown that D1 receptors in the prefrontal cortex may play an opposing role [29]. Thus, it is possible that the specific effects of D1 receptor agonists may result from the sum of opposing contributions of this receptor across different brain areas. Further studies will be needed to ascertain this possibility. Interestingly, previous studies have shown that the differences between LE and SD rats on the role of dopamine receptors in PPI regulation depends on mechanisms of dopamine receptor signalling in the nucleus accumbens [30-33]. From this perspective, it is worth noting that these results provide a first experimental platform to study the mechanism supporting the independent contributions of D1 and D2 receptor activation to dopaminergic PPI deficits in different rat strains.

In contrast with the effects of SKF82958, SKF38393 failed to impair PPI in any strain. The inability of the latter drug to produce PPI deficits confirms previous data from our group and others

[19], and is likely to result from its partial efficacy in activating the adenyl cyclase coupled to D1 receptors, which corresponds approximately to 50–70% of that of dopamine [34-36] and is markedly lower than that of SKF82958, a full D1 receptor agonist [34, 37]. Indeed, comparative analyses of these two benzazepine derivatives have shown that SKF82958 elicits a number of phenotypes not typically observed following administration of SKF38393, including activation of early-response genes [38-42], tyrosine phosphorylation of NMDA receptor subunits [43] and activation of vertical locomotor activity [44]. Thus, these data may signify that, in LE rats, PPI deficits may be triggered only by the full stimulation of D1 receptors and its downstream signalling machinery.

Pharmacological and genetic studies have shown that D1 receptors play a predominant role in the dopaminergic modulation of dopamine in mice [18, 45]. While these findings initially suggested a potential dichotomy between mice and rats with respect to the regulation of sensorimotor gating, this conclusion was later challenged by further studies, which showed that D2 receptor activation could disrupt PPI in other mouse strains used less commonly in research [21]. The present results further expand on these observations, and indicate that, at least within specific setting conditions, PPI may be independently regulated by both D1 and D2 receptors in mice and rats, and that inter-strain variations may play a critical role in the relative importance of each target in sensorimotor gating. In line with this conclusion, previous studies have shown that the dopaminergic regulation of startle reactivity and PPI is strongly influenced by differences in strains and genetic background in rats [32, 46].

We have shown here that, while SKF82958 failed to affect startle response across all rat strains, SCH23390 had apparently opposing intrinsic roles in affecting startle amplitude. Indeed, while this parameter was mildly, yet significantly reduced by D1 receptor blockade in SD and WIS

rats, it was modestly increased in LE animals. While this discrepancy cannot account for the observed differences in PPI responses across these strains, our data may suggest that the role of D1 receptors in LE rats may also diverge with respect to the regulation of startle reactivity. Our data are apparently in partial contrast with previous reports, which documented that D1 receptor activation enhances startle response in SD rats [47]. A likely explanation for this apparent divergence lies in the characteristics of our testing protocol, which was optimized for the assessment of dopaminergic effects on PPI, rather than startle reactivity. Irrespective of these considerations, future comparative studies are warranted to evaluate the specific impact of D1 receptors in the modulation of acoustic startle amplitude across different rat strains.

Although the present studies do not provide any direct mechanism to account for the differential responsiveness of LE rats to D1 receptor activation with respect to PPI, several data indicate that the dopaminergic system in this strain is distinctly different from that of albino rats. In rats, albinism is primarily due to a genetic defect in tyrosinase [48], leading to low melanin production. In the presence of tyrosinase, dopamine and its precursor l-DOPA inactivate the rate-limiting enzyme for dopamine synthesis, tyrosine hydroxylase [49]. Accordingly, intracerebral infusion of tyrosinase leads to enhanced dopamine release [50]. Previous studies have documented that the tyrosinase levels in LE rats were associated with higher dopamine turnover in comparison with SD rats [51] and, indeed, LE rats display higher corticolimbic levels of the dopamine metabolic enzyme catechol-O-methyl transferase [31]. Furthermore, fur pigmentation in LE rats is negatively correlated with the effects of apomorphine on PPI [32], suggesting that the activity of dopamine receptors is also influenced by different levels of tyrosinase. Taken together, these data suggest that the differences in PPI regulation between albino and LE rats may be underpinned by changes in D1 receptor responsiveness. The possibility that albino rats may present alterations

in D1 response is also supported by evidence showing that the domestication process of rats has led to significant differences in dopaminergic responses [52], likely to be due to the active selection of tameness and exclusion of aggressive traits [53].

Given that LE rats were originally obtained by crossing a WIS dam with a wild sire [54, 55], our data raise the possibility that wild rats or other fully pigmented strains may exhibit an even greater responsiveness to D1 receptor agonists than that of LE animals. Although logistical and safety considerations pose important problems in behavioural testing of wild rats, further studies are warranted to analyse the differential impact of albino and pigmented strains with respect to the dopaminergic regulation of sensorimotor gating. As previously indicated, it should be emphasized that the PPI-disrupting effects of D1 receptor activation in LE rats were revealed under specific protocol settings. Different testing conditions and protocol indices can greatly influence PPI, such as the loudness of the startle-eliciting pulse and the prepulses (with respect to the background noise), the duration of the inter-stimulus and inter-trial intervals, as well as the resilience of the accelerometric platform [56]. In this respect, it is important to notice that our results were paralleled by Swerdlow and colleagues at the University of California San Diego (N. Swerdlow, pers. comm.), who found that, when tested with inter-stimulus intervals of 120 ms, LE rats exhibited a %PPI baseline of approximately 80%, and responded to the full D1 receptor agonist SKF81927 with a significant reduction of PPI to about 60%. However, in the presence of shorter inter-stimulus intervals, the same D1 receptor agonist elicited either no significant effect or even enhancements of PPI (depending on the specific duration of the interval). It is also possible that the high baseline levels in our experiment may have facilitated the detection of D1-mediated PPI deficits. Accordingly, previous studies have shown that baseline PPI values play a fundamental role in influencing the susceptibility to the effects of pharmacological treatments on PPI

modulation. Indeed, similar conclusions were recently drawn in human studies [6]. In light of these considerations, it is possible that, while the conditions of our testing protocol may be optimal to capture the contribution of D1 receptors to PPI regulation, extreme caution should be advocated in the interpretation and generalization of these results, as they are likely to refer to a relatively narrow range of experimental conditions, whose biological significance remains to be determined.

We also confirmed that, under the same settings, LE rats exhibited PPI deficits also in response to the D2 receptor agonist quinpirole (which were selectively reversed by the highly selective D2 receptor antagonist L741626) and the D1/D2 non-selective agonist apomorphine. Conversely, although the same startle protocol evoked PPI impairments in SD and WIS rats in response to quinpirole and apomorphine, no significant D1-dependent PPI deficits were identified in either strain. Indeed, while SKF82958 elicited PPI deficits in both strains, as previously published [20, 57], these impairments were prevented by the selective D2 receptor blocker L741626, rather than by the D1 receptor antagonist SCH23390. The sensitivity of all tested strains to quinpirole confirms that, in rats, D2 receptors serve a prominent role in the regulation of PPI.

As mentioned in the Introduction, PPI has gained wide acceptance as the main operational paradigm for sensorimotor gating testing, because of its cross-species validity. Deficits in this index have been documented across several neuropsychiatric disorders, including schizophrenia and Tourette syndrome [2]. Building from this observation, it is interesting to observe that D1 receptors have been implicated in the pathophysiology of both conditions. In schizophrenia, these targets have been widely implicated in the modulation of cognitive deficits and negative symptoms [58]. In particular, both overstimulation and suppression of D1 receptors may result in impairments of working memory [59], a core cognitive deficit of schizophrenia. Furthermore, while stimulation of D1 receptors has been largely advocated as a potential therapeutic strategy to reduce the severity

of negative and cognitive symptoms, preliminary studies provided anecdotal support for an efficacy of D1 receptor blockers in the reduction of negative symptoms ([60, 61]; but see also [62]for contrasting data). While the role of D1 receptors in the pathophysiology of Tourette syndrome is not as well established, emerging evidence has pointed to this receptor as a promising therapeutic target; indeed, the selective D1 receptor antagonist ecopipam has been recently shown to be effective in reducing tic severity [63].

The identification of a strain-specific role of D1 receptors in PPI and startle regulation suggests that the specific interactions between this receptor and genetic factors may be essential in influencing PPI and, potentially, the pathophysiology of schizophrenia and Tourette syndrome. This concept is in keeping with ample evidence emphasizing the genetic roots of both disorders [64-67].

Several limitations of our study should be acknowledged. First, our analyses did not include molecular studies to evaluate the mechanisms underpinning the observed interstrain differences with respect to the role of D1 receptor in startle and PPI regulation. Secondly, unlike the studies on D1 receptors, our experiments on the effects of quinpirole were only performed in animals subjected to a pretreatment; thus, we cannot rule out that some of the observed findings may be affected by the stress related to the pretreatment injection. Finally, although our experiments were performed on equivalent experimental protocols and apparatuses, it is worth noting that the experiments were performed in two different laboratories (SD and WIS at the University of Cagliari, and LE at the University of Kansas). Thus, we cannot completely exclude that these logistic differences, or divergences in the colonies from the suppliers. Accordingly, previous reports have shown that differences in PPI can reflect differences in substrains based on the specific location of the supplier [57]. Nevertheless, these potential concerns are tempered by

preliminary studies in both laboratories, which essentially confirmed our findings on the three rat strains irrespective of the locations and source of the animals.

In summary, our study has identified a heuristic experimental platform to test the selective role of D1 receptors in producing gating deficits in rats. As stated above, PPI deficits are an endophenotypic feature of several neuropsychiatric disorders, including schizophrenia and Tourette syndrome. Thus, our results may prove valuable in the testing of specific hypotheses on the direct involvement of D1 receptors in rat models of these disorders.

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Author Contributions

L. J. M. executed part of the experiments, analyzed the data and wrote the first draft of the manuscript. R. F., A. P. and R. P. executed part of the experiments. P. D. designed the studies and

provided the funds for part of the experiments. M. B. designed the studies, provided the funds for the study, analysed the data and wrote the final version of the manuscript.

Conflict of Interest

The authors certify that they have no conflict of interest in relation to the content of this article.

Figures

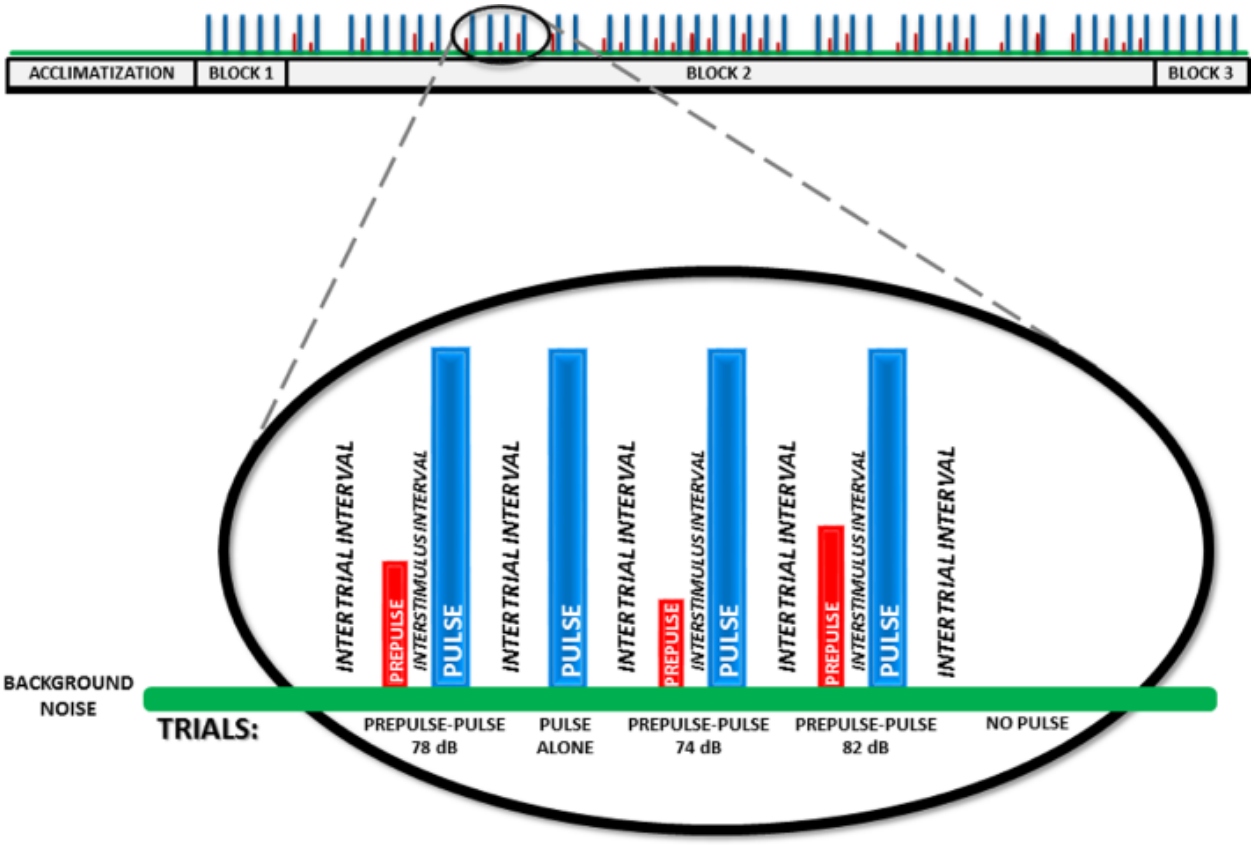

Figure A4.1 Scheme of the PPI paradigm. The green horizontal bar represents the 70 dB background noise; the blue vertical bars represent the 120 dB pulse trials; the red vertical bars represent the three prepulse levels of 74 dB, 78 dB and 82 dB. The complete session is outlined at the top with a portion enlarged to detail a subset of the trials. The acclimatization represents 5 min of exposure to the background noise; block 1 represents five pulse-alone trials; block 2 represents 50 trials containing a pseudorandom sequence of no stimulus trials, pulse alone trials and prepulse-pulse trials; block 3 represents five pulse-alone trials.

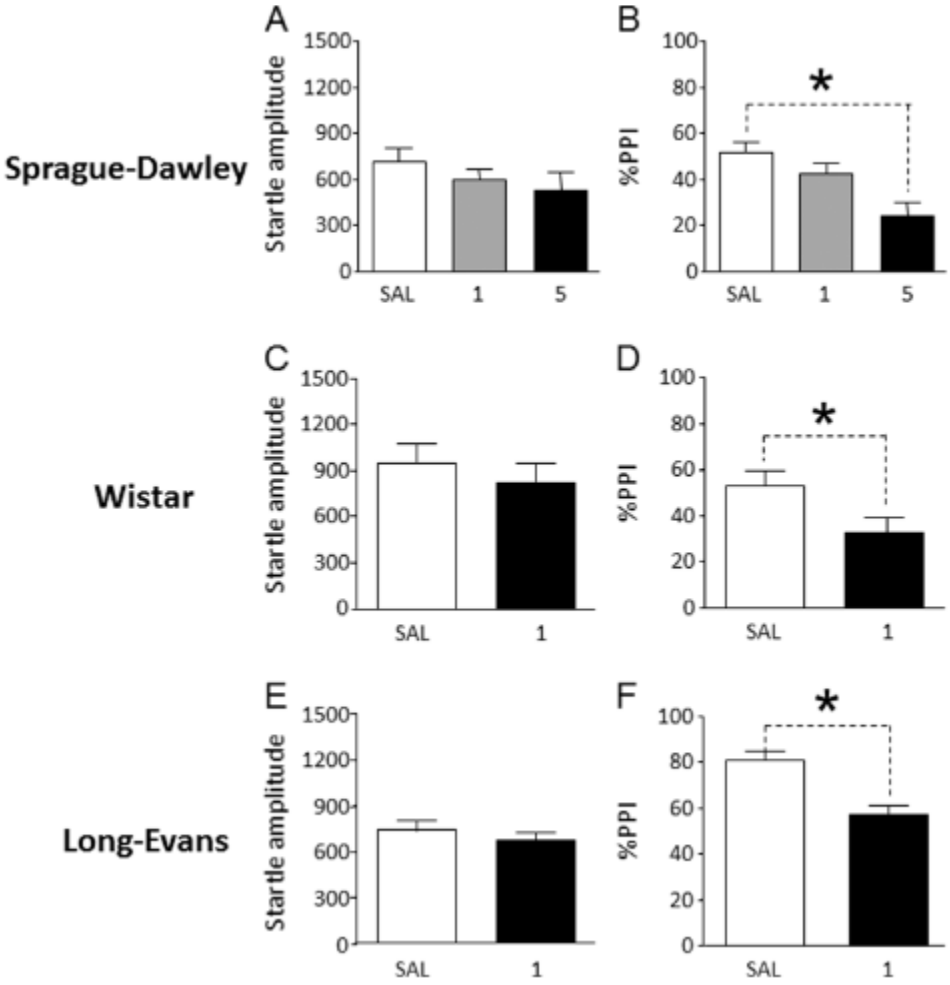

Figure A4.2 Effects of the D1 receptor full agonist SKF82958 or its vehicle, saline (SAL), on startle reflex and PPI of the startle in SD, WIS and LE rats. Values represent mean ± SEM for each experimental group. Doses of SKF82958 (1 or 5 mg/ml, s.c.) are indicated below the horizontal axis. *P < 0.05 in comparison with saline-treated controls. For more details, see text.

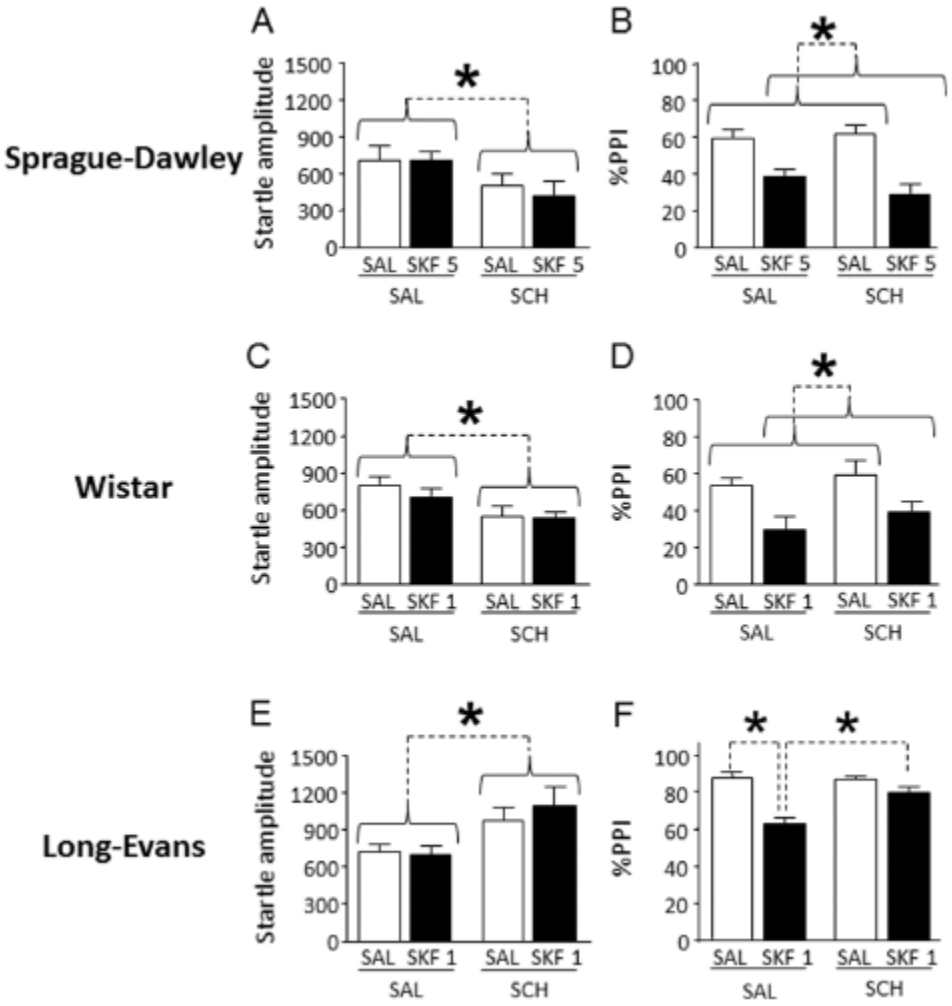

Figure A4.3 Involvement of D_1 receptors in the effects of SKF82958 (SKF; 5 mg/ml, s.c.) in SD rats and 1 mg/ml, s.c.) in WIS and LE rats) on the regulation of startle reflex and PPI in different rat strains, as tested through the combined treatment with the selective D_1 receptor antagonist SCH23390 (SCH; 0.1 mg/ml, IP). Values represent mean ± SEM for each experimental group. Doses of SKF are indicated in mg/ml. *$P < 0.05$, significantly different as indicated. For more details, see text.

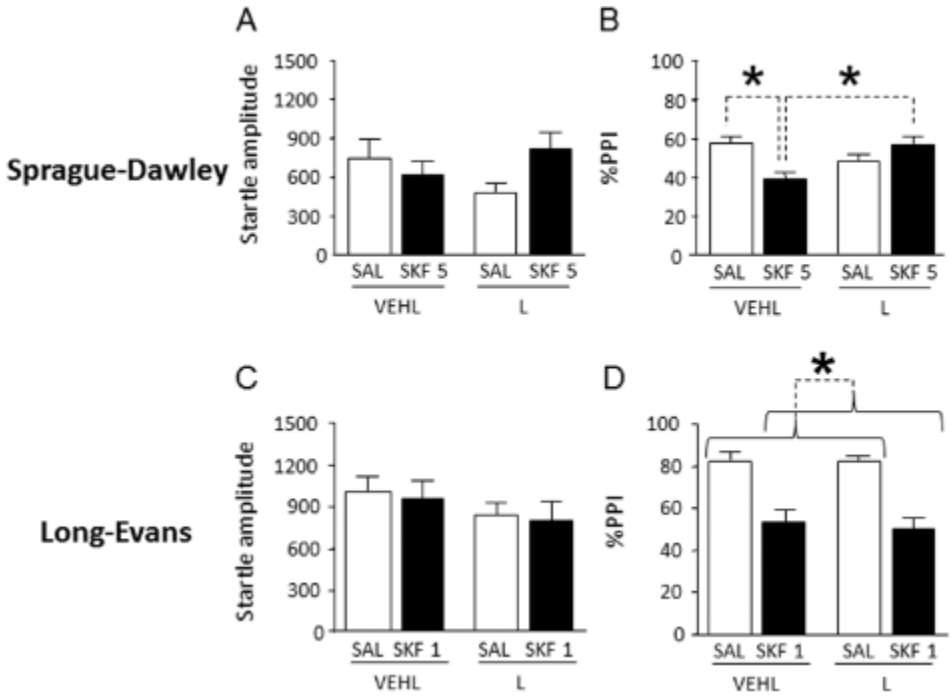

Figure A4.4 Involvement of D_2 receptors in the effects of SKF82958 (SKF) on the regulation of startle reflex and PPI in different rat strains, as tested through the combined treatment with the selective D_2 receptor antagonist L741,626 (L; 1 mg/ml, s.c.). Values represent mean ± SEM for each experimental group. PPI values are represented as the means of all prepulse-loudness values. Doses of SKF are indicated in mg/ml. VEHL, vehicle for L741,626; *$P < 0.05$, significantly different as indicated. For more details, see text.

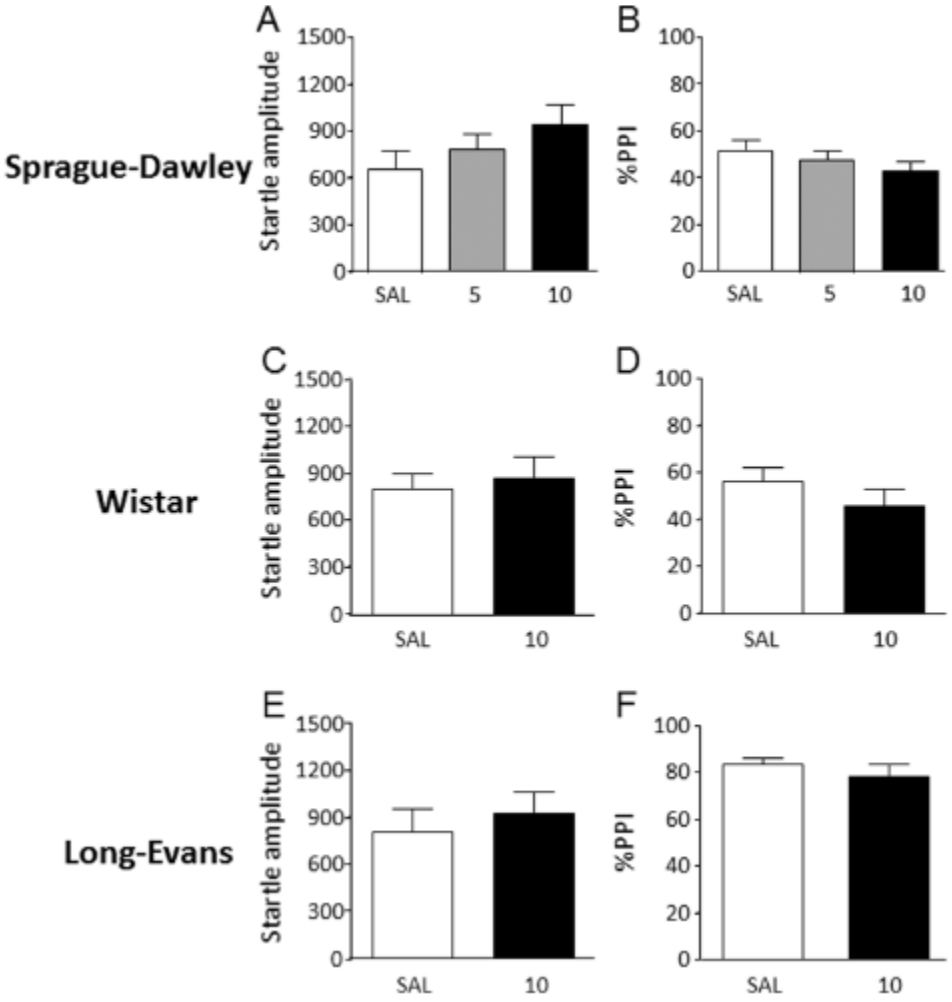

Figure A4.5 Effects of the D_1 receptor partial agonist SKF38393 or its vehicle,saline (SAL), on startle reflex and PPI of the startle in SD, WIS and LE rats. Values represent mean ± SEM for each experimental group. Doses of SKF38393 (in mg/ml, s.c.) are indicated below the horizontal axis. For more details, see text.

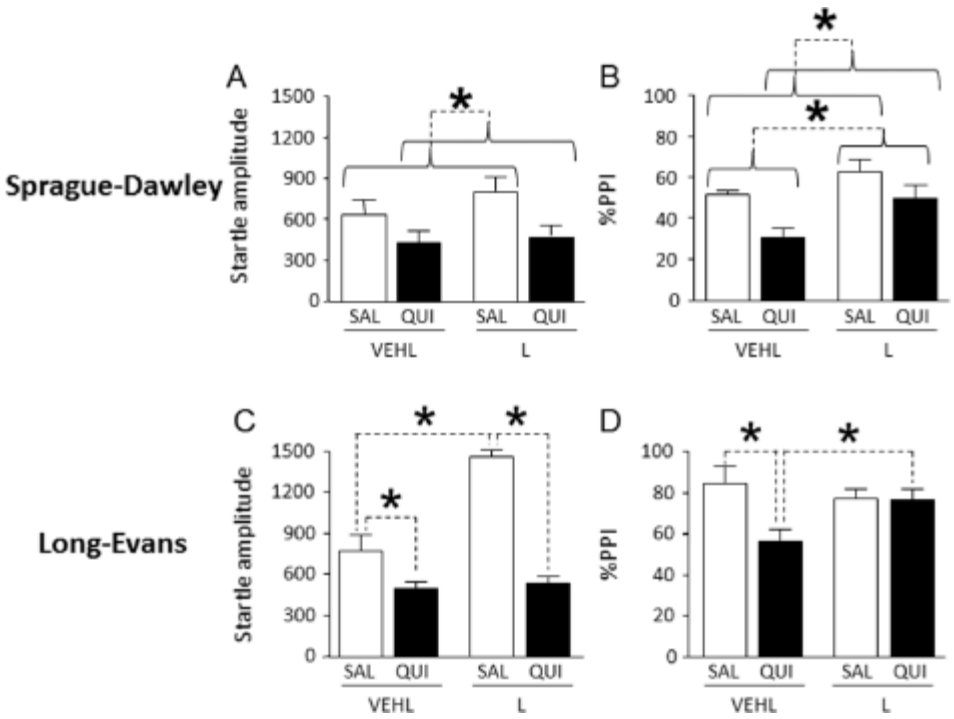

Figure A4.6 Involvement of D_2 receptors in the effects of quinpirole (QUI) on the regulation of startle reflex and PPI in different rat strains, as tested through the combined treatment with the selective D_2 receptor antagonist L741,626 (L; 1 mg/ml, s.c.). Values represent mean ± SEM for each experimental group. PPI values are represented as the means of all prepulse-loudness values. VEHL, vehicle for L741,626; $*P < 0.05$, significantly different as indicated. For more details, see text.

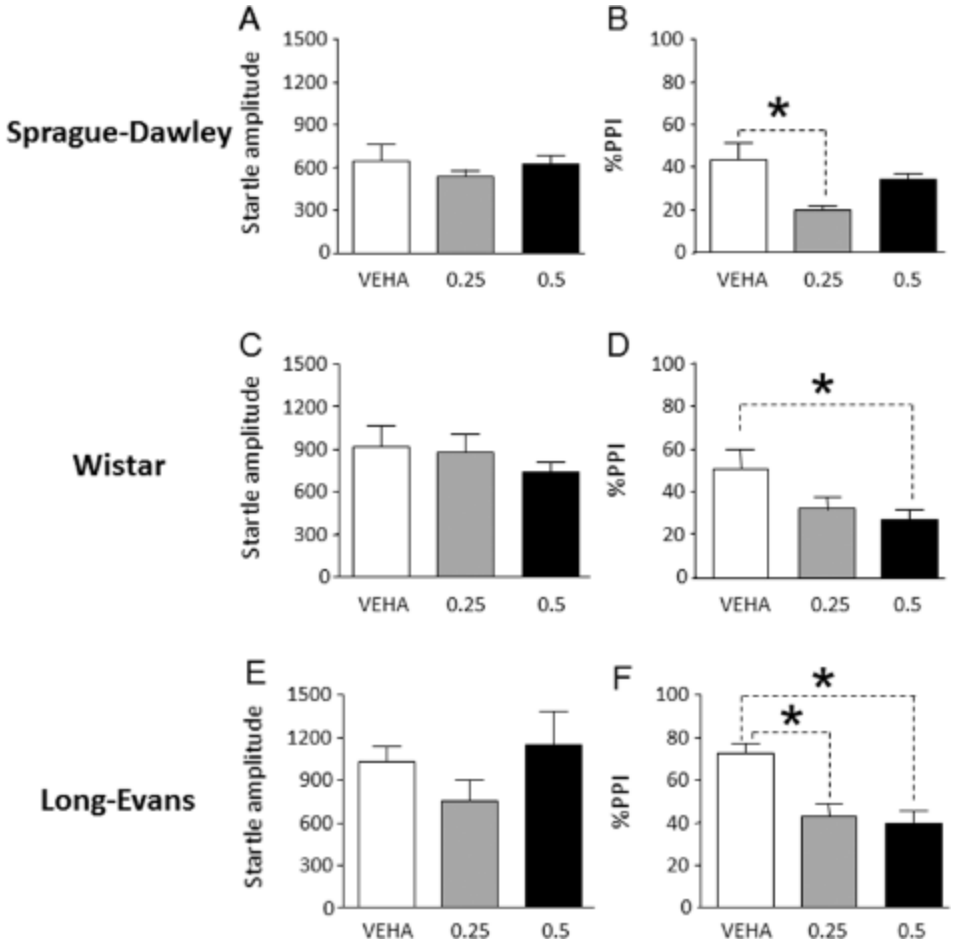

Figure A4.7 Effects of the non-selective dopaminergic agonist apomorphine or its vehicle (VEHA) on startle reflex and PPI of the startle in SD, WIS and LE rats. Values represent mean ± SEM for each experimental group. Doses of apomorphine (in mg/ml, s.c.) are indicated below the horizontal axis. *$P < 0.05$, in comparison to vehicle-treated controls. For more details, see text.

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A.5 Steroid 5α-reductase 2 deficiency leads to reduced dominance-related and impulse-control behaviors

Mosher, L.J., Godar, S.C., Morissette, M., McFarlin, K.M., Scheggi, S., Gambarana, C., Fowler, S.C., Di Paolo, T., Bortolato, M., *Steroid 5α-reductase 2 deficiency leads to reduced dominance-related and impulse-control behaviors.* Psychoneuroendocrinology, 2018. **91**: p. 95-104.

Introduction

The steroid 5α-reductase (5αR) family includes several enzymes catalyzing the saturation of the 4,5-double bond of the A ring of several 3-ketosteroids [1, 2]; in particular, 5αRs convert testosterone and progesterone into 5α-dihydroprogesterone (DHP) and 5α-dihydrotestosterone (DHT). These products are further metabolized into neuroactive steroids that play key roles in behavioral regulation, such as 3α,5α-tetrahydroprogesterone (allopregnanolone; AP) and 5α-androstan-3α,17β-diol (3α-diol), respectively [3-5]. In addition, 5αRs serve the degradation of glucocorticoids, such as corticosterone and cortisol, into their 5α-reduced derivatives [6].

The two best-characterized members of the 5αR family, type 1 (5αR1) and 2 (5αR2), differ by anatomical distribution and substrate specificity. While 5αR1 is abundantly expressed in the CNS throughout all developmental stages, 5αR2 is the predominant type in the prostate and male accessory sex glands [1, 7]. In addition, 5αR2 plays a primary role in the conversion of testosterone into the potent androgen DHT [1]. The brain distribution of 5αR2 was initially reported to be mainly limited to perinatal periods [8]. Recent investigations, however, have shown that, in adult rats, 5αR2 is expressed in the output neurons of brain regions involved in emotional and sensorimotor regulation, including the prefrontal and somatosensory cortices, striatum, thalamus, amygdala, hippocampus and cerebellum [9]. Furthermore, unlike 5αR1, 5αR2 is not expressed in

small neurons and glial cells, pointing to cell-specific patterns in the expression of this enzyme throughout the brain [10, 11].

This neuroanatomical distribution raises critical questions about the role of 5αR2 in behavioral regulation. A useful experimental approach to grapple with this issue is afforded by the characterization of the neurobehavioral phenotypes associated with the congenital deficiency of this enzyme. In men, non-functional mutations of the gene encoding 5αR2 (SRD5A2) result in Imperato-McGinley syndrome, a rare disorder characterized by a dramatic reduction in DHT synthesis, which leads to ambiguous genitalia at birth [12]. The affected individuals are often raised as girls, but experience virilization at puberty, with testicular descent, hirsutism and enlargement of the clitoris [13]. In C57BL/6 mice, the lack of 5αR2 leads to a large reduction of plasma DHT levels, as well as a reduction in prostate size and mating efficiency; however, this mutation does not affect the formation of internal and external genitalia [14].

To the best of our knowledge, although 5αR2-deficient individuals do not exhibit any major psychiatric disturbance [12], the behavioral and brain-functional changes associated with this mutation have not been fully characterized. Thus, the present study aimed at the investigation of the behavioral repertoire of 5αR2 knockout (KO) mice – in comparison with their heterozygous (HZ) and wild-type (WT) littermates – as well as its neurochemical underpinnings. Given the role of 5αR2 in the conversion of testosterone into the more potent androgen agonist DHT, we speculated that the lack of DHT in 5αR2-deficient mice may compromise some of the behavioral paradigms affected by testosterone and DHT through the activation of androgen receptors. Our studies were particularly focused on behaviors that have been related to testosterone levels and androgen receptor activation, including aggression, dominance, sexual behavior and sensation-seeking [15-19]. Furthermore, since previous work has shown that social dominance is associated

with increased D2-D3 receptor binding in the nucleus accumbens [20-22], we also analyzed the levels of dopamine and dopamine receptor binding in this region.

Materials and methods

Animals. The experiments included in this study were performed on adult (3–5-month old), experimentally naïve male 5αR2KO, HZ and WT mice (strain: C57BL/6), obtained from breeding colonies at the Universities of Kansas and Utah. All mice were generated from HZ x HZ crosses. Progenitors were obtained by Dr. Mala Mahendroo (Southwestern University). Unless stated otherwise for specific experimental purposes, all mice were housed in groups of 4–5/cage, with at least 1 mouse/ genotype, and had ad libitum access to food and water. Housing facilities were maintained at 22 °C with a reverse light/dark cycle (lights off at 08:00 AM hours and on at 08:00 PM). Whenever the same mice were used for multiple behavioral paradigms, the order of animals in each test was counterbalanced throughout the study. Tests were arranged from least to most stressful (separated by at least one week) to minimize carry-over stress. Experimental manipulations were carried out in the animals' dark cycle between 10:00 AM and 6:00 PM. All handling and experimental procedures were performed in compliance with the National Institute of Health guidelines and approved by the local Institutional Animal Care and Use Committees.

Genotyping. Mouse genotyping was performed by PCR. Samples of genomic DNA were extracted from tail biopsies acquired from mice at weaning (postnatal day 21). The following primers were used to identify 5αR2 KO mice: 1) GAT GAC CTC TCC GGG CTT CC 2) GAA TGT TCC AAG TCA CAG GC 3) CGC TTC TGA GGA GAG AAC TGA CTG A. Reaction conditions were: 94 °C x 2 min, 94 °C x 40s, 55 °C x 40s, 72 °C x 5 min, 4 °C x ∞, as previously described [14].

Behavioral testing. Mice were tested in an extensive array of behavioral tasks, aimed at evaluating motoric, sensory, and anxiety-related responses. Except when specifically stated, all tests were performed in group-caged animals. All tests and analyses were consistently performed by personnel blinded to genotypes, using littermates from all three genotypes. Thus, none of the genotypes underwent more testing than the other groups. Furthermore, no animal was tested in more than 3 paradigms.

Locomotor activity. Locomotor behaviors were measured in a square force-plate actometer as previously described [23]. The apparatus consisted of a white load plate (28 × 28 cm) surrounded on all four sides and covered by a clear Plexiglas box (30 cm tall). Four force transducers placed at the corners of each load plate were sampled 100 times s−1 , yielding a 0.01 s temporal resolution, a 0.2-g force resolution and a 2-mm spatial resolution. Custom software directed the timing and data-logging processes via a LabMaster interface (Scientific Solutions Inc., Mentor, OH, USA). Additional algorithms were used to extract macrobehavioral variables, such as distance travelled. Overall distance was calculated as the sum of the distances between coordinates of the location of center of force recorded every 0.50 s over the recording session. Low-mobility bouts were defined as periods of 5 s during which mice confined their movements to a 15-mm radius virtual circle. Velocity was defined as the distance coved by a run divided by the duration of that run and expressed as mm/s (equivalent to the product of stride length and stride rate). Distance to the nearest wall was used as an index of thigmotaxis. Mice (n = 13/genotype) were placed in the center and their behavior was monitored for 30 min.

Hot plate. Pain sensitivity was measured in the hot plate task, as previously described [24, 25]. Mice (n = 12/genotype) were individually exposed to a hot plate (IITC Life Science,

Woodland Hills, CA) at 47.5 °C, 50 °C and 52.5 °C, and the latency to lick their paws was measured. The cut-off time was set at 40 s.

Sticky tape removal test. Sensorimotor integration was tested using the sticky tape test as previously described elsewhere [24, 25]. Mice (n = 12/genotype) were briefly restrained and a circular piece of tape was placed on each forepaw. The latency to remove both pieces of tape was recorded.

Olfactory discrimination. Olfactory discrimination was tested as previously described [26]. Mice (n = 13/genotype) underwent five training trials of 5-min exposure to two identical objects of the same scent. The objects were cylinders wrapped in tape and evenly scented with diluted almond or lemon oil. On the subsequent (sixth) test trial, one of the cylinders was replaced with an identical one, sprayed with a novel scent, in counterbalanced order. Testing was performed in dim light (5 lx) and the behavior was video-recorded and olfactory discrimination was measured as a novel exploration index (NEI), calculated as the ratio of the time spent sniffing the object imbued with the novel scent and the total time spent sniffing both the novel and the familiar scent.

Acoustic startle reflex and prepulse inhibition (PPI) of the startle. Acoustic startle reflex and PPI were tested as previously described in chapter 3.

Resident-Intruder aggression. Testing was conducted as previously described [27]. Two separate cohorts of mice (n = 9-12/genotype) were isolated for 4 and 8 weeks. After this period, they were exposed to age- and weight-matched WT males from different litters, for 10 min within the resident's home cage. The test was performed with light levels maintained at 10 lx. Measures included the number and duration of fighting bouts. Other aggressive behaviors scored included biting, tail rattling, and chasing.

Tube dominance test. Testing was performed as previously described [28]. The apparatus consisted of clear PVS tubing (3 cm in diameter and 45 cm long), anchored to a solid base. All testing was performed at 30 lux. In the first experiment mice were consistently paired with cage mates carrying a different genotype. All tests within a social group were randomized across cage mates so that a given mouse did not encounter the same animal twice in succession. The submissive mouse was identified as that animal which first withdrew from the tube. If no animal exited the tube, the trial was coded as a tie. In a subsequent experiment with a new cohort of mice, males kept in isolation for 4 weeks (during adulthood) were tested against non-cage mates (n = 16-19/genotype pairing).

Mating responses. To analyze mating behaviors, males (n = 8/genotype) were isolated for 24 h. WT female mice were placed in the male's cage for 15 min and the behaviors were video-recorded. All females were in estrus, as assessed by the cell morphology of vaginal smears [29]. Mounting responses were monitored as previously described [30].

Novel-object exploration. Novel-object exploration was tested in an experiment modified from [31]. Mice (n = 12/genotype) were individually exposed to two identical novel objects, placed equidistant in their home cage after a 2-day isolation. Light was maintained at 10 lx. Mice were placed in the center of the cage between the two objects, facing away from the objects. The behavior of each mouse was recorded for 15 min to attenuate any potential confounds related to neophobia. Sniffing behavior was scored as number of approaches and duration of exploration with the novel object. Test sessions were also video recorded and the behavior scored as the novel object frequency and duration.

Wire-beam bridge test. The wire-beam bridge test was conducted with slight modification as previously described [31]. The apparatus consisted of a 30-cm high Plexiglas platform and a

50-cm high Plexiglas wall, oppositely placed at 30 cm distance. The edge of the platform and the wall were connected by a horizontal, unrailed bridge (1.25 × 30 cm), made in black aluminum wire. The bridge consisted of 2 parallel beams (0.01 cm thick) perpendicularly connected by 24 equally distanced cross-ties (1.25 cm long). It was modestly flexible, with a downward deflection of 1 cm per 100-g load at the center point. Mice (N = 8/genotype) were placed on the end of the wire-beam bridge by the Plexiglas wall and their behavior was recorded for 5 min. Behavioral measures included the latency for the mouse to cross the bridge to the safe platform. Testing was conducted in dim (10 lux) light conditions.

Light-Dark box. Testing was performed as previously described [32]. The apparatus consisted of a Plexiglas cage (20 × 30 × 20 cm) comprising of a dark area (20 × 10 × 20 cm) and an adjacent brightly lit compartment (20 × 20 × 20 cm; illumination: 250 lx). The two compartments were separated by a Plexiglas divider, providing a 7 × 4 cm opening. Briefly, mice (N = 11/genotype) were individually placed in the corner of the light area, and allowed to freely explore either compartment for 10 min. Mice were video-recorded, and the latency to exit the light compartment as well as the number and total duration of light compartment-entries were scored.

Marble burying. Marble burying was conducted as previously described [31]. Briefly, mice (N = 6-7/genotype) were individually placed in a dimly lit (10 lx) cage (35 × 28 cm), with 5 cm of fine sawdust, for a 30-min acclimatization period. Subsequently, mice were briefly removed and 20 marbles (1 cm diameter) were placed in the cage, on top of the sawdust, arranged in 5 equidistant rows (each consisting of 4 marbles). Mice were then returned to the cages, and their behavior was video recorded for the following 30 min. A marble was considered buried if at least two thirds of its surface area was covered in sawdust.

Saccharin consumption and preference. Mice (N = 12-13/genotype) were isolated for 2 weeks, and given ad libitum access to food throughout the remainder of this test. They were then given free access to two water bottles, containing either water or saccharin (at one of the following concentrations: 0.025%; 0.05%, 0.1%) for 48 h, as described by Tordoff et al. [33]. The overall consumption and preference (calculated as the ratio of saccharin solution/ total liquid consumed by each mouse) were measured.

Biochemical testing. Striatal biogenic amines determination. The left striata were dissected at coordinates anterior to Bregma −0.10 mm according to the mouse brain atlas of Franklin and Paxinos [34]. Tissues were homogenized at 4 °C in 250 μl of 0.1 N HClO4 and centrifuged at 13,200xg for 20 min to precipitate proteins. The supernatants were stored at −80 °C in small polyethylene tubes until they were assayed. The pellet was dissolved in 250 μl of 1 M NaOH for the determination of proteins content using a Micro BCA Protein Assay kit (Thermo Scientific, Rockford, IL). The content of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT) were measured by high performance liquid chromatography (HPLC) with electrochemical detection [35]. Supernatants of striatal tissue were directly injected into the chromatograph consisting of a Waters 717 plus autosampler automatic injector, a Waters 515 pump equipped with a C-18 column (Waters Nova-Pak C18, 3 μm, 3.9 mm × 150 cm), a BAS LC-4C electrochemical detector and a glassy carbon electrode. The mobile phase consisted of 0.025 M citric acid, 1.7 mM 1-heptane-sulfonic acid, and 10% methanol, in filtered distilled water, delivered at a flow rate of 0.8 ml/min. The final pH of 4.0 was obtained by addition of NaOH. The electrochemical potential was set at 0.8 V with respect to an Ag/AgCl reference electrode. Results were expressed in ng of amine per mg of protein.

[3 H] SCH 23390 and [3 H] spiperone autoradiography. Right hemispheres were immersed in ShandonTM M-1 embedding matrix (Thermo Scientific, Rockford, IL) at −20 °C, mounted on cryostat chucks and cut into 12-μm-thick coronal slices for autoradiography at three rostro-caudal levels (anterior: bregma 1.70 at 1.34 mm; medial: 0.98 at 0.38 mm; posterior: 0.02 at −0.94 mm). Slices were thawmounted on superfrost pre-cleaned slides (Thermo Scientific). Slices and dissections were kept at −80 °C until use for assays. D1- and D2- like receptor antagonist sites were labeled using the radioligands [3 H] SCH 23390 (81.9 Ci/mmol; PerkinElmer, MA, USA) and [3 H]spiperone (81.2 Ci/mmol; PerkinElmer, MA, USA) respectively [36]. Two slides containing each 3–6 mounted coronal brain sections per animal for each level for D1 and D2 receptor binding were preincubated 15 min at room temperature in sodium phosphate buffer (PBS, 100 mM, pH 7.4) containing MgCl2 2 nM and in a Tris[hydroxymethyl]aminomethane (TRIS) buffer solution (Tris-HCl 50 mM containing KCl 5 mM, CaCl2 2 mM, MgCl2 2 mM, pH 7.4) respectively. Sections were then incubated for 60 min at room temperature in their respective buffer containing either 1 nM of [3 H] SCH 23390 and 50 nM ketanserin (to block 5-HT2A receptors) or 1 nM of [3 H] spiperone and 50 nM ketanserin. Non-specific binding was defined in the presence of SKF-38393 1 μM and (+)-butaclamol 1 μM for D1 and D2 receptors respectively. After two 5-min washes in respective buffer at room temperature, sections were then rinsed briefly (10 s) in ice-cold distilled water. Finally, the slide-mounted tissue sections were exposed to BioMax MR films (Kodak, Rochester, NY) along with tritium standards [3 H]-microscales (Amersham, Arlington Heights, IL) for 16 and 29 days at room temperature for D1- and D2-like receptor binding experiments respectively. Films were developed and quantification of autoradiograms was performed on a Power Mac G4 computer connected to a video camera (XC-77, Sony, Tokyo, Japan) with a constant illumination light table

using computerized densitometry and the public domain Image J processing software from NIH (v. 1.63). Optical gray densities were transformed into nCi/mg of tissue equivalent by using a [3 H] standard curve and then converted into fmol/mg of tissue using the specific activity of the radioligand. For densitometric measurements of striatal D1-like receptors, the optical density of the total area of the brain structure was measured since it was previously reported that specific binding was evenly distributed in the striatum [37]. For D2-like receptors, a striatal lateral/medial gradient was observed with higher specific binding in the lateral versus the medial part; hence, these subregions were quantified separately [37, 38]. The nucleus accumbens is differentiated into at least two anatomically and functionally distinct regions, the core and the shell [39]. Thus, densitometric measurements of D1- and D2- like receptors were measured separately in these two nucleus accumbens subregions. Analyses were conducted by personnel blinded to group genotypes.

Statistical analyses. Data distributed binomially (such as the results of the tube test) were analyzed by a binomial test. Continuously distributed data were tested for normality and homoscedasticity by the Kolmogorov-Smirnov and Bartlett's test. Based on these results, parametric and non-parametric statistical analyses were performed by a one-way ANOVA and Kruskal-Wallis test, followed by Neuman-Keuls' or Nemenyi's tests for post-hoc comparisons, respectively. Significance was set at $P = .05$.

Results

Behavioral characterization of 5αR2 mutant mice. Neither 5αR2 KO nor HZ mice exhibited any overt abnormality in physical appearance and body weight (both across development and in adulthood), as compared with WT littermates. Similarly, the analysis of locomotor activity did not point to differences in any index, including total distance travelled (Fig. A5.1A), number

of low-mobility bouts (Fig. A5.1B), maximum velocity (Fig. A5.1C), mean velocity of top ten runs (Fig. A5.1D), or average distance from the walls (Fig. A5.1E), an index of thigmotaxis. 5αR2 KO mice failed to exhibit any deficit in thermic pain responsiveness and sensorimotor integration, as measured by the latencies to lick the paws in the hot-plate test (Fig. A5.1F) and to remove sticky tape from the paws (Fig. A5.1G). Finally, no alterations were found in olfactory discrimination (Fig. A5.1H), acoustic startle response (Fig. A5.1I), and PPI (Fig. A5.1J).

We then proceeded to assess whether 5αR2 genotype may affect aggressive and dominance-related behaviors. Resident 5αR2 KO and HZ mice were less aggressive than WT littermates toward WT intruders after 4 weeks of social isolation (Fig. A5.2A–C). Indeed, both genotypes were found to engage in aggressive behavior for a significantly lower duration [Fig. A5.2A; H (2), = 14.09, $P < 0.001$; multiple comparisons: WT vs HZ, $P < 0.01$; WT vs KO, $P < 0.05$]. Mutants also displayed a lower number of aggressive bouts [Fig. A5.2B; H (2) = 13.76, $P < 0.01$; multiple comparisons: WT vs HZ, $P < 0.01$; WT vs KO, $P < 0.05$] and a greater latency to engage in aggression [Fig. A5.2C; H (2) = 14.50, $P < 0.001$; multiple comparisons: WT vs HZ, $P < 0.01$; WT vs KO, $P < 0.05$]. After 8 weeks of isolation, different cohorts of both HZ and KO mice showed significant reductions in the duration of aggressive behaviors [Fig. A5.2D; $F(2,24) = 8.99$, $P < 0.01$; post hoc comparisons: WT vs HZ, $P < 0.01$; WT vs KO, $P < 0.01$] as well as in the number of aggressive behaviors [Fig. A5.2E; $F(2,24) = 11.55$, $P < 0.001$; post hoc comparisons: WT vs HZ $P < 0.01$; WT vs KO $P < 0.001$]; furthermore, the latency to the first aggressive interaction was longer than those exhibited by WT [Fig. A5.2F; $H(2) = 18.09$, $P < 0.001$; multiple comparisons: WT vs HZ, $P < 0.01$; WT vs KO, $P < 0.001$]. Throughout both experiments, only a small percentage of KO mice (22.2% after 4 weeks of isolation and 33.3% after 8 weeks of

isolation) initiated occasional, brief attacks, which were not conducive to victories (i.e., the opponents did not assume a subordinate posture after fighting).

Building on this finding, we tested 5αR2KO mice for potential changes in behavioral domains relevant to social dominance. First, we tested the behavior of 5αR2-deficient mice in the tube dominance test. 5αR2KO mice consistently retreated when confronted with WT cage mates (Fig. A5.2G; $P < 0.001$). To verify whether this behavior was indeed related to social ranking with respect to cage mates, we studied whether the tendency to retreat in the tube was ablated in confrontations among isolated mice. Indeed, KO mice did not exhibit any significant differences in tube dominance against non-littermates after a 4- week social isolation (Fig. 2H). We next measured the mounting behavior of 5αR2-deficient mice towards estrous WT females (Fig. A5.3A-B); in comparison with WT males, 5αR2 KO mice exhibited a trend toward a reduction in the frequency of mounting bouts [$H(2) = 8.422$, $P < 0.05$; multiple comparisons: WT vs KO = 0.08, HZ vs KO < 0.05] and a significant overall duration of active mounting [$H(2) = 8.67$, $P < 0.05$; multiple comparisons: WT vs KO < 0.05; HZ vs KO < 0.05]. Females were equally receptive to males of all genotypes.

Given that androgens have been described to be associated with higher novelty-seeking and risk-taking proclivity [40], we verified whether 5αR2 deficiency could affect these impulsivity constructs using a novel-object exploration task and a wirebeam bridge paradigm, respectively. In comparison with WT mice, 5αR2 KO littermates explored novel objects for significantly less time [Fig. A5.3C; $F(2,33) = 3.37$, $P < 0.05$; post hoc comparisons: WT vs KO, $P < 0.05$], and with a reduced number of approaches [Fig. A5.3D; $F(2,33) = 8.55$, $P < 0.01$; post hoc comparisons: WT vs HZ, $P < 0.05$; WT vs KO, $P < 0.001$]. Finally, both 5αR2 KO and HZ mice crossed a wire-beam

suspended bridge with a significantly increased latency [Fig. A5.3E; F (2, 21) = 6.25, P < 0.01; post hoc comparisons: WT vs HZ, P < 0.01; WT vs KO, P < 0.05].

To verify whether these changes were reflective of changes in anxiety- and reward-related responses we next assessed the reactivity of 5αR2-deficient mice to environmental stimuli. 5αR2KO mice failed to exhibit anxiety-like behaviors in the light-dark box (Fig. A5.4A–C) and in the marble-burying task (Fig. A5.4D), Furthermore, 5αR2-deficient mice displayed no overt alterations in saccharin preference and consumption (irrespective of the concentration) over 48 h (Supplementary Fig. A5.S1).

DAergic neurotransmission. Given that dominance is characterized by increased binding of D2- like receptors in the nucleus accumbens, we performed these analyses in 5αR2 mutant mice, as compared with WT littermates (Supplementary Fig. A5.S2). 5αR2 KO mice exhibited a significant reduction in the binding of D2-like receptors in the rostral shell of this region (between 1.70 mm and 1.10 mm from the bregma) [Fig. A5.5A; F (2,15) = 4.53, P < 0.05; post hoc comparisons: WT vs KO, P < 0.01]. No significant difference was found in either the rostral shell or the core of the nucleus accumbens (Fig. A5.5B-D). Finally, no significant changes in D1-like receptor binding were identified (Fig. 5E-H), although a statistical trend toward a decrease in D1 receptor binding in the 5αR2 HZ and KO mice was observed in the caudal portion of the core [Fig. A5.5H; F(2, 15) = 3.56; P = .051]. Finally, 5αR2 HZ, but not KO mice, exhibited decreased levels of DA in the striatum [Fig. A5.6A; F (2,21) = 6.11, P < 0.01; post hoc comparisons: WT vs HZ, P < 0.05; KO vs HZ, P < 0.01], DOPAC [Fig A5.6B; F(2,21) = 5.55, P < 0.05; post hoc comparisons: WT vs HZ, P < 0.05; KO vs HZ, P < 0.05], HVA [Fig A5.6C; F(2,21) = 5.73, P < 0.05; post hoc comparisons: WT vs HZ, P < 0.01; KO vs HZ, P < 0.05], and 3-MT [Fig. A5.6D; F(2,21) = 3.5, P < 0.05; post hoc comparisons: WT vs HZ, P < 0.05]. However, there were no alterations in DA

metabolism as evidenced by the ratios between DOPAC/DA (Fig. A5.6E), HVA/DA (Fig. A5.6F) or 3-MT/DA (Fig. A5.6G).

Discussion

The main results of this study show that 5αR2-deficient mice exhibit a reduction in dominance-related behavioral phenotypes, including aggression against intruders, tube-dominance and mating with receptive females. These changes are not accompanied by sensorimotor deficits or abnormalities in anxiety- or reward-related responses, pointing to a specific importance of 5αR2 in dominance-related behaviors.

Confrontations between WT and 5αR2 KO mice in the tube-dominance test resulted in the consistent predominance of the former. This paradigm [41, 42] is often used to study social dominance hierarchies and their genetic underpinnings [43], and has been recently validated as a reliable paradigm to investigate social dynamics [44]. Although the relation between social dominance and tube dominance is not linear [45, 46], the latter index has been shown to be consistently associated with multiple components of social dominance, including the Dalila barbering effect [47], urine marking [48] and emission of ultrasonic vocalizations during courtship [49]. Notably, the reduction in tube dominance in 5αR2 KO mice was only observed in group-housed mutants, indicating that this phenotype is not intrinsically due to the genotype − as shown by the different results of the encounters after a 4-week isolation period − but rather to the effects of the genetic mutation on social interaction with cage mates. Extensive evidence has shown that, in mice, social isolation enhances aggression and other dominance-related behaviors [50, 51]. These results indicate that, while 5αR2 contributes to shaping social hierarchies, its influence on the escalation to dominance is mediated by interactions with environmental factors (such as the exposure to social interactions). From this perspective, it is likely that 5αR2-deficient mice may

exhibit alterations in dominance following social housing. Future studies will be necessary to examine the impact of these and other environmental variables (including stress and progressive habituation to social contexts) on 5αR2-deficient mice.

Another key result of our study was that the reduction in dominance-related behaviors in 5αR2-deficient mice was accompanied by lower impulsivity-related responses, such as novel-object exploration and risk-taking (measured as the proclivity to cross a suspended wirebeam bridge). These behavioral domains are instrumental to the allocation of environmental resources and, ultimately, the development of social stratification [52, 53], and, thus, may be related to social dominance. Accordingly, previous work has shown that dominant rodents have higher risk-taking propensity [54]; furthermore, high novelty-seeking has been shown to serve as a robust predictor of aggression [55].

Rich evidence has shown that the activation of androgen receptors by testosterone influences the formation of hierarchical ranks, as well as the ontogeny of aggression, mating, risk-taking, and novelty-seeking behaviors, in men and other animals [16, 56-62]. Given that 5αR2-deficient males exhibit normal to high plasma testosterone [14, 63], our findings suggest that the conversion of testosterone into DHT is instrumental in the acquisition of dominance. In line with this notion, prior research has documented that the relationship between testosterone and dominance and aggression is not direct, but rather modulated by environmental factors, including the presence of social stressors or the uncertainty of resources [60]. Several findings suggest that 5αR2 may mediate this environmental influence on the behavioral effects of testosterone on social dominance. First, 5αR2 synthesis in the brain is dependent on stress exposure; specifically, the brain expression of this enzyme is increased by acute or short-term stress [64, 65], and reduced by chronic, inescapable stress [32]. Second, the conversion of testosterone into DHT by 5αR2

amplifies androgen signaling, given that DHT activates androgen receptors more potently than its precursor [66, 67]. Third, DHT promotes the synthesis of 5αR2 by a unique feed-forward mechanism [68], which may be instrumental for the behavioral escalation to dominance. In fact, androgens have been posited to increase aggressive proclivity following a successful confrontation with a competitor, and this process is finalized to the acquisition of a higher status in social hierarchy [60, 69-71].

In addition to the reduction in dominance-related and impulse control behaviors, 5αR2 KO mice displayed a significant reduction in D2/D3 DA receptor binding in the rostral shell of the nucleus accumbens. Reductions in D2/D3 receptor binding have been associated with social subordinate status in rats [20] and non-human primates [21, 22]. Furthermore, D2-like receptor availability has been associated with trait extraversion in humans [72] which is believed to reflect aspects of social dominance [73]. Accordingly, PET studies have shown lower uptake of D2/D3 receptor ligands in the striatum of subordinate cynomolgus monkeys, as compared with dominant ones [21, 74]. These behavioral deficits were not accompanied by changes in the levels of DA or its receptors; however, 5αR2 KO mice showed a reduction in D2/D3 receptor binding in the rostral shell of the nucleus accumbens. Taken together, these results strongly suggest that 5αR2 is an important substrate for the regulation of dominance through the modulation of DAergic signaling in the mesolimbic system.

Our studies did not include direct mechanistic experiments to probe the mechanisms underlying the reduction in D2-like receptor binding in the rostral shell of the nucleus accumbens; thus, it remains unclear whether this phenotype is a direct consequence of 5αR2 deficiency, or rather a mere epiphenomenon of reduced dominance. While further research is needed to explore this critical issue, it is worth noting that previous data from our group point to a link between the

functions of 5αR2 and the synthesis/regulation of DA receptors. For example, we found that, the 5αR2 inhibitor finasteride curbed the risk-taking effects of pramipexole [75], a dopaminergic agonist with high affinity for D3 receptors [76]. Furthermore, we showed that finasteride suppresses behavioral responses mediated by D1 and D3 DA receptors [77, 78] through its effects in the nucleus accumbens [79].

Although the involvement of DA in the behavioral profile of 5αR2- deficient mice remains unclear, it is tempting to speculate that changes in DA receptor binding in the nucleus accumbens may contribute to the reduced aggression, mating, sensation seeking and risk-taking observed in these mutants, in view of the well-documented role of dopamine in these behavioral domains [80-83].

The complexity of the relationship between DA receptor availability and these domains of behavioral disinhibition may be related to the balance between presynaptic and postsynaptic receptors. Overexpression of postsynaptic D2-like receptors, for example, increases motivation without altering consummatory behavior [84]. This connection may be mediated by the reduction in DHT synthesis. Indeed, DHT increases DA synthesis and stimulates the transcription of DA D2 receptors, as well as molecules involved in the signaling pathway in the nigrostriatal pathway, including DA and vesicular monoamine transporter [85, 86]. The possibility that 5αR2 controls DAergic responses is also in line with our findings on the behavioral effects of finasteride. This drug is currently approved for the therapy of conditions linked to excess DHT, such as benign prostatic hyperplasia and androgenetic alopecia. In addition to these applications, finasteride may have therapeutic properties for several neuropsychiatric conditions characterized by poor impulse control and externalizing manifestations [1]. Studies in rat models suggest that these effects may

be underpinned by anti-DAergic properties [78, 87]. Taken together, these results suggest that 5αR2 may play a key role in the organization of behavioral responses related to DA.

In summary, the results of this study document that 5αR2 deficiency in mice results in reduced social dominance and related behavioral traits, ranging from aggression and mating to sensation seeking and risk taking. These results complement previous findings on the role of androgens and novelty-seeking personality, suggesting that the changes observed in 5αR2 KO mice are reflective of changes in androgen profile. Nevertheless, the interpretation that 5αR2 deficiency may have similar effect in humans should be considered with caution, in view of potential differences in the role of this enzyme between mice and humans. Additional limitations should be acknowledged. First, we did not identify the steroid mechanisms responsible for these changes. Previous studies have documented that plasma of 5αR2 KO mice displays no detectable DHT levels, and slightly increased levels of testosterone. Understanding whether alterations of the brain steroid profile may contribute to the observed phenotypes is extremely important; unfortunately, previous analyses of brain-regional changes in DHT have proven unsatisfactory, given the detection limits posed by current techniques and the small size of mouse brain regions. To overcome this barrier, we are in the process of developing a novel line of 5αR2 KO rats. The analysis of steroid levels in these animals will also enable to confirm whether DHT replenishment (either in adulthood or in earlier developmental stages) may restore the ability to achieve a dominant status and normalize the related behavioral traits, including aggressiveness, mating efficiency, risk-taking and novelty seeking. Given that 5αR2 KO mice are characterized by low DHT levels, it is possible that DHT replenishment (either in adulthood or in earlier developmental stages) may restore the ability to achieve a dominant status and normalize the related behavioral traits, including aggressiveness, mating efficiency, risk-taking and novelty seeking. Second, our

studies were only limited to male rodents; it is possible that, in consideration of the contributions of androgens to the synthesis of 5αR2, there may be sex differences in the expression of these behaviors. Irrespective of these potential limitations, these findings suggest the role of 5αR2 in the achievement of social dominance, and in the regulation of impulse control, possibly through the modulation of dopaminergic mesolimbic signaling. These results warrant further ethological investigations on the function of this enzyme in social dominance and hierarchical ranking, as well as with endophenotypes related to sensation seeking and risk taking (such as delay and probability discounting). Finally, future studies will be needed to understand the mechanisms whereby steroids can influence dopaminergic signaling in the nucleus accumbens.

Conflict of interest

The authors declare no conflict of interest.

Contributors

LM monitored data collection, analyzed behavioral data, performed statistical analyses and wrote the first draft of the manuscript. SG and KM performed behavioral tests and performed statistical analyses. MM and SS performed biochemical testing and related statistical analyses. CG, SF and TDP designed the experiments, analyzed data and discussed the paper. MB designed the experiments, supervised the experimental execution, monitored data collection, wrote and revised the manuscript.

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Figures

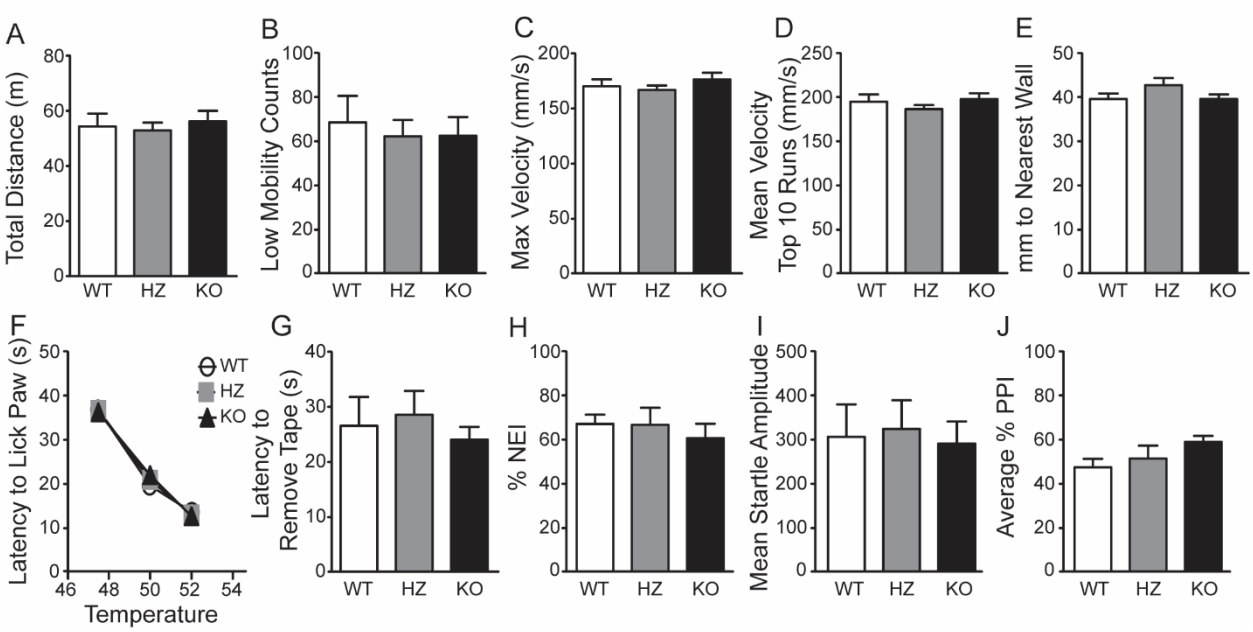

Figure A5.1. 5αR2-deficient mice do not display deficits in motoric and sensory functions. Mice (n = 13/genotype) tested on a force-plate actometer revealed no differences among genotypes in (A) total distance travelled, (B) number of low-mobility episodes, (C) maximum velocity, (D) mean velocity of the fastest 10 runs, and (E) average distance to the nearest wall. Similarly, no differences were found in pain sensitivity and sensorimotor integration, as revealed by the latencies to (F) lick paws in the hot-plate test (n = 12/genotype) and (G) remove sticky tape from the forepaws (n = 12/genotype). No alterations were found in (H) odor discrimination, as measured by% novel exploration index (%NEI) (n = 13/genotype), (I) acoustic startle response, and (J) % prepulse inhibition (PPI) of the startle reflex (n = 8/genotype). Data are shown as mean ± SEM.

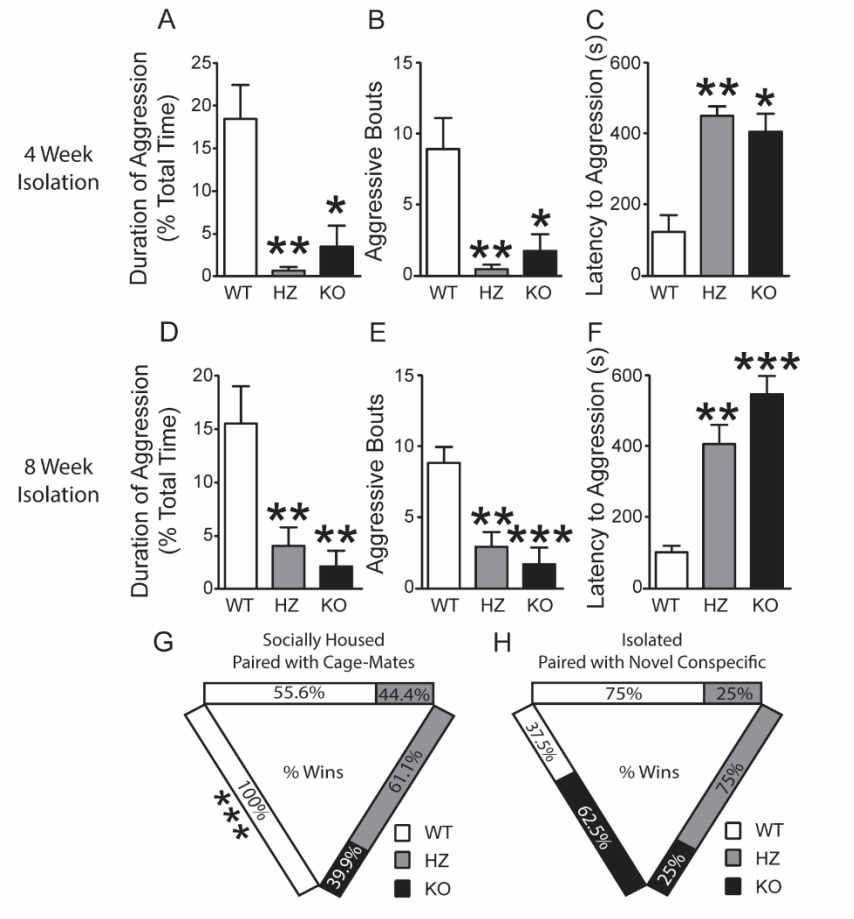

Figure A5.2. 5αR2 knockout mice display decreases in aggression and tube dominance. Aggressive behaviors were measured in the resident intruder paradigm following either a 4 week (A-C) or 8 week (D-F) isolation. The total time of aggressive behaviors (A,D), the number of aggressive bouts (B,E), and the latency to aggression (C,F) were evaluated. N = 9-12/group. Dominance behaviors were measured in the tube test while the mice were (G) socially caged or (H) socially isolated. N = 16-19/genotype. Data are shown as mean ± SEM. Abbreviations: WT, wildtype; HZ, 5αR2 heterozygous; KO, 5αR2 knockout. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to WT littermates.

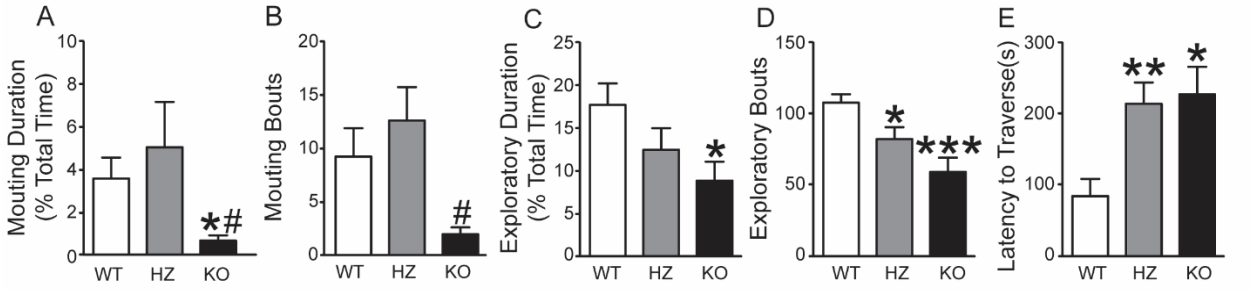

Figure A5.3. 5αR2 knockout mice display decreases in mating behaviors and exploration. (A) The total time engaged in mounting behaviors and (B) the number of encounters were measured when males were exposed to WT females in estrous. N = 8/genotype. (C) The duration of exploration of novel objects expressed as% of total time, and (I) the bouts of exploration were measured during the object exploration task. N = 12/genotype. (J) Risk-taking behaviors were measured as the latency to traverse a wire-beam bridge. N = 8/genotype. Data are shown as mean ± SEM. Abbreviations: WT, wildtype; HZ, 5αR2 heterozygous; KO, 5αR2 knockout. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to WT littermates. #P < 0.05 compared to HZ mice.

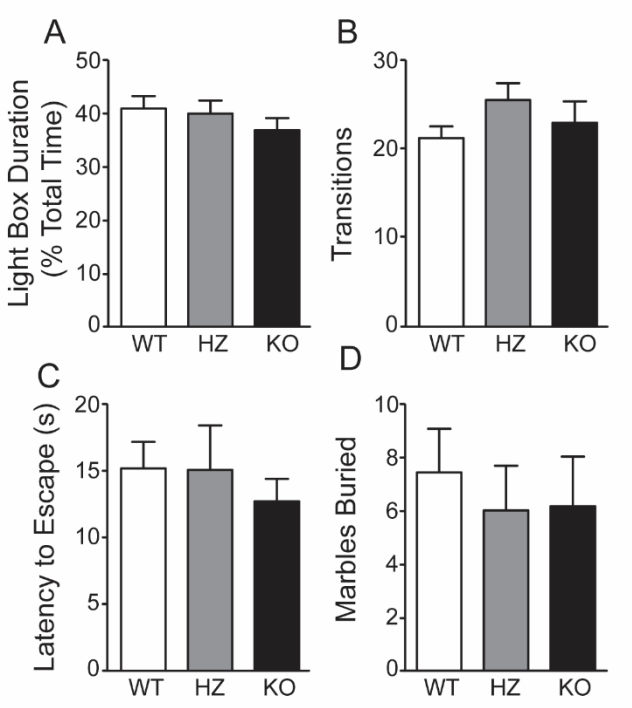

Figure A5.4. 5αR2-deficient mice do not display anxiety-like manifestations. In the light-dark box (n = 11/genotype), no differences were found in the (A) total duration of time (expressed as% of total time) spent by mice in the lit compartment, (B) number of transitions between the two compartments, and (C) latency to enter the dark compartment. Similarly, no differences among genotypes were detected in either the (D) number of marbles buried in a marble-burying task (n = 6-7 genotype). Data are shown as mean ± SEM. Abbreviations: WT, wildtype; HZ, 5αR2 heterozygous mice; KO, 5αR2 knockout mice

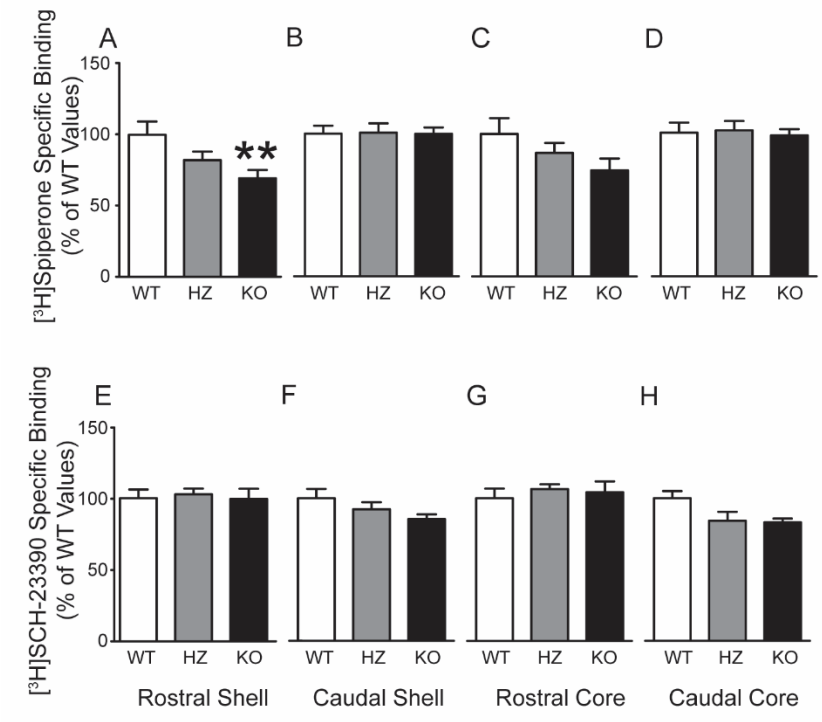

Figure A5.5. D1- and D2-like dopamine receptor binding in the nucleus accumbens of 5αR2-deficient mice. Binding was measured in (A; E) the rostral and (B; F) the caudal shell as well as in (C; G) the rostral and (D; H) caudal core of the nucleus accumbens. Data are shown as mean ± SEM. N = 5-8/genotype. Abbreviations: WT, wildtype; HZ, 5αR2 heterozygous; KO, 5αR2 knockout. **P < 0.01 compared to WT littermates.

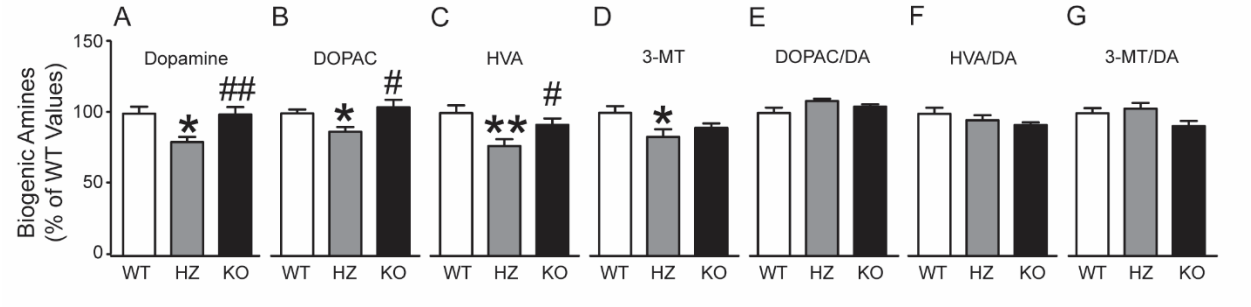

Figure A5.6. (A) Dopamine, (B) DOPAC, (C) HVA, (D) 3-MT, (E) DOPAC/DA, (F) HVA/DA, (G) 3-MT/DA levels in the striatum. N = 8/genotype. Abbreviations: WT, wildtype; HZ, 5αR2 heterozygous; KO, 5αR2 knockout. *P < 0.05 and **P < 0.01 compared to WT littermates. #P < 0.05 and ##P < 0.01 compared to HZ mice.

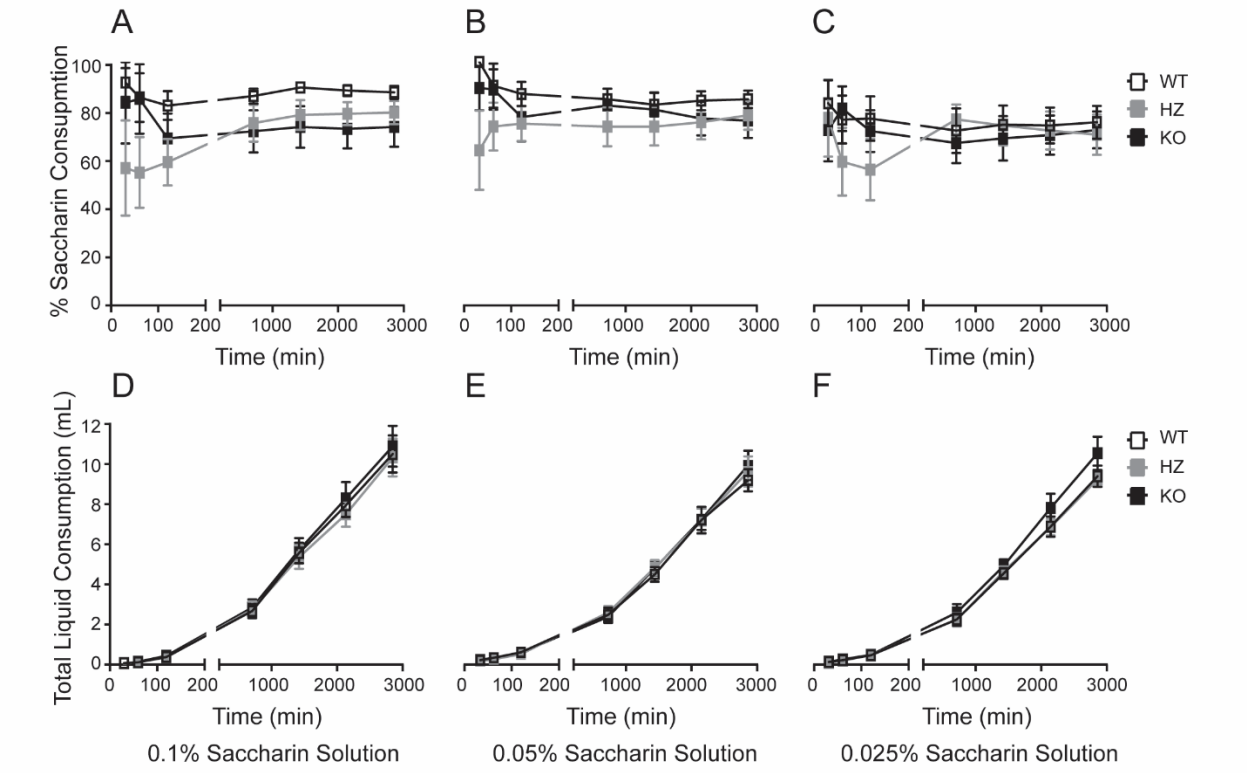

Figure A5.S1. 5αR2 knockout mice do not display alterations in saccharin preference. % saccharin preference was measured at (A) 0.1% saccharin solution (B) 0.05% saccharin solution (C) 0.025% saccharin solution. The total consumption of both water and saccharin solution was measured at (D) 0.1% saccharin solution (E) 0.05% saccharin solution (F) 0.025% saccharin solution. N=12-13/genotype. Data are shown as mean ± SEM. Abbreviations: WT, wildtype; HZ, 5αR2 heterozygous; KO, 5αR2 knockout.

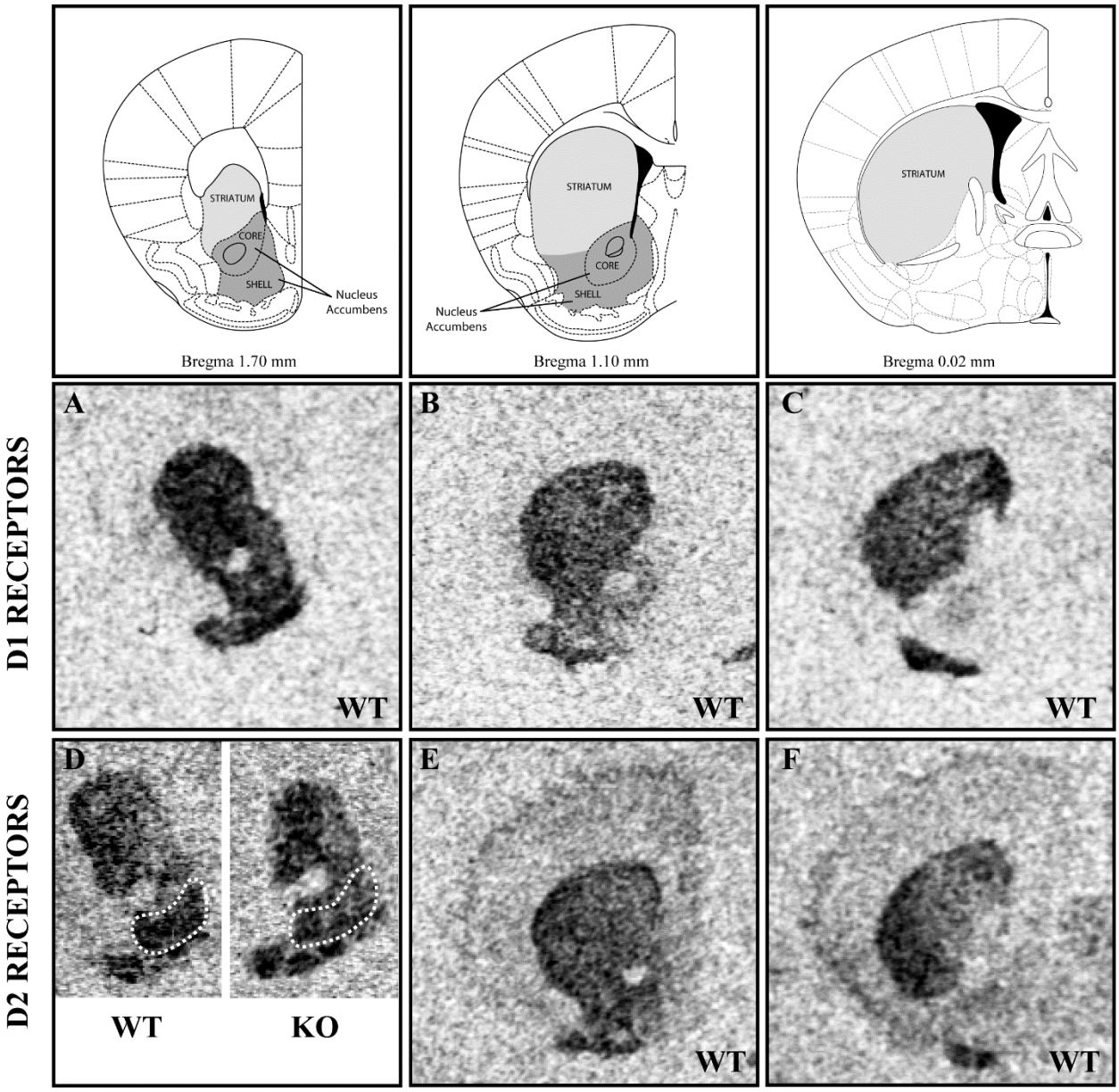

Figure A5.S2. Representative images of D1 and D2 binding in 5αR2 knockout mice and wildtype littermates. Images of D1 receptor binding in a representative WT mouse is described at (A) Bregma 1.70 mm, (B) Bregma 1.10 mm and (C) Bregma 0.02 mm. (D) Contrasting images of D2 receptor binding in a WT vs a 5αR2 KO mouse at Bregma 1.70 mm. Representative images of D2 binding in a WT mouse is also included at (E) Bregma 1.10 mm and (F) Bregma 0.02.

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