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VULNERABILITY FACTORS IN STRESS-RELATED DISORDERS: ROLE OF 5α-REDUCTASE IN THE NEUROBIOLOGY OF DEPRESSIVE AND ANTISOCIAL BEHAVIORS

ACADEMIC DISCIPLINE: **Pharmacology** (BIO/14)

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To Ginetta, Fortunato, Vasco and Bruna.

My roots, my strength.

ABSTRACT

The inhibitor of 5α-reductase enzymes (5αRs), finasteride (FIN), has potent depressogenic effects and can decrease impulsive-like behaviors in rodents. In light of this evidence, changes in genes transcription of 5αR enzyme 1 (SRD5A1) and 5αR enzyme 2 (SRD5A2) could be indicative of whether these genes are implicated in depressive- and aggressive-like behaviors. Transcriptomics analyses performed in human brain samples of Major Depressive Disorder (MDD) patients or rodent models of depression did not demonstrate changes in SRD5A1 and SRD5A2. Transcriptomics data from brain samples of subjects with an Antisocial Behavior (ASB) diagnosis are not presently available. Here, I performed the first transcriptomic study in brain samples of ASB subjects. However, these analyses did not show differences between the experimental groups in terms of SRD5A1 and SRD5A2 mRNA levels. One plausible explanation could be the restricted cell-specific pattern of 5αRs in the brain. Therefore, I collected human brain samples from NIH NeuroBioBank and performed western blot analyses in both experimental groups (MDD and ASB subjects) and their respective control groups. Protein analyses revealed reductions in 5αR2 levels in MDD samples, suggesting that 5αR2 levels could be correlated to MDD development or expression, although no differences were detected in RNA levels. One of the most relevant conditions related to the onset of MDD disorders is a stressful environment across the entire lifespan. To further investigate whether stress is directly linked to a reduction in the levels of 5αRs, I evaluated the expression of these enzymes in three commonly used models of depression in rats, the Chronic Mild Stress, Social Isolation and Social Defeat models. Interestingly, the expression of 5αR2 was reduced in the prefrontal cortex (PFC) and nucleus accumbens (NAc) of rats exposed to long term stress, that is, in the same areas where a decrease in 5αR2

levels was detected in MDD subjects. However, a correlation between stress and altered expression levels of 5αR2 is not sufficient to prove that this enzyme is involved in the onset of the disorder. Therefore, I knocked down the expression of SRD5A2 in the PFC or NAc of rats and then I evaluated the depressive-like behaviors to assess the induction of a depression-like condition. Indeed, the knock down of SRD5A2 in the PFC and NAc was accompanied by the development of distinct behavioral modifications akin to depressive symptoms. I also performed knock-down experiments of SRD5A1 in the PFC and NAc to determine whether 5αR1 is also involved in the development of depressive phenotypes or if depressive symptoms are mainly related to reduced expression of 5αR2. Interestingly, rats with reduced expression of 5αR1 did not display depressive-like behaviors.

To summarize, I studied:

1- 5αR1 and 5αR2 expression, in terms of RNA levels by transcriptomic analysis and protein expression levels by immunoblotting, in the orbitofrontal cortex (OFC) of human samples of ASB subjects;
2- 5αR1 and 5αR2 protein expression levels in human samples of MDD in the OFC, anterior cingulate cortex (ACC), NAc, hippocampus (HIPPO) and amygdala (AMY) by immunoblotting;
3- 5αR1 and 5αR2 protein expression levels in the PFC, NAc, HIPPO, and AMY of rats in three different animal models of MDD: the social defeat, social isolation, and chronic mild stress models, accompanied by the assessment of depressive-like behaviors (novelty-induced hypophagia test, sucrose preference test and forced swim test);

4- behavioral consequences of the knock-down of 5αR1 or 5αR2 in the PFC and NAc of rats, assessing depressive-like behaviors with novelty-induced hypophagia test, sucrose preference test, and forced swim test.

CONTENTS

CHAPTER 1
5-ALPHA REDUCTASE ENZYMES: AN INSIGHT

NEUROACTIVE STEROIDS AND NEUROSTEROIDS:

Steroids belong to the class of biochemicals named lipids. They are water-insoluble, organic compounds that are highly soluble in nonpolar organic solvent. The backbone of steroids is the compound gonane (17-carbon molecule composed of 4 rings). The three cyclohexane rings are called together phenanthrene (composed by rings A, B and C). The fourth ring (ring D) is a cyclopentane. Typically, steroids have an alkyl side chain (R) at carbons C-17 (Fig. 1). Different steroids vary in the functional groups attached to these rings. Common categories of steroids in vertebrates are sex steroids (including androgens, estrogens, and progestogens); corticosteroids (glucocorticoids and mineralocorticoids). Neurosteroids, or neuroactive steroids, could include all the above and be synthetized within the brain; they

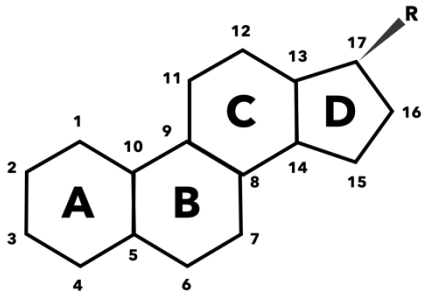


Figure 1. Basic structure of a steroid.

Table 1. Different examples of steroids.

Category	Example
Glucocortocoids	Cortisol
Mineralcoirticoids	Aldosterone
Progestogens	Progesterone
Androgens	Testosterone
Estrogens	Estradiol
Neurosteroids	Allopregnanolone

can be natural or synthetic steroids and rapidly alter neural excitability by binding to neurotransmitter membrane receptors (Tab. 1).

They can also act as a transcriptional factor in the regulation of gene expression (Rupprecht, 2003). Briefly, neuroactive steroids can be classified into three categories: pregnane neurosteroids (consisting of progesterone derivates such as allopregnanolone, and allotetrahydrodeoxycorticosterone); androstane neurosteroids (including androstanediol and etiocholanone); and sulfated neurosteroids (dehydroepiandrosterone sulfate and pregnenolone sulfate). In 1980 it was demonstrated for the first time that natural neuroactive steroids were not exclusively peripherally synthesized, yet they could be produced from cholesterol within the central nervous system (CNS). Such steroids were called neurosteroids (Corpechot et al., 1981; Morfin et al., 1992). Neurosteroids are implicated in proliferation, differentiation, activity, and survival of neurons (McEwen, 1992). Besides, they are also involved in neuroendocrine, metabolic, and behavioral processes such as response to novelty, food consumption, sexual activity, aggressiveness, anxiety, depression (do Rego & Vaudry, 2016). The physiologic effects of neurosteroids are mediated through direct interaction with neurotransmitter receptors, transporter, or indirectly via promotion of second messenger signaling cascades (Kostakis et al., 2013; Ratner et al., 2019). Their action could be exerted via the allosteric modulation of receptors located on the surface of the membrane, such as γ-aminobutyric acid receptors type A ($GABA_ARs$) (Ratner et al., 2019). Since neurosteroids have high affinity to ligan-gated ion channels, they can rapidly modulate neuronal excitability. Hence, they have been proposed as biomarkers (Almeida et al., 2020) or potential therapeutic target (Schüle et al., 2014) for some

psychiatric disorders. A key role of neurosteroids has been demonstrated in several neuropathophysiological processes. They have been discovered to play a crucial role in the onset of aggressive behaviors (Soma et al., 2015), schizophrenia (Cai et al., 2018), mood disorders (Bäckström & Bixo, 2014.), learning and memory processes (Mayo et al., 2001), anxiety and depression (Schüle et al., 2014).

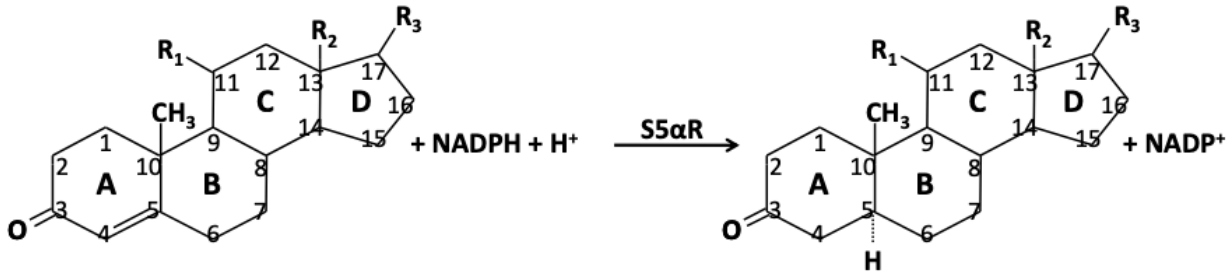
Neurosteroids concentrations fluctuate in response to physiological conditions such as development, ovarian cycle, and stress exposure. In fact, after an acute stress neurosteroids are increased both in rats and humans, and this effect could represent a homeostatic mechanism to restore the altered hypothalamic-pituitary-adrenal (HPA) axis function (Porcu et al., 2016). Alteration in progesterone and its metabolites, that are primarily responsible for reproductive functions in women, contribute to stress-related disorders (anxiety, depression, and post-traumatic stress disorder). In particular, level of progesterone is lower in individuals with depressive disorders and symptomatology and increases in relation to decreasing HPA axis hormone levels (Peltier et al., 2021). Allopregnanolone (AP), is implicated in mood disorders and depression, probably due to its capacity to directly bind $GABA_A$ receptors. Moreover, AP may promote externalizing and impulsive reactions to acute stress in subjects with high AP baseline levels. Conversely, its administration to individuals with low AP baseline levels, may help to reduce internalizing responses to stress (such as depressive and anxious symptoms) by promoting euthymia and eudaimonia (Bortolato et al., 2022). In fact, brexanolone, analog of AP, is approved for post-partum depression (Meltzer-Brody et al., 2018). Nevertheless, the mechanisms underlying the activation of neurosteroidogenesis induced by stress are still unclear. It is plausible that AP synthesis is promoted by upregulation of 5α reductase (5αR) enzyme. In experimental animal models,

after exposure to acute stress (such as sleep deprivation) 5αR enzymes and AP are upregulated (Frau et al., 2020), suggesting that neurosteroidogenic enzyme and neurosteroids are strictly linked to stress responses and the pathological conditions emerged from a stressful environmental condition.

The expression of enzymes involved in *de novo* steroidogenesis in the brain (neurosteroidogenesis) have been demonstrated by immunohistochemistry and *in situ* hybridization in various brain regions such as amygdala (AMY), cerebral cortex, cingulate cortex (AAC), dentate gyrus, cerebellum, and olfactory bulbs (do Rego et al., 2009). The main neurosteroidogenic enzymes are: 3β-hydroxysteroid dehydrogenase (3β-HSD) which is responsible for the conversion of pregnenolone in progesterone and androstenediol in testosterone; 3α-hydroxysteroid dehydrogenase (3α-HSD) that converts dihydroprogesterone in allopregnanolone; steroid 5αR which catalyzes the transformation of progesterone and testosterone respectively in dihydroprogesterone and dihydrotestosterone.

STEROID 5α-REDUCTASE FAMILY:

The enzyme steroid 5αR catalyzes the conversion of Δ-3-ketosteroid precursors (such as testosterone, progesterone, corticosterone, aldosterone, deoxycorticosterone) into their 5-α-reduced metabolites. This process, which requires nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor, consists in the formation of an enolate intermediate on the C3 position of the substrate ketosteroid, which consequently results in the protonation of C4 and direct transfer of a hydride ion from NADPH to C5 (Fig. 2).



SUBSTRATE	$-R_1$	$-R_2$	$-R_3$	PRODUCT
CHOLEST-4-EN-3-ONE	-H	$-CH_3$	$-CHCH_3-(CH_2)_4-(CH_3)_2$	5α-CHOLESTAN-3-ONE
PROGESTERONE	-H	$-CH_3$	$-CO-CH_3$	5α-DIHYDROPROGESTERONE
DEOXYCORTICOSTERONE	-H	$-CH_3$	$-CO-CH_2OH$	5α-DIHYDRODEOXYCORTICOSTERONE
CORTICOSTERONE	-OH	$-CH_3$	$-CO-CH_2OH$	5α-DIHYDROCORTICOSTERONE
ALDOSTERONE	-OH	-CHO	$-CO-CH_2OH$	5α-DIHYDROALDOSTERONE
ANDROSTENEDIONE	-H	$-CH_3$	=O	5α-ANDROSTANEDIONE
TESTOSTERONE	-H	$-CH_3$	-OH	5α-DIHYDROTESTOSTERONE

Figure 2. Scheme of the reaction catalyzed by 5αR (from Paba et al., 2011).

The first functional characterization of 5-α-reduction processes was reported in the early 1950s by several independent groups of researchers, in reference to the conversion of deoxycorticosterone and androgenic Δ-3-ketosteroids into their 5-α-reduced metabolites (Samuels, 1951; Schneider & Horstmann, 1951). 5αRs were identified and characterized primarily in rat liver homogenates (Forchielli & Dorfman, 1956; Schneider & Horstmann, 1951). The current molecular and genetic evidence on the existence of multiple types of 5αR enzymes, and the two major isoforms involved in a key role in steroidogenesis are 5αR1 and 5αR2 (Paba et al., 2011). The employment of high-resolution sequence, high-throughput cloning and characterization and modeling of protein allowed to identify and characterize the members of 5αR family.

The 5αR family is composed of 3 subfamilies and 5 members (isozymes, different proteins that perform the same function):

1) 5αR1 and 5αR2
2) 5αR3
3) TECR and TECR-like (TECRL).

STEROID 5αR1:

The first isolated 5αR isozyme, 5αR1 is encoded by the gene SRD5A1 organized in 5 exons and 4 introns. SRD5A1 encodes for a highly lipophilic, non-glycosylated protein, featuring 5 predicted transmembrane helices. The protein is typically found in the lipidic bilayer of the endoplasmic reticulum membrane (microsomal enzyme), frequently in perinuclear localization (Savory et al., 1995; Span et al., 1996). 5αR1 is highly expressed in keratinocytes, melanocytes, sweat glands, neurons of central and peripheral nervous system, fibroblasts, hepatocytes, and in several organs such as prostate, lung, colon, and kidney (Chen et al., 1998; Finn et al., 2006; Habib et al., 1998; Russell & Wilson, 2003; Thigpen et al., 1993a; Titus et al., 2005; Tsuruo et al., 1996; Yokoi et al., 1998). In the adult brain 5αR1 is the major isozyme expressed, hence it is speculated that 5αR1 accounts for the majority of the 5α-reduction reactions (Martini et al., 1996; Melcangi et al., 1993; Negri-Cesi et al., 1996; Thigpen et al., 1993b). 5αR1 has been shown to be present in the temporal cortex, hippocampus, hypothalamus, pons, and cerebellum after post-mortem studies in humans (Stoffel-Wagner et al., 2000; Thigpen et al., 1993b). 5αR1 is a NAPDH-dependent enzyme, composed of 259 ammino acids, and has molecular weight of ~ 25 kDa (Langlois et al., 2010; Russell & Wilson, 2003).

STEROID 5αR2:

5αR2 is encoded by the gene SRD5A2 (organized in 5 exons and 4 introns). This gene results from the duplication of a common ancestor with SRD5A1 (Paba et al., 2011). Therefore, a relatively high level of identity is shared by both genes SRD5A1 and SRD5A2 (Langlois et al., 2010). 5αR2 is a NADPH-dependent, membrane-associated enzyme (4 transmembrane helices) composed of 254 amino acids (molecular weight of ~ 25 kDa) (Azzouni et al., 2012). Even though 5αR1 and 5αR2 are intrinsic membrane proteins and catalyze the same reaction, they only share a limited degree of homology in protein sequences, are located on different chromosome (5p15 and 2p23 respectively) and possess distinctive biochemical properties such as optimal pH (6-8 and 5 respectively), and substrate specificity (5αR2 has a higher affinity to testosterone and progesterone than 5αR1) (Azzouni et al., 2012; Paba et al., 2011). Also, the pattern of distribution of 5αR2 is notably different from that of 5αR1. 5αR2 is predominantly expressed in the male urogenital tract, skin, hair follicles and liver (Finn et al., 2006) In the brain, 5αR2 has been found ubiquitously in early developmental stages (Poletti et al., 1998) and becomes restricted to several regions of the adult brain, e.g., hypothalamus, olfactory bulb, thalamic nuclei, hippocampus, basolateral amygdala, cerebellum, prefrontal cortex (PFC), and nucleus accumbens (NAc) (Castelli et al., 2013; Poletti et al., 1998; Sánchez et al., 2008).

STEROID 5αR3:

5αR3 is encoded by SRD5A3 located at 4q12, it is composed of 318 amino acids and has only 19-20% homology with 5αR1 and 5αR2 (Azzouni et al., 2012). 5αR3 was discovered in 2007 through the analysis of genome-wide gene expression of prostate cancer cells (Tamura et al.,

2007). The 5αR3 has an ubiquitous distribution throughout the organism (Paba et al., 2011). Cantagrel et al., 2010 identified 5αR3 as necessary for and promoting the reduction of polyprenol to dolichol in human, mouse, and yeast: a key process that enables the N-glycosylation of proteins.

TECR AND TECRL:

Trans-2,3-Enoyl-CoA Reductase (TECR) or synaptic glycoprotein 2 (GPSN2) encodes the enzyme trans-2,3-enoyl-CoA reductase, a multi-pass membrane protein (3 helices) composed of 363 amino acids and with a molecular weight of 42 kDa (Paba et al., 2011). Trans-2,3-Enoyl-CoA Reductase-Like (TECRL) is also called GPSN2-like or SRD5A2-like 2. TECR function is to catalyze the last step for the elongation of fatty acids, consisting in the reduction of the double bond of a trans-enoyl-CoA (Moon & Horton, 2003). While it is likely that TECRL may catalyze a similar enzymatic reaction, its substrate remains unknown to date. No information is available on the ability of these enzymes to process the 5-reduction of ketosteroids (Paba et al., 2011).

CHAPTER 2
5ARS IN PSYCHOPATHOLOGIES

STEROID 5αR AND PSYCHIATRIC DISORDERS: FOCUS ON DEPRESSION, AGGRESSION, DOMINANCE, AND IMPULSIVITY:

The brain concentration of neurosteroids with quite different molecular mechanisms of action may be affected by either activation or inhibition of 5αR enzymes. For instance, neurosteroid levels, such as progesterone, are changed in animal models of anxiety, depression, and post-traumatic stress disorder (PTSD) (Pibiri et al., 2008; Serra et al., 2000; Uzunova et al., 2006). Moreover, several pharmacological and epidemiological studies have revealed a strong correlation between the use of 5αRs inhibitors and the development of depression, an increase of suicide attempts and suicidal thoughts (Dacso, 2017; Kim et al., 2020; Pompili et al., 2021; Traish, 2020; Traish et al., 2010; Welk et al., 2017). Additionally, pharmacological treatment with antidepressants, antipsychotics, and mood stabilizers restores central and peripheral concentration of neuroactive steroids in both animal models of psychiatric diseases and patients (Porcu et al., 2016).

Finasteride (FIN; N-(2-methyl-2-propyl)-3-oxo-4-aza-5α-androst-1-ene-17β carboxamide) is the prototypical inhibitor of 5αR (a potent inhibitor of 5αR2, less effective on 5αR1). FIN is clinically approved for the treatment of benign prostatic hyperplasia and male baldness (Paba et al., 2011). Although FIN is generally well-tolerated, post-marketing reports of adverse psychological events have led to growing concerns about the safety profile of this drug. Several studies have substantiated that FIN increases the risk for depressive symptoms

in a subset of vulnerable patients (Altomare & Capella, 2002; Rahimi-Ardabili et al., 2006; Traish et al., 2015). Based on this evidence, Godar et al., 2019 tested the behavioral effects of FIN in a broad battery of standardized behavioral tests in Long-Evans rats, in order to assess its effects on complementary aspects of mood regulation, anxiety, impulse control, and stress reactivity. The main results of this study show that FIN increases the duration of immobility in the force swim test, a behavioral parameter that measures stress coping abilities. Moreover, FIN reduces saccharin preference, a well-characterized index of reward sensitivity and one of the most robust predictors of depressive-like behavior in animal models. The possible mechanism that underlies the onset of depressive-like behavior could be the inhibition of the synthesis of 5-α-reduced neurosteroids (Godar et al., 2019). These steroids promote corticotropin releasing hormone (CRH) synthesis by inactivating GABAergic neurons in the paraventricular nucleus (PVN) of hypothalamus. Moreover, FIN increases levels of progesterone, which exerts negative effects on CRH synthesis in the PVN (Godar et al., 2019). Considering this evidence, it is likely that changes in neurosteroids may be primarily responsible for depressive-like behavior in FIN treated rats.

In men, non-functional mutations of the gene SRD5A2 result in Imperato-McGinley syndrome: a rare disorder characterized by a dramatic reduction in the synthesis of 5α-dihydrotestosterone (DHT, an androgen derived from the metabolization of testosterone by 5αR2, and further metabolized into 5α-androstan-3α,17β-diol a potent neuroactive steroid), which in turn leads to ambiguous genitalia at birth (Imperato-McGinley et al., 1974). Although it is well known that this disorder affects the reproductive system, the behavioral and brain-functional changes are still not fully characterized. Thus, Mosher et al., 2018

investigated the behavioral repertoire of a 5αR2 knockout (KO) mice compared to their wild-type (WT) littermates. Their study was centered on behaviors that have been related to increased testosterone levels, comprising of aggression, dominance, sexual behavior, and sensation-seeking. The 5αR2 KO mice exhibit a reduction in aggressive and dominance-related behavioral phenotypes and lower impulsivity-related behavior (Mosher et al., 2018).

Copious evidence has demonstrated that neuroactive steroids have psychopathological implications (Dubrovsky, 2005) and could be involved in the pathophysiology of schizophrenia and bipolar disorder (Marx et al., 2009). In light of these premises, Frau et al., 2017 studied the implications of 5αR enzymes in the psychopathology of psychosis- and mania-related behaviors in rodents. The results of this study provide the first evidence that increased levels of 5αR2 mediate several psychotic- and manic-like behaviors, as well as risk-taking behaviors (Frau et al., 2017).

FIN has been recently discovered as one of the most effective compounds to reduce opioid and fentanyl self-administration in animal models (Bosse et al., 2021), thus it could be speculated that 5αR and neurosteroids synthetized by those enzymes are also implicated in addiction and substance use disorders as element of impulsive behavior.

Agís-Balboa et al., 2007 showed that social-isolated mice express lower mRNA levels of 5αR1 in some brain regions (hippocampus, amygdala, and cortex). These mice are also more aggressive than the control group. These findings imply the role of 5αR1 in mediating responses to stressful conditions (social isolation) and in the onset of aggressive behavior.

Although it is still uncertain whether the altered neurosteroidogenesis is the result of impaired brain homeostasis or if it could be a contributing factor to the pathological outcome, evaluating the implication of neurosteroidogenic enzymes in the onset of depression could be a critical point to further understand the role of neurosteroids in neuropsychiatric disorders. Yet, this issue needs to be further investigated.

However, it is clear from all these preclinical studies that 5αR enzymes are potentially involved in various psychiatric disorders. Then, the first goal of my thesis project was to investigate whether SRD5A1 and SRD5A2 were involved in Major Depressive Disorder (MDD) and Antisocial Behavior (ASB) since they seem more implied in mood and personality disorders. Moreover, MDD and ASB have been shown to have a high level of comorbidity (especially in adolescent individuals) (Ritakallio et al., 2008) and both are strictly linked to the exposure to a stressful environment.

CHAPTER 3
TRANSCRIPTOMICS ANALYSES IN MDD AND ASB

OVERVIEW CHAPTER 3:

Based on premises from chapter 1 and 2, we performed transcriptomics analyses in human samples of MDD and ASB subjects to assess whether SRD5A1 and SRD5A2 genes were involved in these disorders. Transcriptomics aims to study all RNA molecules in a cell, in other words it reveals how active genes are in different cell populations. This analysis provides a connection between the genome and the proteome. It is particularly helpful for revealing the molecular constituents of cells and tissues, and consequently for understanding the development of diseases. In light of these premises, my first approach was to examine the literature.

Transcriptomics analyses have revealed a dysregulation in SRD5A1 gene expression in various cancer cell populations such as breast, and colorectal cancer (Dou et al., 2021; Wei et al., 2020); while SRD5A2 is frequently associated with developmental gonadal disorders (Nash et al., 2019). However, very little is known about imbalances of SRD5A1 and SRD5A2 gene expression and psychiatric diseases.

Transcriptomic studies in MDD highlight significant overlaps between transcriptional alterations in the PFC and NAc of human subjects (Scarpa et al., 2020). In particular, these analyses indicate a different expression of GABAergic markers which suggests a disruption

in terms of neural activation. Additionally, Labonté et al., 2017 found a dramatic sexual dimorphism at the transcriptional level in MDD male and female subject in genes related to the GABAergic, glutamatergic, and serotoninergic systems. Similar results have been obtained in mouse models of MDD (Touchant & Labonté, 2022). Since MDD has a huge impact on modern societies, acquiring a deep knowledge about its etiological mechanism and its gender-related differences should be a top priority for healthcare system. As discussed above, neurosteroids directly bind $GABA_A$ receptors, consequently we can speculate that modifications in 5αRs levels should result in different levels of neurosteroids and, finally, in altered activation of prefrontal cortices and NAc of MDD patients. Nevertheless, transcriptomics imbalances of SRD5A1 and SRD5A2 genes have not been discovered yet. One plausible explanation could be the localization of the 5αRs. In fact, 5αR2 is highly expressed in the pyramidal cells, but not in other neuronal or glial cells, pointing to a strictly cell-specific expression pattern of these enzymes within the brain (Castelli et al., 2013). Thus, it is unlikely that a sufficient number of pyramidal cells can be extracted from post-mortem human brain samples in order to perform a single cell transcriptomic analysis.

A completely different scenario is displayed in psychiatric diseases where the principal components are impulsivity and aggression such as ASB. ASB is a term used to describe a great variety of attitudes and behaviors that violate societal norms, values, property or rights of others, and inevitably laws. ASB is a key feature of several neuropsychiatric disorders, including conduct disorders (CD) in children or adolescents and antisocial personality disorders (ASPD) in adults. Thus far, transcriptomic studies of subjects with ASB have not been performed or published. Performing this type of analysis in ASB subjects could

contribute to clarify the molecular mechanisms underlying this disorder, which are still largely elusive.

ASB is a persistent pattern of violation of societal norms, often entailing damage to the rights and properties of others, as well as covert and overt hostility (Vitiello & Stoff, 1997). The lifetime prevalence of CD and ASPD in the general population is estimated at 9.5% (Nock et al., 2006) and 4.3% (Goldstein et al., 2017), respectively. However, these rates reach 47-78% among incarcerated individuals (Fazel & Danesh, 2002; Rotter et al., 2002), given the strong association between these disorders and criminal behavior. ASB is also characterized by a marked male preponderance, with male-to-female ratios ranging from 3:1 to 7:1 (Grant et al., 2004; Hamdi & Iacono, 2014; Maughan et al., 2004).

ASB is highly comorbid with several psychiatric disorders, the most prominent being substance use disorder (SUD) (Black, 2015). Consensual evidence indicates that the rate of antisocial individuals with a lifetime history of substance use (including alcohol) is 90-95% (Hatzitaskos et al., 1999; Messina et al., 1999). In keeping with these data, 68% of jail inmates in the US meet the diagnostic criteria for SUD (Karberg & James, 2005). A complex etiological and pathophysiological relationship supports the association between ASB and SUD. On the one hand, this comorbidity has shared genetic and environmental vulnerabilities; on the other hand, each of these two disorders can exacerbate the symptom severity of the other, leading to a vicious circle (Nichita & Buckley, 2020). The comorbidity of ASB and SUD is a staple of the spectrum of externalizing psychopathology (Forbes et al., 2017; Krueger et al., 2007), characterized by poor self-regulation as well as outward, disruptive, and hyperactive behavior.

Several authors have shown that the maladaptive social responses in ASB are rooted in deficits in facial affect processing (Blair, 2003; Marsh & Blair, 2008; Montagne et al., 2005) directly contributed by dysfunctions of the orbitofrontal cortex (OFC). This brain area regulates the interpretation of social cues and the enactment of appropriate emotional reactions (Bechara et al., 2000; Blair, 2004; Blair & Cipolotti, 2000; Lavarco et al., 2022) as well as reward processing and response inhibition (Bechara et al., 2000; Fan et al., 2003; Krawczyk, 2002). OFC dysfunctions play a pivotal role in the pathophysiology of ASB (Séguin, 2004) and related personality traits, including aggression, disinhibition, impulsivity, lack of insight, and sense of guilt (Blair & Cipolotti, 2000; Funayama et al., 2019; Gansler et al., 2009; Grafman et al., 1996; Hofhansel et al., 2020; Raine et al., 2000; Rolls et al., 1994). In addition, converging evidence indicates that the OFC is critically implicated in the ontogeny of SUD (Dom et al., 2005), with particular reference to craving and continued drug use despite harmful consequences and relapse (Goldstein & Volkow, 2002; Yücel et al., 2004).

Transcriptomics analyses have already been performed in MDD human samples. Thus, I performed these analyses in OFC from ASB human samples, since this is the area primarily correlated with aggression and impulsivity.

MATERIAL AND METHODS

ASB SAMPLES:

OFC tissues were obtained from the NIH NeuroBioBank (NBB) Brain and Tissue Repository (BTR) at the University of Pittsburgh. The OFCs were harvested from right hemisphere of each brain blocked coronally, immediately frozen, and stored at -80°C in accordance with the policies and procedures utilized by the BTRs participating in the NIH NBB. Analyses were conducted in 27 post-mortem samples belonging to 3 experimental groups (n=9 for each group): CTL group (subjects without history of psychiatric disorders); SUD group (subjects with SUD diagnoses, since it is unlikely to find patients with ASB diagnoses without SUD, due to its high comorbidity); ASB group (subjects with diagnoses of ASB and SUD). The transcriptomic analyses were performed in post-mortem OFC tissue and these results were analyzed together with the clinical data, including age, sex, brain pH, and post-mortem interval (PMI) for age- and sex-matched cohorts of nine individuals.

RNA EXTRACTION AND SEQUENCING:

RNA was extracted from 30 mg of brain tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) in an elution volume of 40 μl of RNase-free water. RNA purity was assessed by the A260/A280 absorbance ratio using NanoDropTM spectrophotometer (Thermofisher Scientific). RNA extracted from OFC samples was sequenced using NovaSeq Reagent Kit v1.5_150x150 bp (100 M read-pairs) Sequencing (Illumina ® Inc, San Diego CA, USA), and sequencing libraries were prepared using Illumina TruSeq Stranded Total RNA Library Prep Ribo-Zero Gold kit (Illumina ® Inc, San Diego, CA, USA) at the High Throughput Genomics core (Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA).

TRANSCRIPTOMICS:

Libraries were sent to Tgen (The Translational Genomics Research Institute, Phoenix, AZ, USA) where transcriptomics analyses were performed as follows. Raw reads were aligned using STAR v2.7.5b (Dobin et al., 2013), and the count table was generated using the function featureCounts, as implemented in the R-package subread (Liao et al., 2014). Read mapping was assessed using MultiQC v1.12 (Ewels et al., 2016), and samples with less than 60% of uniquely mapped reads were removed before downstream analysis. Raw counts were then imported on DESeq2 (Love et al., 2014), and after removing genes with less than ten total counts, data were transformed with the variance stabilizing method (Anders & Huber, 2010) before running principal components analysis (PCA). Outliers were defined as samples with PC1 or PC2 values above plus or minus four standard deviations from the mean in the first round of PCA. The relationship between confounding factors (RNA integrity number-RIN, PMI, and age) and gene expression was conducted by correlating the top two principal components using Pearson's r. Raw counts were normalized using DESeq2, and differential expression analysis was conducted for all three pairwise comparisons, including the covariates sex, RIN, and PMI. P values were adjusted for multiple testing using the False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995). Genes with adjusted-$p < 0.05$ were considered statistically significant differentially expressed genes (DEGs). Pathway analysis was conducted on the DEGs using clusterProfiler R-package (Yu et al., 2012), referencing to the Gene Ontology database, and adjusting the p-values with the FDR method. Cell-specific expression enrichment was computed across the DEGs using the cell-specific gene markers obtained according to the workflow described in previous studies (Piras et al., 2022). The enrichment test was conducted by hypergeometric statistics using the function

enrichment from the R-package bc3net (de Matos Simoes & Emmert-Streib, 2012). We conducted coexpression analysis using Multiscale Embedded Gene Co-expression Network Analysis (MEGENA), which has been demonstrated to outperform existing methods such as WGCNA (Song & Zhang, 2015). The matrix of raw counts was filtered, excluding genes with less than ten total counts, and normalized by the voom method (Law et al., 2014). Then, 50% of the genes with lower median absolute deviation (MAD) were excluded. Coexpression network generation was conducted by means of the MEGENA R-package (Song & Zhang, 2015) according to the following workflow. First, signed pairwise gene correlations were conducted using Pearson's method with 1,000 permutations, retaining correlation significant at the 5% FDR level (function: calculate.correlation). Then, significantly correlated gene pairs (FDR < 0.05) were ranked and iteratively tested for planarity to grow a Planar Filtered Network using the Planar Maximally Filtered Graph (PMFG) algorithm (function: calculate.PFN). Finally, we conducted a multiscale clustering analysis to identify coexpression modules at different network scale topology, as well as their hub genes (function: do.MEGENA). Significant coexpression modules (permuted $p < 0.05$) were retained for downstream analysis. We extracted module eigengenes (the first principal component of the genes module) using the function moduleEigengenes from the WGCNA R-package (Langfelder & Horvath, 2008), and we computed pairwise differential expression by means of the limma R-package (Ritchie et al., 2015), adjusting for age, PMI and RIN. Significantly associated modules were annotated for cell-specific enrichment and GO functional classes using the same workflow as described for differentially expressed genes. Relevant coexpression modules were represented using the R-package ggnet.

RESULTS

TRANSCRIPTOMIC ANALYSIS OF HUMAN SAMPLES OF ASB:

All the 27 samples derived from male subjects belonging to three experimental groups: 1) CTL, with no diagnoses; 2) SUD, with diagnoses of Substance Use Disorder, used as an internal control, and 3) ASB group, with diagnoses of Substance Use Disorder and ASB. Subjects had an average age ± standard deviation of 43.1 ± 9.9 years (range: 21 – 59 years old). Average PMI was 18.1 ± 6.6 hours (range: 7.1 – 31 hours). Average RIN was 7.8 ± 1.04 (range: 5.2 – 9.3). Only one sample displayed a RIN value lower than 6. We sequenced all 27 samples for a total of 755.8 M reads (average: 28.0 M; range: 20.5 M – 36.0 M). The average percentage mapping rate (uniquely mapped reads) was 70.4% (range: 45.3% - 78.7%) (Fig. 3). Thirteen samples out of 27 (48.1%) showed a mapping rate > 70.0%, and twenty-four samples (88.9%) showed a mapping rate > 65%. All the samples, but one, had a mapping rate larger than 60%. The sample with the lowest mapping rate (45.3%) was excluded from the downstream analysis, being also the sample with RIN < 6. Additionally, the same sample was also a significant outlier (Fig. 4A). After the removal of that sample, no further outliers were identified (Fig. 4B). We assessed the relationship of the gene expression with the confounding factors (PMI, age, and RIN) using the two top PCs, by Pearson's r. We detected

Figure 3

Figure 4A

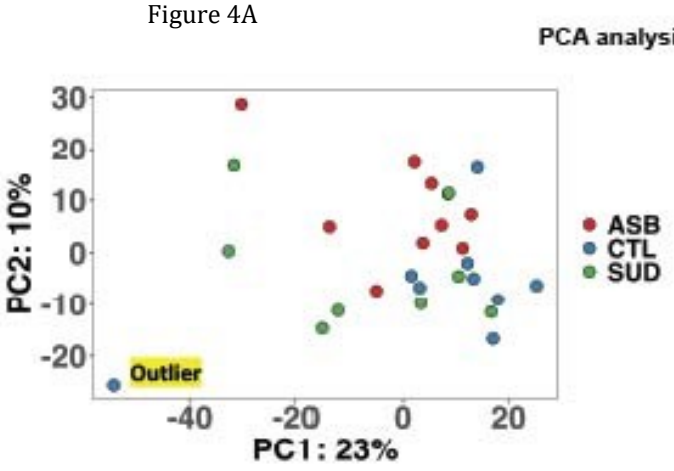


Figure 4B

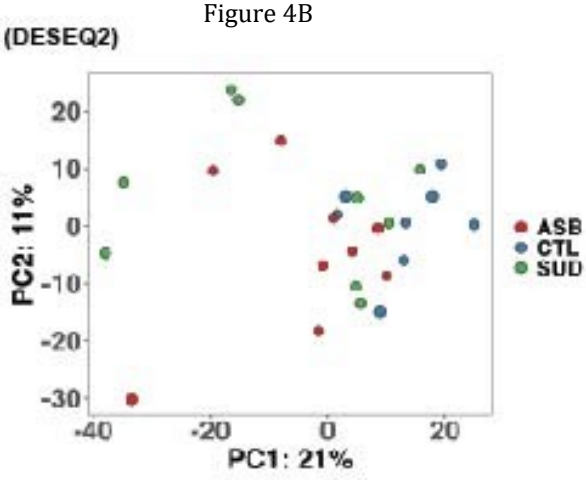


Figure 5

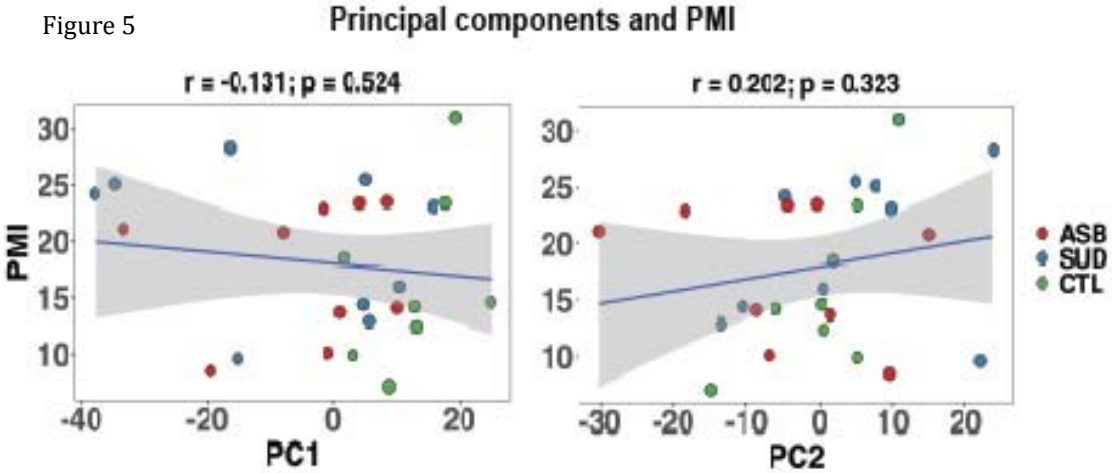


Figure 3. Mapping rate.
Figures 4A-B. Identification of one significant outlier.
Figure 5. Correlation between PMI and gene expressions.

a significant correlation between at least one of the PCs with age (p = 0.038) and RIN (p =

1.4-03). No significant correlation was observed between PCs and PMI ($p \geq 0.323$). (Figs 5, 6, and 7). We conducted differential expression analysis adjusting for all three confounding factors. The first pairwise comparison (ASB versus CTL) showed 276 DEGs (Fig. 8). The second comparison (ASB versus SUD) yielded only two DEGs, both downregulated in ASB: SEMA7A and MICAL2 (Fig. 8). Finally, the third comparison (SUD versus CTL) did not show any DEG (Fig. 8). We further explored the results for the comparison with more genes (ASB versus CTL; n = 276 DEGs). The heatmap in Fig. 9, generated from the DEGs using the Euclidean distance with the Manhattan clustering method, showed a clear separation

Figure 6

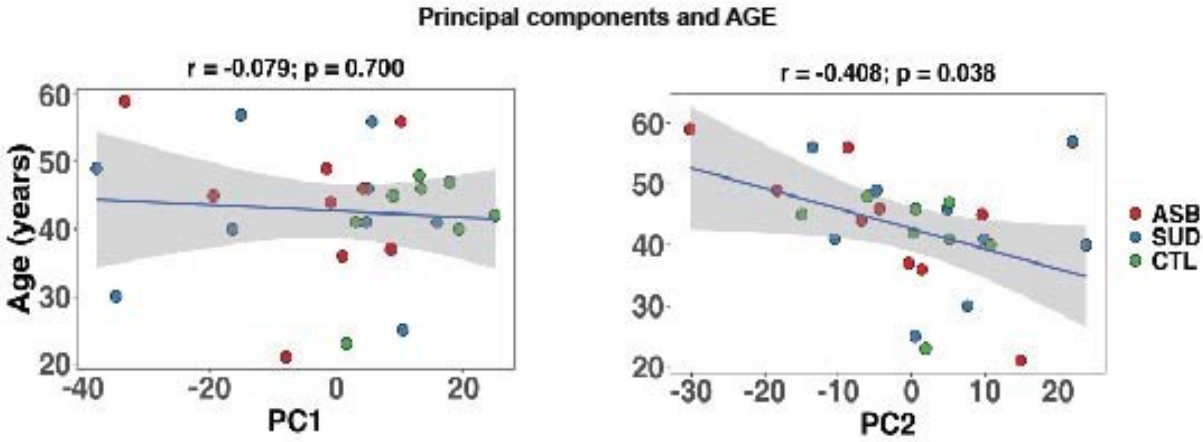


Figure 7

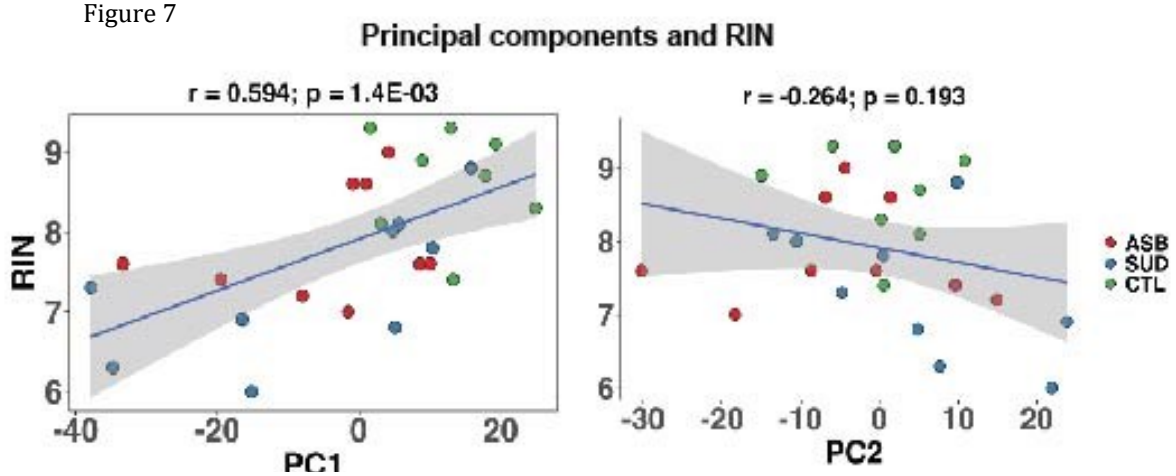


Figure 6. Correlation between age and gene expressions.

Figure 7. Correlation between RIN and gene expressions.

between ASB and CTLs. These analyses did not show relevant alterations in gene expression of SRDA5A1 and SRDA5A2. Interestingly, gene expression analyses in ASB as well as MDD did not show alteration of SRD5A1 expression and, since SRD5A2 was undetectable, its role remains elusive.

Figure 8

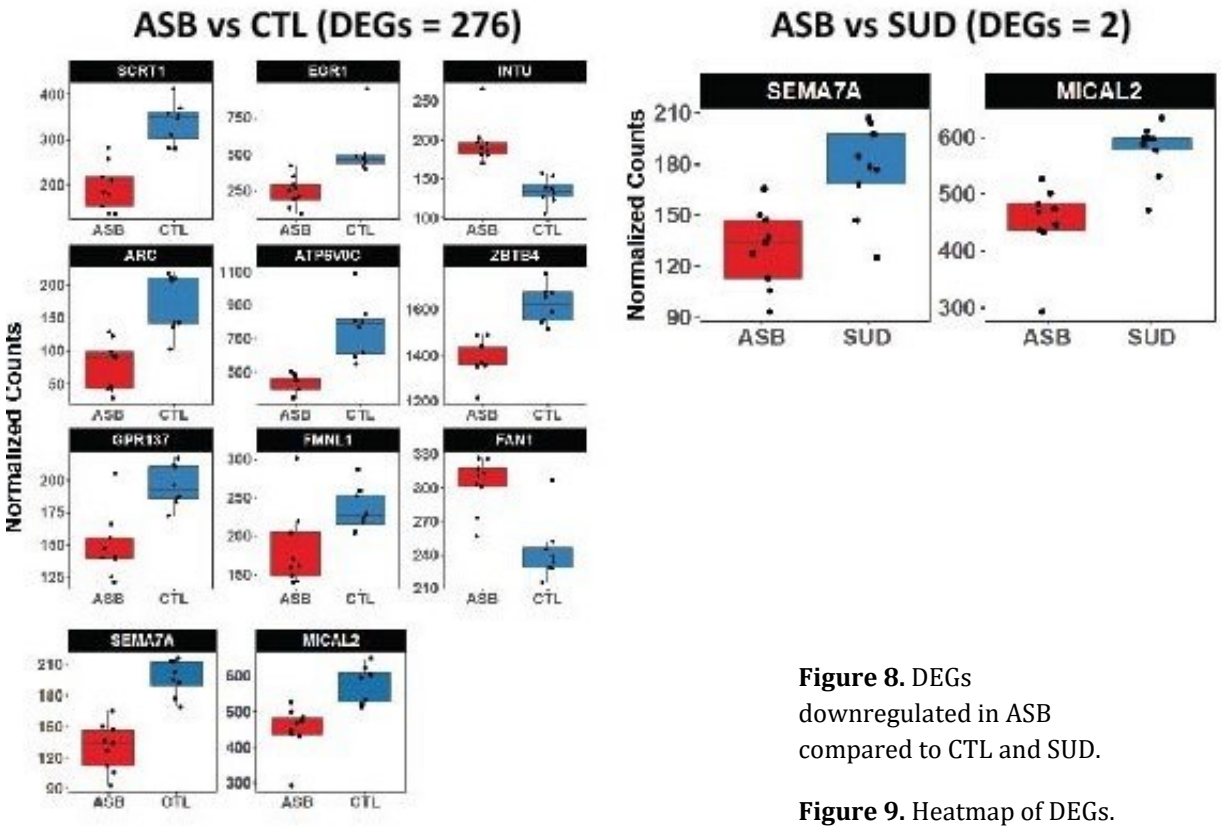
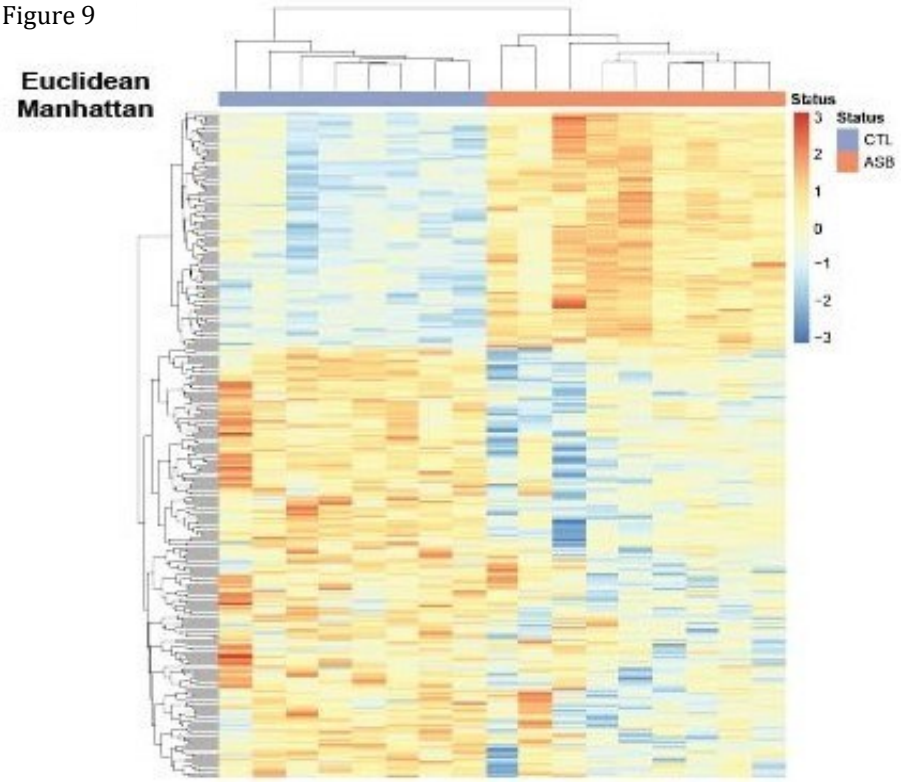


Figure 8. DEGs downregulated in ASB compared to CTL and SUD.

Figure 9. Heatmap of DEGs.

Figure 9



DISCUSSION

To our knowledge, this is the first unbiased and comprehensive characterization of gene expression changes in postmortem OFC samples from antisocial subjects. In line with epidemiological evidence showing an extremely high prevalence of SUD in individuals with lifetime diagnoses of ASPD and CD, all the subjects in the ASB group had an history of substance use (including alcohol). Thus, they were compared with two different sets of matching controls from the same brain bank: SUD-only and unaffected individuals.

The comparison of OFC samples from ASB+SUD and unaffected matched controls revealed the differential expression of 276 genes. The list of the genes identified by our analyses did not substantially overlap with other sets of genes of interest in ASB or externalizing behavior, as highlighted by GWAS (Linnér et al., 2021; Tielbeek et al., 2017). We were also unable to detect transcriptomic changes akin to those identified in induced pluripotent stem cells (iPSC)-derived cortical neurons and astrocytes obtained from psychopathic subjects (Tiihonen et al., 2019). However, while psychopathic individuals engage in extreme manifestations of ASPD, they should not be regarded as representative of the whole ASB spectrum. Even with these limitations, our analyses pointed to the downregulation of some genes previously implicated in traits related to ASB and SUD. For example, we detected a reduced expression of GABRA2 transcript (data not shown). This molecule has been repeatedly implicated in the ontogeny of ASB (Deak et al., 2019), alcohol use disorder (D. Li et al., 2013), and externalizing behavior (Dick et al., 2010; Karlsson Linnér et al., 2021). We also identified the downregulation of several genes implicated in ubiquitination and proteasomal degradation, including UBQLN4 (the gene encoding ubiquitin 4), UBA1 (which

catalyzes the first step in ubiquitin conjugation), RAD23A (which delivers ubiquitinated proteins to the proteasome), ADRM1 (a component of the 26S proteasome, which degrades ubiquitinated proteins), and PSMC3 (a regulator subunit of the 26S proteasome) (data not shown). The ubiquitin/proteasome pathway allows for the degradation of misfolded or accumulated proteins (Lecker et al., 2006); importantly, impairments of this machinery have been implicated in the pathophysiology of other developmental conditions associated with social deficits, such as autism-spectrum disorder (Kasherman et al., 2020). Future studies will be needed to assess the implication of ubiquitination in the pathophysiology of ASB and externalizing behavior.

GO analyses revealed significant downregulations of genes associated with excitatory neurons (which, in the context of the cortex, correspond to glutamatergic pyramidal neurons) and glutamatergic synapses, spines, and neurotransmitter release. GO analyses revealed significant downregulations in 32 GO classes, but no upregulation. The top downregulated cellular component (CC) pathways were "dendritic spine" (GO:0043197), "neuron spine" (GO:0044309), "glutamatergic synapse" (GO:0098978), "transport vesicle" (GO: 0030133), "cation channel complex" (GO:0034703), "postsynaptic density" (GO:0014069), and "asymmetric synapse" (GO:0032279). The top downregulated biological process (BP) pathways were "modulation of chemical synaptic transmission" (GO:0050804), "regulation of trans-synaptic signaling" (GO:0099177), "regulation of neurotransmitter receptor activity" (GO:0099601), and "regulation of neuronal death" (GO:1901214) (data not shown). These results suggest that the combination of ASB and SUD is characterized by glutamatergic deficits. Given that pyramidal neurons are responsible for the functional output of the cortex, and their dysfunction corresponds to functional deficits of this brain

region, these data suggest that antisocial individuals with comorbid SUD feature an impairment in the output of the OFC. These data align with the well-established concept that functional deficits in the OFC lead to several traits directly connected with ASB and SUD, including aggression and several impulsivity constructs (Kuniishi et al., 2016; Mobini et al., 2002; Torregrossa et al., 2008). Indeed, externalizing behavior is negatively associated with cortical thickness in the OFC (Ameis et al., 2014).

MEGENA identified 23 downregulated modules and 5 upregulated modules in ASB+SUD. Of the downregulated modules, the largest module by size, M81, was characterized for cellular components related to the glutamatergic synapse with a cell enrichment in excitatory neurons and a hub on SBCB (data not shown). This gene encodes β-synuclein, a protein with neuroprotective functions (Hashimoto et al., 2001) that inhibits apoptosis and enables proteasomal activity (Hayashi & Carver, 2022). Thus, the downregulation of this transcript in excitatory neurons may signify an increased degeneration of pyramidal cells. This interpretation is consistent with a general functional reduction of the projection neurons of the OFC.

The fact that both DEG and MEGENA point to deficits of pyramidal neurons strongly supports the possibility of an impairment in the output of the OFC in externalizing disorders. Ample evidence has shown that the OFC is critical to enable reasoning related to empathy and theory of mind (Adolphs, 2001; Happé et al., 2001; Heinrichs, 1989; Schoenbaum et al., 2000; Stone et al., 2003), and decision making in relation to salience and craving (Goldstein & Volkow, 2002). Evidence has also shown that OFC focal lesions lead to disinhibition, impulsivity, lack of insight, lack of concern with the consequences of one's actions,

irresponsibility, social inappropriateness, and deficits in social behavior interpretations (Grafman et al., 1996; Rolls et al., 1994). In line with this perspective, dysfunctions of the OFC have been associated with the pathophysiology of ASB (Séguin, 2004) and SUD (Dom et al., 2005). Our results point to the possibility that specific glutamatergic deficits may be responsible for these impairments. Future proteomic, functional, and histological studies will be necessary to verify whether ASB is associated with decreased functions or numbers of pyramidal cells in the OFC, and what specific trait components may be associated with specific transcriptomic signatures.

The comparison between ASB+SUD and SUD-only samples revealed differences only in the transcripts of two genes, SEMA7A and MICAL2, the expression of which was also significantly reduced in ASB+SUD subjects compared to unaffected controls. The proteins encoded by these two genes are abundantly expressed in the cortex, as documented by the Human Protein Atlas portal (www.proteinatlas.org). SEMA7A (Semaphorin 7A) is a protein anchored to the membrane via a glycosylphosphatidylinositol (GPI) linkage. The semaphorin family includes more than 20 members, falling into 8 classes (Wannemacher et al., 2011), which bind to plexins to promote their dimerization for signal transduction (Janssen et al., 2010; Nogi et al., 2010; Wannemacher et al., 2011). In turn, the activation of plexins regulates multiple functions, such as synaptic neurotransmission. In particular, the interaction of SEMA7A with either Plexin C1 or an RGD-dependent α1β1-integrin (Pasterkamp et al., 2007) promotes axon growth and guidance (Pasterkamp et al., 2003). Animal experiments have shown that the deficiency of SEMA7A leads to neurodevelopmental problems, such as a defective olfactory tract (Pasterkamp et al., 2003) and the disruption of cortical architecture, with misorientation of dendritic arbors and imbalances of excitatory and inhibitory

connectivity (Carcea et al., 2014). MICAL2 (Microtubule associated monooxygenase, calponin and LIM domain containing 2) is a nuclear enzyme that oxidizes specific methionine residues in F-actin, resulting in its depolarization and disassembly (Lundquist et al., 2014). In general, MICALs regulate microtubule dynamics (Alto & Terman, 2017; Hung et al., 2010; Hung & Terman, 2011) by phosphorylating collapsin responsive mediator proteins (CRMPs) (Hota & Buck, 2012; Pasterkamp et al., 2003; Schmidt et al., 2008; Terman et al., 2002; Vikis et al., 2000). Notably, the activity of MICALs is regulated by plexins, and their interactions have been shown to transduce semaphorin signaling into axon guidance (Schmidt et al., 2008). Taken together, this background suggests that the downregulation of SEMA7A and MICAL2 transcripts documented in antisocial individuals may lead to overlapping deficits in plexin functions and dysregulated synaptic signaling and neurite formation in the OFC. In line with this idea, previous evidence has shown a thinning of the gray matter in the OFC of antisocial individuals (Carlisi et al., 2020; Raine et al., 2000).

Surprisingly, the comparisons of SUD-only and unaffected subjects revealed no significant differences, likely due to the high heterogeneity of substances used in the first group (reflecting different mechanisms of action and molecular outcomes). Thus, the lack of significant differences between these two groups lends support to the possibility that most of the changes identified in the comparison between ASB+SUD and unaffected individuals may primarily reflect ASB-related mechanisms.

The main limitations of this study are the relatively limited number of brain samples analyzed, the lack of ASB-only samples, of female subjects, and the unavailability of diagnostic details on the specific behavioral traits of our subjects. These limitations are

largely due to the extremely low availability of brain samples from antisocial individuals, which was made possible in our case only thanks to the establishment of greater repository systems such as that offered by the NBB system in the US. Future concerted efforts are needed to increase the availability of specimens with more targeted analyses to confirm and expand the present results. As more postmortem brain samples of antisocial individuals become available, we expect that future studies will enable the recognition of more DE transcript associated with ASB and, likely, in relation to specific behavioral traits associated with antisocial conduct.

In summary, the convergent results from DGE and coexpression network analyses of the present transcriptome data strongly implicates dysfunctional glutamatergic signaling and synaptic regulation in the OFC of individuals with comorbid ASB and SUD. These data are particularly interesting, given that previous research has already pointed to glutamate receptors as critical mediators of aggressive behavior in animal models reproducing gene x environment interactions in ASB (Godar et al., 2019). Overall, these results point to the implication of glutamate in the ontogeny of ASB and related behaviors and underscore the possibility that strategies aimed at normalizing glutamate signaling in the OFC may offer new opportunities to manage ASB. Animal models may be particularly relevant to help us decipher the molecular mechanisms underlying glutamatergic impairments in the OFC of antisocial individuals.

The results of transcriptomic analyses in ASB, as those of previous studies in MDD, did not reveal any alterations in terms of SRD5A1 mRNA levels but the role of SRD5A2 is still unclear due to its undetectability in transcriptomics analyses. As previously pointed out, this is

probably due to the highly restricted 5αR2 cell localization that does not allow to extract a sufficient amount of mRNA molecules from post-mortem tissues. Anyhow, 5αR2 is well known to be present in CNS (Melcangi et al., 2021; Reddy, 2013).

Hence, the best approach to investigate whether 5αR1 and 5αR2 expression levels are modified in the brain is via protein analyses. Western blot analyses of 5αR1 and 5αR2 in ASB and MDD subject were then the second goal of this project.

CHAPTER 4
EVALUATING THE ROLE OF 5ARS VIA PROTEIN ANALYSES IN ASB AND MDD

OVERVIEW CHAPTER 4:

In view of the difficulty in detecting SRD5A2 by transcriptomics analyses, I then focused on the investigation of 5αR1 and 5αR2 protein expression levels in ASB and MDD human samples.

ASB and MDD are two disorders which often share a stressful environment during childhood or across the entire lifespan as a cause of onset. In particular, MDD is one of the most prevalent and debilitating psychiatric disorders. In general, about 1 out of every 6 adults will have depression at some time in her/his life, and depression affects about 16 million American adults every year (National Institute of Mental Health). Characterized by impairments in cognition, emotional regulation, memory, motoric function, motivation, and neurovegetative symptoms, MDD can cause severe disability (Otte et al., 2016). In addition to its primary effects, the disorder also causes secondary disability, as patients with depression are more likely to develop chronic medical illnesses. The combination of the primary disability caused by depression and the secondary disability of chronic medical illness makes MDD one of the more costly medical burdens in the world (Proudman et al., 2021). MDD is more prevalent among women than men (13-26% and 7-14% respectively) and its age of onset is around 30 years old (Hasin et al., 2018).

MDD is diagnosed based on symptomatic criteria set forth in the Diagnostic and Statistical Manual of Mental Disorders - DSM-V (American Psychiatric Association, 2013) (Tab. 2).

Table 2	Diagnostic Criteria for Major Depression
	Depressed mood
	Irritability
	Low self esteem
	Feelings of hopelessness, worthlessness and guilt
	Decreased ability to concentrate and think
	Decreased or increased appetite
	Weight loss or weight gain
	Insomnia or hypersomnia
	Low energy, fatigue, or increased agitation
	Decreased interest in pleasurable stimuli
	Recurrent thoughts of death and suicide
see DSM V	

Table 2. Diagnostic criteria for MDD.

According to the variety of symptoms, depression should be viewed as a heterogeneous syndrome comprised of numerous diseases with distinct causes and pathophysiology. Attempts have been made to establish subtypes of depression defined by certain sets of symptoms. These subtypes include melancholic depression, reactive depression, psychotic depression, atypical depression, dysthymia. One of the most common and severe symptoms is anhedonia. This term refers to the reduced ability to experience pleasure. The presence of anhedonia is considered to be a core feature of MDD. Animal models of depression, in fact, are basically built upon an anhedonic phenotype.

Depression has also been shown to be associated with structural alteration and impaired synaptic plasticity (Yang et al., 2020). This evidence points out the role of brain-derived neurotrophic factor (BDNF) in the neurobiology of depression. BDNF is one of the most

important neurotrophic factors which plays a key role in the maintenance and survival of neurons and in synaptic plasticity (Gottmann et al., 2009). Indeed, alterations in levels of BDNF have been shown in depressed patients (Dwivedi, 2010; Yang et al., 2020).

The monoamine hypothesis is crucial for the pathophysiology of depression, and it postulates that depression is originated by a depletion in the levels of one or more of the monoamines in CNS, specifically serotonin (5HT), norepinephrine (NE) and dopamine (DA). From 1950s, when this hypothesis has been postulated, drugs able to increase the brain concentration of monoamine have been investigated (Hirschfeld, 2000). So far, the drugs most commonly used for the treatment of depression are selective or non-selective reuptake inhibitors of 5HT, NE or DA (Tab. 3). Recently, also ketamine (a non-competitive antagonist

Table 3	List of antidepressants
Drug Class	**Mechanism of action**
Selective serotonin reuptake inhibitors (SSRI)	Selectively inhibit the reuptake of 5HT
Serotonin-norepinephrine reuptake inhibitors (SNRI)	Inhibit reuptake of both 5HT and NE, weakly inhibit DA reuptake
Moniamine oxidase inhibitors (MAOI)	Competitively inhibit MAO (differ in their reversibility and their activity against MAOa and MAOb
Serotonin modulators	Selective inhibitor of 5HT reuptake; also act as 5HT2 antagonist
Dopamine-norepinephrine reuptake inhibitors	Inhibit DA and NE reuptake
Noradrenergic and specific serotonergic antidepressant	Block presynaptic α2 autoreceptors and post-synaptic 5HT2-5HT3 receptors
Tricyclic antidepressant (TCA)	Inhibit reuptake of NE and 5HT

Table 3. List of more common antidepressants.

at glutamate N-methyl-D-aspartate (NMDA) receptors, traditionally used as a dissociative anesthetic) has been reported to have antidepressant properties with rapid onset, probably involving NMDA and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazoleprionic acid) receptors as well as downstream activation of BDNF to potentiate synaptic plasticity

(Matveychuk et al., 2020). Additionally, several forms of psychotherapy could be effective. However, it could happen that there is no inversion of the mood state and consequently a therapeutic failure. Since the treatment of depression should be built on rational approaches involving the understanding of pathophysiology, a better characterization of the neurobiological mechanism involved in the onset of depression is still necessarily required.

Targeting the neurosteroid system may represent a novel therapeutic strategy for the treatment of depressive disorders. There is considerable preclinical and clinical evidence that neuroactive steroids are fundamental endogenous modulators of mood, and they could adjuvate the effect of antidepressants typically used in clinic (Eser et al., 2014). Brexanolone, an intravenous formulation of allopregnanolone, is the only treatment specifically approved for postpartum depression by the FDA (Epperson et al., 2023). Clinical and therapeutical experiences, where allopregnanolone (AP) and other neurosteroids (such as DHEA, dehydroepiandrosterone) were associated with conventional antidepressants, have revealed a higher therapeutic effect (Longone et al., 2007; van Broekhoven & Verkes, 2003). Also, treatment with some antidepressants is associated with increases in AP levels (Zorumski et al., 2013). It is now clear that there is a link between neurosteroids and antidepressant effects, even though the specific connection is still unknow. We can speculate that this association is represented by the 5αR enzymes, since they are required for the synthesis of neurosteroids. Over and above that, this hypothesis could help to clarify why women are more susceptible than man to depression: since neurosteroids levels are altered by ovarian cycle and hormonal changes, women could be more susceptible to stressful stimuli and develop a depressive state more easily than a man. Sex differences in expression

levels of 5αR in human, may underlay the different vulnerability to the effect of stress and the higher risk of women to develop depression.

It is likely that many brain regions intervene in the symptomatology of depression. This is supported by human brain imaging and anatomical studies, which demonstrate that the major brain areas implicated in depression are the PFC, OFC, AAC, hippocampus (HIPPO), striatum (STR), AMY, and thalamus (TH) (Liotti & Mayberg, 2010; Manji et al., 2001; Rajkowska, 2000; Zhu et al., 1999). It may be speculated that cortices and hippocampus could mediate cognitive aspects such as memory impairment, suicidality and feelings of worthlessness, guilt, doom; conversely, the striatum and amygdala could mediate anhedonia, anxiety, and reduced motivation. In particular, reduced activation of OFC, ACC, and NAc has been observed in MDD subjects (Pizzagalli & Roberts, 2021). This suggests an impairment in the reward-stimuli pathway which can ultimately lead to anhedonia or anhedonic-like symptoms.

Since 5αRs are the rate-limiting step for the synthesis of neurosteroids, I measured the expression levels of 5αR1 and 5αR2 in the OFC, AAC, NAc, AMY, and HIPPO of post-mortem samples of MDD patients. I also evaluated the expression levels of these enzymes in samples of ASB subjects, to further investigate the role of 5αRs in both psychopathologies.

MATERIAL AND METHODS

ASB HUMAN SAMPLES:

OFC tissues were obtained from the NIH NeuroBioBank (NBB) Brain and Tissue Repository (BTR) at the University of Pittsburgh. The OFCs (defined by the medial orbital gyrus) were harvested from the right hemisphere of each brain blocked coronally, immediately frozen, and stored at -80°C in accordance with the policies and procedures utilized by the BTRs participating in the NIH NBB. The analysis was performed in post-mortem OFC tissue and associated clinical data, including age, sex, brain pH, and PMI for age- and sex-matched cohorts of nine individuals. Analyses were conducted in 27 post-mortem samples belonging to 3 experimental groups (*n*=9): CTL group (subjects without history of psychiatric disorders); SUD group (subjects with Substance Use Disorder diagnoses, since it is unlikely to find patients with ASB diagnoses without SUD due to its high comorbidity); ASB group (subjects with diagnoses of ASB and SUD) (Tab. 4).

MDD HUMAN SAMPLES:

OFC, ACC, NAc, AMY and HIPPO tissues were obtained from the NIH NBB BTR at the University of Pittsburgh. Tissues were harvested from the right hemisphere of each brain, dissected in the coronal plane, immediately frozen and stored at -80°C in accordance with the policies and procedures utilized by the BTRs participating in the NIH NBB. The analysis was performed in post-mortem tissue and associated clinical data including age, sex, brain pH and PMI for age- and sex-matched cohorts of 7 individuals with MDD diagnoses and 7 individuals with no history of psychiatric disease (Tab. 5).

Table 4. Demographic characteristic of ASB subjects.

Group	ID	Age	Ethnicity	Manner of Death	SUD diagnoses	ASB diagnoses	Other comorbid disorders
Unaffected Controls	A1	48	White	Accidental	/	/	/
	A2	53	White	Natural	/	/	/
	A3	40	White	Natural	/	/	/
	A4	23	Black	Accidental	/	/	/
	A5	42	White	Natural	/	/	/
	A6	46	White	Accidental	/	/	/
	A7	45	White	Natural	/	/	/
	A8	47	Black	Natural	/	/	/
	A9	41	White	Natural	/	/	/
SUD only	B1	57	White	Natural	Alcohol (S); tobacco (M); Opioids (M); Cocaine (M)	/	/
	B2	30	Black	Natural	Alcohol (S); Sedatives (Mi)	/	Anxiety disorder
	B3	41	Black	Natural	Alcohol (Mi)	/	Seizure disorder
	B4	40	White	Accidental	Alcohol (Mi); Cocaine (Mi); Opioids (Mi)	/	/
	B5	49	White	Pending	Alcohol (Mi); Cocaine (S); Cannabis (M)	/	/
	B6	56	White	Accidental	Alcohol (M); Tobacco (M); opioids (Mi)	/	/
	B7	41	White	Natural	Alcohol (M); Cannabis (M); Tobacco (M)	/	/
	B8	25	White	Accidental	Alcohol (Mi); Cocaine (Mi); Hallucinogens (Mi)	/	/
	B9	46	White	Accidental	Amphetamines (M); Tobacco (M); Cannabis (Mi)	/	/
SUD+ASB	C1	45	White	Natural	Alcohol (S); Opioids (M); Sedatives (M)	ASPD	/
	C2	37	White	Accidental	Alcohol (M); Cocaine (Mi); Cannabis (Mi)	CD (childhood onset)	Dysthymia
	C3	59	White	Accidental	Alcohol (S); Opioids (Mi)	CD (adolescence onset)	Intermittent explosive disorder
	C4	49	White	Natural	Alcohol (S); Tobacco (S); Sedatives (M); Opioids (M); Amphetamines (Mi)	ASPD	Unspecified depressive disorder Borderline personality disorder
	C5	44	Black	Accidental	Alcohol (S); Cocaine (S); Cannabis (M); Opioids (Mi)	ASPD	Major depressive disorder, Single episode
	C6	46	Black	Accidental	Cannabis (M); Alcohol (Mi); Opioids (Mi); Sedatives (Mi); Cocaine (Mi)	ASPD	/
	C7	21	White	Suicide	Cannabis (Mi)	CD (adolescence onset); ASPD	Major depressive disorder, Single episode; learning disorders; Borderline personality disorder
	C8	36	Black	Accidental	Tobacco, opioids (S); Cannabis (M); Alcohol (Mi)	CD (unspecified onset); ASPD	Unspecified depressive disorder; Gambling disorder; ADHD; trauma and stress-related disorder
	C9	56	White	Accidental	Opioids, Tobacco (S); Alcohol (M); Cocaine (Mi)	CD (unspecified onset)	Disruptive, impulse-control disorder

Acronyms: ADHD: Attention deficit hyperactivity disorder; ASB: antisocial behavior; ASPD: antisocial personality disorder; CD: conduct disorder; M: moderate; Mi: mild; S: severe; SUD: substance use disorder

Table 5. Demographic characteristic of MDD subjects.

Subject	Age	Sex	Official Psychiatric Diagnosis	HAM-D31 - Hamilton Rating Scale for Depression
69	53	Female	296.30-Major Depressive Disorder	18
87	47	Male	296.30-Major Depressive Disorder; 300.21-Panic Disorder with Agoraphobia	7
147	75	Male	296.30-Major Depressive Disorder	29
153	52	Female	Normal Control, No Diagnosis	0
162	54	Female	Normal Control, No Diagnosis	Lost contact with NOK- NO NOK Interview
192	73	Male	Normal Control, No Diagnosis	2
194	63	Female	Normal Control, No Diagnosis	4
198	52	Female	296.32-Major Depressive Disorder; 300.01-Panic Disorder Without Agoraphobia	41
207	74	Male	Normal Control, No Diagnosis	2
216	23	Female	309.28-Adjustment Disorder with Mixed Anxiety and Depressed Mood	0
228	60	Female	296.23-Major Depressive Disorder; 301.40-Obsessive-Compulsive Personality Disorder	13
232	57	Male	296.32-Major Depressive Disorder; 300.02-Generalized Anxiety Disorder	4
264	47	Male	Normal Control, No Diagnosis	0
270	53	Male	Normal Control, No Diagnosis	0

IMMUNOBLOTTING:

Samples from human tissues were stored at -80°C until assayed. To analyze the expression levels of 5α1 and 5αR2, tissues were weighted and diluted (10 µg/10 µl) in RIPA buffer containing 20mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, 1 µg/ml leupeptin and protease inhibitor cocktail. Small aliquots of the homogenate were used for protein determination using a modified Lowry protein assay method (DC protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Samples containing 15 µg of total proteins were run in duplicate onto 4-15% Criterion™ TGX Stain-free™ precast gels (Bio-Rad Laboratories) and transferred into nitrocellulose membranes (Bio-Rad Laboratories). Stain-free™ gel formulation includes a trihalo compound that, when exposed to ultraviolet (UV) irradiation, generates a covalent reaction with tryptophan residues of proteins and allows them to be visualized within the gel or after transfer to a blotting membrane. Following protein transfer, the membrane was detected by UV and blot image was collected for total protein. Primary antibodies against 5αR1 (#66329, Proteintech, dilution 1:1000) and 5αR2 (#MA537985, Invitrogen, dilution 1:1000) were incubated in TBS-T containing 3% (w/v) BSA buffer overnight at 4 °C. Next, blots were washed in TBS-T and then incubated in TBS-T containing goat anti-rabbit HRP-conjugated (#31462, Thermo Fisher Scientific; dilution 1:10000) or goat anti-mouse HRP-conjugated (#31430, Thermo Fisher Scientific; dilution 1:5000) secondary antibodies, for 90 minutes at room temperature. Chemiluminescence was detected with the ChemiDoc™ XRS+ Imaging System using the Clarity Western ECL substrate (Bio-Rad Laboratories). Bands were quantified in arbitrary units and normalized using the software Image Lab (Bio-Rad Laboratories).

Samples containing the same amounts of total proteins in each experimental group were run on the same immunoblots and then analyzed together. Membranes were stripped and re-probed with primary antibody anti-β-actin (mouse monoclonal #sc47778 Santa Cruz Biotechnology) to control for equal loading. All data are expressed as mean ± SEM and analyses were performed using GraphPad Prism 8 statistical package (GraphPad, San Diego, CA, USA). Neurochemical data were analyzed via T-Test, and One-way ANOVA in order to determine if the means of the groups were significantly different. Post-hoc comparisons were performed via Tukey's multiple comparisons. Regression and correlation analyses were performed to establish the relationship between variables and the densitometry values resulting from western blot analyses of the experimental samples. Significance was set at $p<0.05$.

RESULTS

5αR1 AND 5αR2 EXPRESSION LEVELS IN HUMAN SAMPLES OF ASB:

One-way ANOVA analysis of 5αR1 revealed no significant differences among the three experimental groups (CTL, SUD, ASB) (F=1.17, p=0.05, n.s.). Analysis by One-way ANOVA of 5αR2 expression levels showed a significant increase in this enzyme levels in the SUD group compared to the CTL group (F=7.97, p= 0.002 **: *post-hoc* Tukey's multiple comparisons test: CTL vs SUD p=0.044 *, SUD vs ASB p>0.05, n.s., CTL vs ASB p>0.05 n.s.) (Fig. 10). These results show that both enzymes are probably not involved in the neurobiology of ASB pathology.

Figure 10

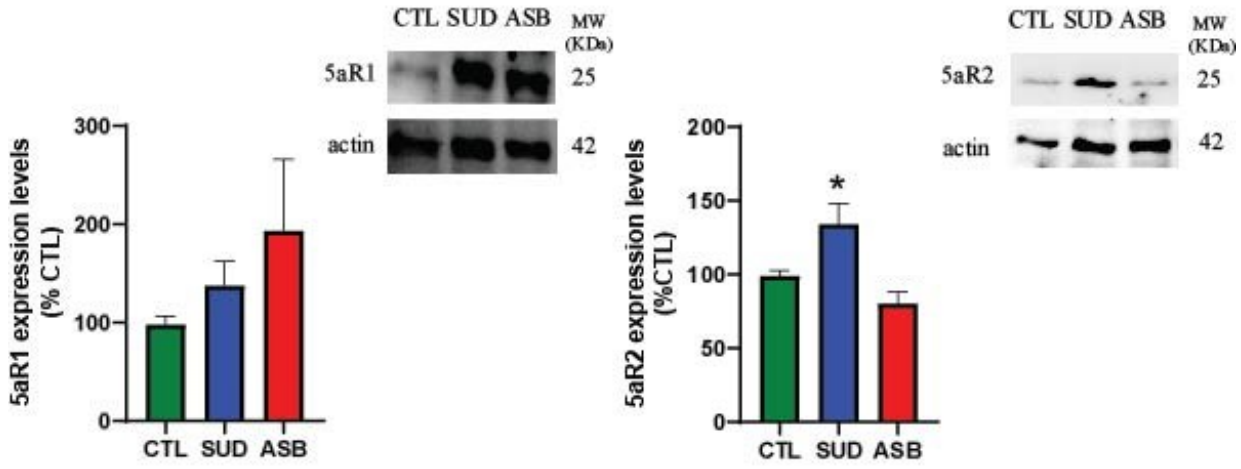


Figure 10. 5αR1 and 5αR2 expression levels in post-mortem OFC samples expressed as mean ± SEM and representative blots.

REGRESSION ANALYSIS:

Univariable regression analysis was used to test whether PMI (expressed in hours) or age significantly modifies brain protein levels. Whenever PMI was associated with protein expression, this index was used as a covariate in multiple regression analyses to assess its effect on potential differences in protein levels across diagnostic groups. Regression analyses did not show statistically significant association between 5αR2 expression and either PMI ($F_{1,25}$=0.04; R^2=0.001; p>0.05 n.s.) or age ($F_{1,25}$=1.30; R^2=0.04; p>0.05 n.s.). Regression analyses of association between 5αR1 expression and PMI, instead, were significantly correlated ($F_{1,25}$=8.18; R^2=0.24; p=0.008**); but no correlation was observed between 5αR1 and age ($F_{1,25}$=0.01; R^2=0.0005; p=0.05 n.s.) (Fig. 11). Since in our samples 5αR1 seems to be highly degraded by post-mortem processes, this element could explain why the expression levels of this enzyme are highly variable in western blot analyses.

Figure 11

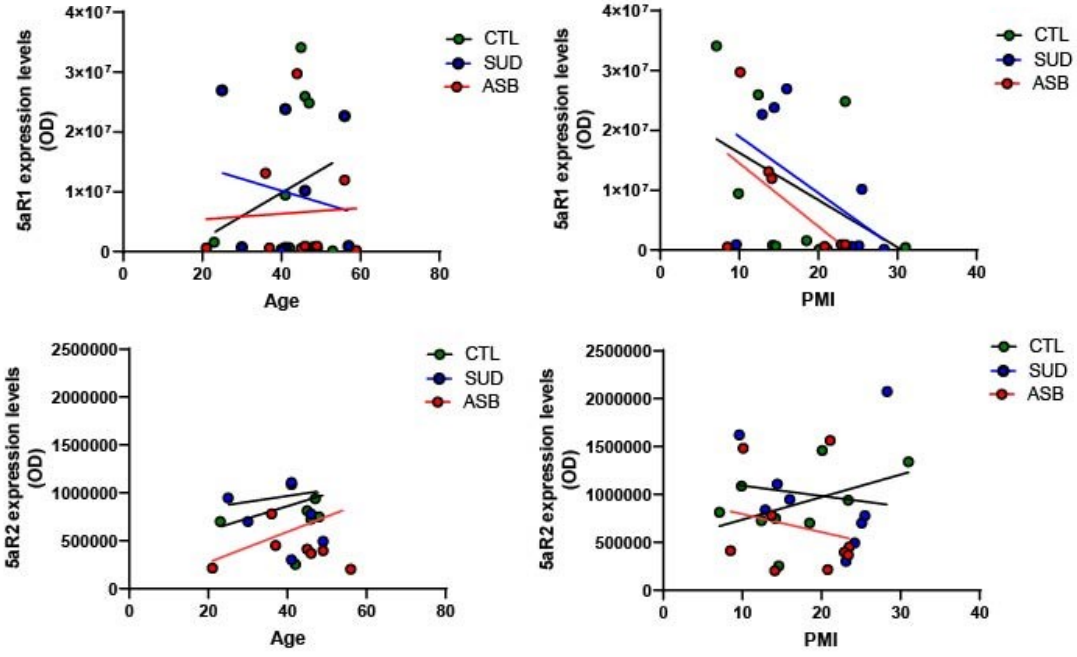


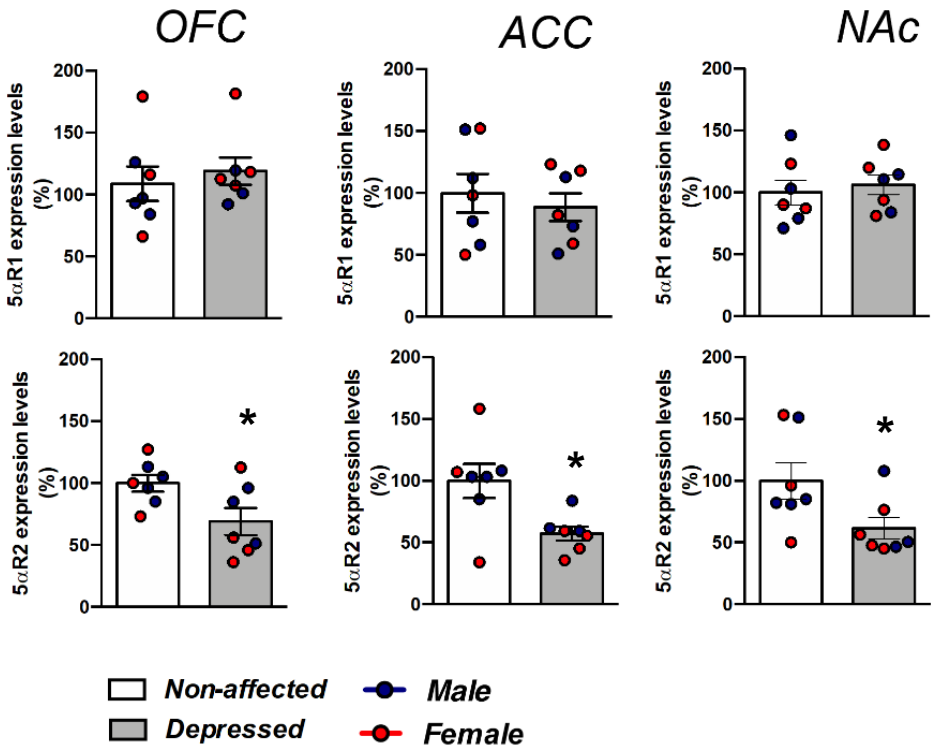
Figure 11. Linear regression between 5αR1 and 5αR2 expression levels expressed and age or PMI.

5AR1 AND 5AR2 EXPRESSION LEVELS IN HUMAN SAMPLES OF MDD:

Various evidence demonstrates that frontal regions of cerebral cortex (such as PFC, OFC and ACC), NAc, AMY, and HIPPO circuitry and structures are altered in depression (Dranovsky & Hen, 2006; Hare & Duman, 2020; Nestler & Carlezon, 2006). We analyzed the protein expression levels of 5αR1 and 5αR2 by immunoblotting to understand whether 5αR enzymes play a key role in the neurobiology of depression.

T-Test analyses did not show significant alteration in the expression levels of 5αR1 in the OFC, ACC, NAc, AMY and HIPPO (OFC: T=0.33, p>0.05, n.s.; AAC: T=0.30, p>0.05, n.s.; NAc: T=0.55, p>0.05, n.s; AMY: T=0.35 p>0.05, n.s.; HIPPO: T=0.29, p>0.05, n.s.). 5αR2 expression levels were, instead, significantly reduced in the OFC, ACC, and NAc of the MDD group compared to the control group (OFC: T=2.24, p=0.04 *; AAC: T=2.67, p=0.02 *; NAc: T=2.32,

Figure 12



p=0.03 *), but not in the AMY and HIPPO (AMY: T=0.01, p>0.05 n.s.; HIPPO: T=0.34, p>0.05, n.s.) (Fig. 12; Fig. 13).

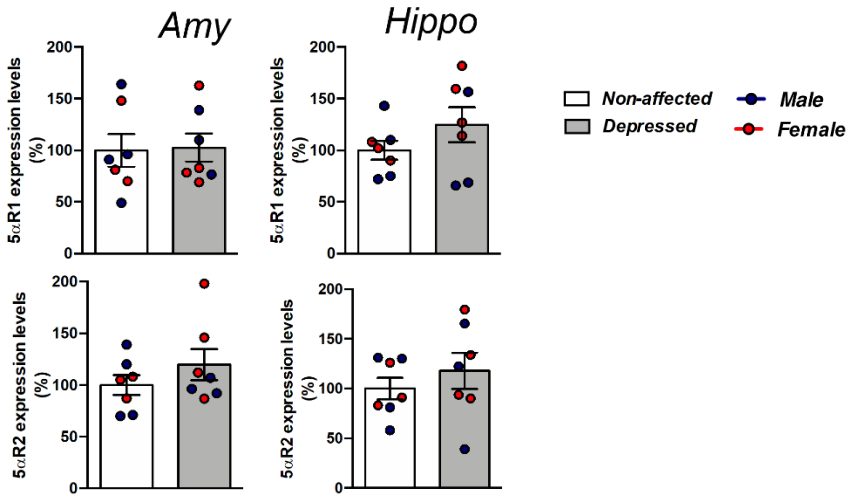


Figure 12. 5αR1 and 5αR2 expression levels expressed as mean ± SEM.

Figure 13

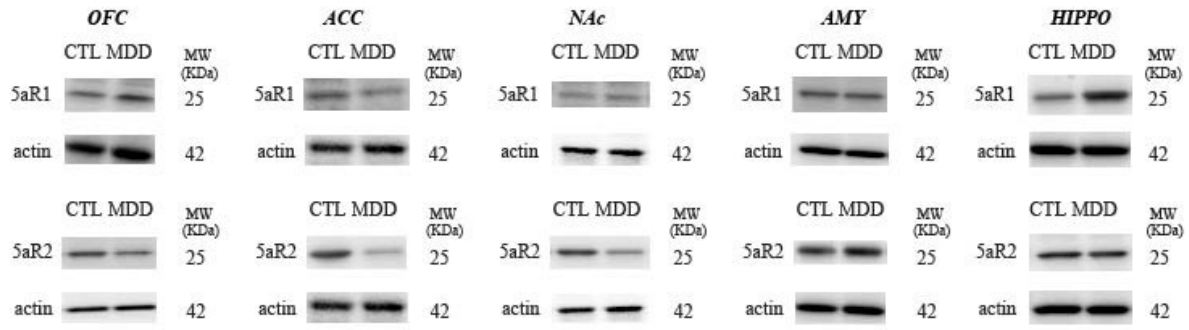


Figure 13. Representative blots of 5αR1 and 5αR2 expression.

CORRELATION BETWEEN 5AR1 AND 5AR2 LEVELS AND HAMILTON RATING SCALE:

The Hamilton Rating Scale for Depression (HAM-D) is used for the assessment of depression severity in patients who were already diagnosed with MDD. With these analyses we wanted to establish whether a correlation exists between the lower expression level of these enzymes and the severity of depression, both in males and females. Linear regression analyses showed a significant negative correlation between 5αR2 expression and the severity of depressive symptoms in males, but not in females, in the OFC (males: $F_{1,5}$=10.58, R^2=0.67, p=0.02 *; females: $F_{1,4}$=0.44, R^2=0.09, p>0.05 n.s.) and ACC (males: $F_{1,5}$=11.28, R^2=0.69, p=0.02 *; females: $F_{1,4}$=0.21, R^2=0.05, p>0.05 n.s). No other significant correlations were observed in the remaining areas for both enzymes (5αR1 OFC males: $F_{1,5}$=0.33, R^2=0.06, p>0.05 n.s.; females: $F_{1,4}$=3.18, R^2=0.44, p>0.05 n.s. 5αR1 ACC males: $F_{1,5}$=0103, R^2=0.02, p>0.05 n.s.; females: $F_{1,4}$=0.21, R^2=0.05, p>0.05 n.s. 5αR1 NAc males: $F_{1,5}$=5.12, R^2=0.50, p>0.05 n.s.; females: $F_{1,4}$=0.36, R^2=0.08, p>0.05 n.s. 5αR2 NAc males: $F_{1,5}$=3.06, R^2=0.37, p>0.05 n.s.; females: $F_{1,4}$=1.79, R^2=0.30, p>0.05 n.s. 5αR1 Amy males: $F_{1,5}$=1.03, R^2=0.17, p>0.05 n.s.; females: $F_{1,4}$=3.92, R^2=0.49, p>0.05 n.s. 5αR2 Amy males:$F_{1,5}$=0.66, R^2=0.11, p>0.05 n.s.; females: $F_{1,4}$=0.21, R^2=0.05, p>0.05 n.s. 5αR1 Hip males: $F_{1,5}$=0.44, R^2=0.08, p>0.05 n.s.; females: $F_{1,4}$=1.19, R^2=0.22, p>0.05 n.s. 5αR2 Hip males: $F_{1,5}$=0.0009, R^2=0.0001, p>0.05 n.s.; females: $F_{1,4}$=4.48, R^2=0.52, p>0.05 n.s.) (Fig. 14).

Figure 14

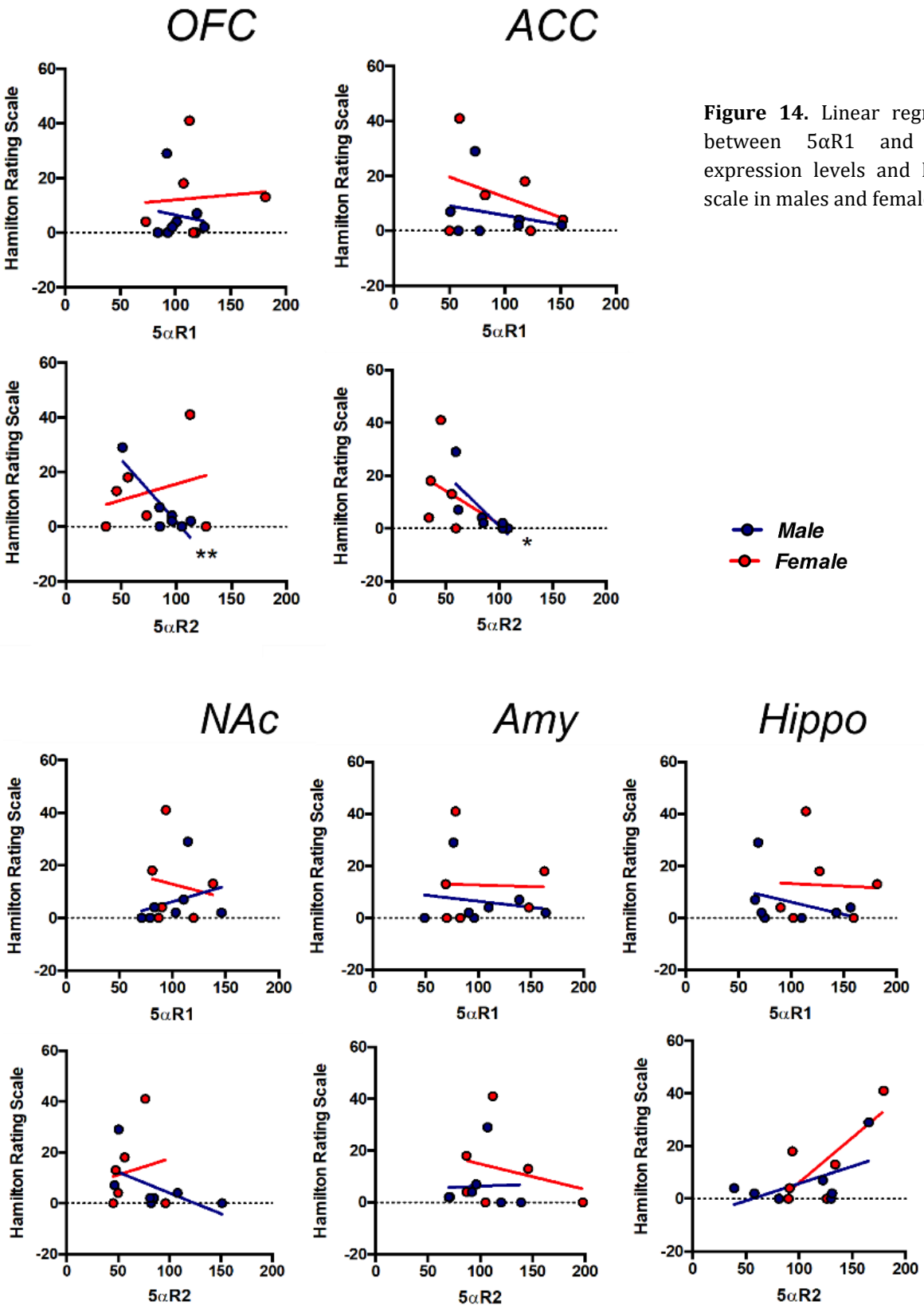


Figure 14. Linear regression between 5αR1 and 5αR2 expression levels and HAM-D scale in males and females.

DISCUSSION

Despite the transcriptomics results, western blot analyses revealed that 5αR2 is implicated in the development of MDD but not in ASB. 5αR1 and 5αR2 levels in ASB human samples were not significantly modified compared to their control groups (unaffected control group and SUD group). These results suggest that the changes in neurosteroid levels observed in subjects diagnosed with these disorders could be due to the modified activity of 5αR enzymes, but not to changes in their expression, or to another enzyme involved in neurosteroid metabolism. It should be pointed out, however, that 5αR2 levels were significantly increased in the SUD group. This is in line with previous studies that showed that DHEA and pregnanolone selectively reduced ethanol-reinforced responding in rats (Gurkovskaya et al., 2009; Hulin et al., 2015). This is probably due to the fact that higher level of 5αR2 contributes to an overall impairment of neurosteroid levels, which could be restored using neurosteroids administration. However, the subjects included in the SUD group had a record of use of different substances of abuse, and it is almost impossible to reconcile our observation to a single underlying mechanism.

Interestingly, the results obtained in the MDD group clearly indicate that 5αR2 plays a significant role in this disorder. In fact, in MDD samples 5αR2 levels are lower in areas, the OFC, AAC, and NAc, that are well known to be relevant in the onset of depression. Moreover, it is definitely fascinating that there were no changes in the expression levels of 5αR1. This strictly points to a critical role of the 5αR2 isoform only in MDD. The limitations of the small number of subjects analyzed and the heterogeneity of the severity of the disorder should be acknowledged, yet it is remarkable that 5αR2 expression levels were negatively correlated

with the HAM-D scale in the OFC and ACC, e.g., the lower the levels of this isoform, the higher the severity of the disorder. It should be pointed out that two males and one female subjects with low HAM-D score (respectively 4, 7 and 18) were treated with Sertraline (a selective serotonin reuptake inhibitor commonly used as antidepressant in clinic). The treatment, then, may have influenced the HAD-D score. Nevertheless, it is intriguing to notice that the same subjects have also a higher expression of 5αR2: first two places of the male subjects and second place of the female subject compared to the subjects of the same sex in both OFC and AAC. This rank is not maintained in the other brain areas (data not shown). Still, we should acknowledge the limited number of subjects analyzed as a caveat about significant inferences from these data.

These data are critically pointing out that 5αR2 is implicated in the depressive disorder and suggest that to further investigate its role in this psychopathology it is a worthy effort.

Then, the next goal of my thesis was to further support these data by studying whether stressful conditions that induce a depressive-like phenotype in the rats can modify the levels of expression of 5αRs, and whether that lack of these enzymes was a necessary condition to induce the expression of a depressive-like phenotype.

CHAPTER 5
STRESS MODELS OF MDD AND EVALUATION OF 5ARS IN THE PATOPHYSIOLOGY OF DEPRESSION

OVERVIEW CHAPTER 5:

As I demonstrated in the previous chapter, 5αR2 is directly implicated in major depressive disorders. Here I aimed to further investigate whether the exposure to a stressful environment (which is one of the most important causes of MDD onset) modified the expression of 5αRs and whether the induction of lower expression of these enzymes was sufficient to reproduce depressive-like symptoms.

Epidemiologic studies show that a 40-50% of the risk for depression is genetic, however depression vulnerability is also due to non-genetic factors such as exposure to stressful environment, viral infection, or stochastic processes during brain development (Fava & Kendler, 2000; Nestler et al., 2002). Depression is often described as a stress-related disorder, yet stress *per se* is not sufficient to cause depression. This underscores that depression in most people is caused by interactions between a genetic predisposition and environmental factors. As matter of fact, stressful life events could enhance depressive episodes in vulnerable individuals (Kendler et al., 1999), and childhood stress in the form of abuse or neglect increases the risk of depression in adult life (Pechtel & Pizzagalli, 2011). A prominent mechanism by which the brain reacts to acute and chronic stress is activation of the HPA axis. Neurons in the PVN of the hypothalamus secrete CRF, which stimulates the

synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. Glucocorticoids exert profound effects on general metabolism and dramatically affect behavior via direct actions on numerous brain regions. The levels of cortisol seen in some depressed patients, particularly over sustained periods of time, might be high enough to be toxic to hippocampal neurons (e.g., reduction in dendritic arborization or birth of new neurons), then impaired hippocampal function might be expected to contribute to some of the cognitive abnormalities of depression (McEwen, 2000; Nestler et al., 2002; Sapolsky, 2000).

As mentioned above, stressful stimuli change the levels of neurosteroids in the brain and they are strictly correlated with the onset of depression. In fact, several studies have documented decreases levels of AP in individuals with MDD (Rasmusson et al., 2006; Uzunova et al., 1998). Moreover, effects of neurosteroids in easing depressive-like behaviors were demonstrated in animal model of depression (Evans et al., 2012; Maayan et al., 2005). Nonetheless, the mechanisms through which neurosteroids impact depression are still unknown and the use of animal models could be a helpful tool to individuate the best therapeutical strategy to be applied in clinics.

Our previous results in humans affected by MDD, showed a significant drop in the levels of 5αR2 in the OFC, AAC, and NAc. Since the reduction of this enzyme leads to a decrease in the levels of neurosteroids (such as AP), it is quite likely that decreased neurosteroid transmission could result in a general inactivation of reduced neuronal activity in these areas or a disrupted connectivity between them. Hence, I evaluated whether the development of

depressive-like behaviors in stress models of MDD was correlated with reduced levels of expression of the 5αR enzymes and whether knock-down rats for 5αR1 and 5αR2 in the PFC and NAc showed depressive-like behaviors.

ANIMAL MODELS OF DEPRESSION:

All animal models of depressive disorders rely on the action of known antidepressants or responses to stress. With regard to animal models of depression, the term "model" is often employed to describe the methods used to assess depressive-like behaviors (tests) as well as the protocols or paradigms that induce the depressive-like phenotype. The distinction between a "model" and a "test" is an important one as the "model" is the complex phenotypic construct that can only be revealed by "tests" of depressive-like responses (Scheggi et al., 2018). In fact, while tests measure an informative behavioral or physiological response in animals, models aim to recreate the human disorder in other species, and they should rely on three criteria: face validity (phenomenological or morphological appearances), construct validity (similar etiology), and predictive validity (therapeutic similarities) (Willner, 1984).

Some of the animal models of depression are created using the following paradigms:

1- *Learned Helplessness*. It refers to the behavioral consequences of repeated exposures to stressful events over which the organism has no control.
2- *Chronic Mild Stress.* In this model, animals are exposed chronically to a constant unpredictable micro-stressor, resulting in the development of a plethora of behavioral changes (including anhedonia).

3- *Social Stress.* It consists in changing the dynamics of social interactions in rodents, such as social isolation. Social defeat is another example of this paradigm, where rodents are exposed constantly to a dominant conspecific, that, via a combination of direct physical contact and indirect sensory contact, results in a stressful and subordinate relation.

Whereas the most common tests used to measure depressive-like behavior in animals are the following:

1- *Forced Swim test:* this method is based on the observation that a rodent, when forced to swim in a situation from which there is no escape, will, after an initial period of vigorous activity, eventually cease to move altogether making only those movements necessary to keep its head above water.
2- *Tail Suspension test:* rodents are suspended by their tails with tape, in such a position that it cannot escape or hold on to nearby surfaces. During this test, the resulting escape-oriented behaviors are quantified.
3- *Sucrose preference test:* which is intended to measure the level of anhedonia in rodents, it basically consists in making the animal choose between a saccharin solution and tap water.
4- *Novelty induced hypophagia test*: in which the animal needs to eat a familiar and palatable food provided in an aversive environment in order to study anxiety responses, since anxiety and depression are highly correlated in humans.

Nevertheless, many of the core symptoms of depression (such as self-denial, dysthymia, and suicide predisposition) cannot be easily measured in laboratory animals. Hence, we need a

more specific animal model to better understand the disorder, yet this model cannot be developed before profoundly understanding the human disorder.

MATERIAL AND METHODS

ANIMALS:

The experiments in this study were performed using Long-Evans males and female rats (Charles River Laboratories, Raleigh, NC, USA), weighing 250-350 g and housed in groups of 3-4 per cage (Fig. 15). Unless otherwise stated for specific experimental manipulations, rats were kept with ad libitum access to food and water. For all tests, animals were used only once. Experimental manipulations were carried out in the animals' dark cycle between 10:00 AM and 06:00 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 of the University of Utah (Protocol 19-05005).

Figure 15. Long-Evans adult rat.

5αR1 AND 5αR2 KNOCK-DOWN (KD) RATS:

Adeno-associated viral (AAV) constructs were obtained from Vector Biolabs (Malvern, PA, USA). The construct used to knock-down 5αR1 was the following: AAV5-GFP-U6-rSrd5a1-shRNA. The eGFP was used as reporter/marker, driven by a citomegalovirus (CMV) promoter, while the promoter used to drive expression was U6. Constructs were packaged into AAV5 capsid, with a titer of $1.5x10^{12}$ genomic copies/ml. We designed 3 shRNA for rat 5αR1 and used mixture of 3 shRNA plasmids for this AAV packaging.

Selected sequences were:

5‘-ACC GCTATGTACAGAGCAGATACTCTCGAGAGTATCTGCTCTGTACATAGC TTTTT-3‘;

5‘-ACC GCACCATCAGTGGTACCATGACTCGAGTCATGGTACCACTGATGGTGC TTTTT-3‘;

5‘-ACC GGGAAACTGGATACAAGATACCTCGAGGTATCTTGTATCCAGTTTCCCT TTTT-3‘.

Similarly, the construct used to knock-down 5αR2 was the following: AAV5-GFP-U6-rSrd5a2-shRNA. The eGFP was the reporter/marker driven by a CMV, and U6 the promoter used to drive expression. Constructs were packaged into AAV5 capsid, with a titer of $2.1x10^{12}$ ($4.4x10^{12}$ GC/ml) genomic copies/ml. The AAV was packaged with mixture of 3 rat-Srd5a2 shRNA plasmids:

5‘-ACCG TACTTCCACAGGACATTTATT CTCGAG AATAAATGTCCTGTGGAAGTA TTTTT-3‘;

5‘-ACC GGTACACAGATGTGCGGTTTA CTCGAG TAAACCGCACATCTGTGTACC TTTTT-3‘;

5 -ACC GCAGGAGTTGCCTTCCTTTGT CTCGAG ACAAAGGAAGGCAACTCCTGC TTTTT-3‘.

AAV5-GFP-U6-scrmb-shRNA ($1.2x10^{12}$ GC/ml) was used as control solution.

Long Evans rats were anesthetized with xylazine/ketamine (20/80 mg/kg^{-1}, IP) and then placed onto a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Blunt ear bars were used to avoid damage to the tympanic membrane. Under aseptic conditions, rats were shaved, and their scalp was retracted. Bilateral craniotomies were made above the target site and a 5µL Hamilton Neuros syringe (Reno, NV, USA) was positioned above bregma (Fig. 16). The target locations for brain infusions were: mPFC: AP + 3.0 mm, ML ± 0.5 mm, DV − 3.0 mm from the dura mater; NAc: AP + 1.7 mm, ML ± 0.8 mm, DV = − 7.8 mm from the dura mater. Coordinates were taken from bregma, according to the stereotaxic brain atlas (Paxinos, 2001). The needle was slowly lowered into the injection site, and 1 µL of adeno-associated virus (AAV) (or scramble shRNA) was infused into one hemisphere. The syringe was left in place for at least 5 minutes after completion of the infusion to eliminate back-flow and then slowly withdrawn. This process was repeated on the other side to produce bilateral viral injections. After completing injections into both hemispheres, rats were placed back on a warming pad until body temperature returned to normal and recovery of normal movement. Rats were given antibiotic therapy for two days (enrofloxacin, Bayer HealthCare, Shawnee Mission, KS, USA) and were allowed to recover in their home cages (single-housed) with food and water available. Behavioral testing started 14 days after surgery.

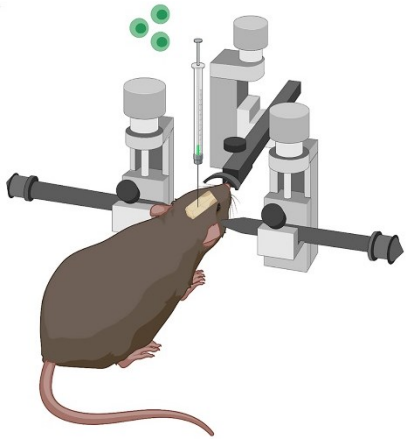


Figure 16. Schematic representation of into a stereotaxic frame (modified from Taylor et al. 2021).

BEHAVIORAL MODELS:

SOCIAL DEFEAT (SD):

The social defeat (SD) stress consists of the exposure of a single rat to a dominant one. Before starting the procedure, experimental rats were single housed and tested for sucrose consumption. Meantime, dominant rats were single housed and exposed regularly to a female in estrus to establish a prominent dominant behavior. After a stable preference for sucrose (2% w/v) was accomplished in each experimental rat, SD procedure started. Experimental rats were divided into two groups: social defeated and non-social defeated. SD protocol consists in 4 sessions performed (one session per day) and repeated for a total of 35 days. On day 1 rats in the social-defeated group were individually placed into the cage of the dominant rat. After 30 minutes of direct exposure, the dominant rat was separated from the social defeated rat by a perforated grid in order to keep olfactory, tactile and visual contact between them for another 30 minutes. Then, the social-defeated rat was placed again in his home-cage. Rats from the control group were only handled by an experimenter. On day 2 all rats were tested for sucrose consumption. On the third day, rats were again exposed to a social defeat session (or handled), and on day 4 they were exposed to social defeat (or handled) and then tested for sucrose consumption. On day 35, we observed a critical drop on sucrose consumption in social defeated rats compared to control rats. Then, behavioral tests were started.

SOCIAL ISOLATION (SI):

Rats were divided into 2 experimental groups: group-housed non stress rats (GH) and single-house (SH) rats. GH rats were maintained in the standard housing condition. SH rats were

kept for 4 weeks individually in a cage with the same dimension and bedding as the home cage and ad libitum access to food and water. Rats were monitored weekly for the 2% (w/v) sucrose consumption. After 35 days, behavioral tests started.

Chronic mild stress (CMS):

Rats were single housed during all the procedure. The unpredictable chronic mild stress regimen used in this study was based on the procedure originally designed by Willner et al., (1992). Rats were subjected several times a day for 5 weeks to one of the following stressors (or a combination of more of them) such as stroboscopic lights, placement in an empty cage, placement in an empty cage with wet betting on the bottom, overcrowded cages, cage tilting (45°), white noises, inversion of light/dark cycle, lights on for a short time during the dark phase. To prevent habituation and to provide an unpredictable feature to the stressors, all the stressors and/or sequences were administered at different time points every week. Rats were divided into 2 experimental groups: single-housed non-stress (SH) and single-housed chronic mild stressed (CMS). Rats were monitored weekly for the 2% (w/v) sucrose consumption. After 35 days, we observed a critical drop on sucrose consumption in chronic-mild stressed rats compared to control rats. Then, behavioral tests were started.

After a four-week exposure to the SD, SI, and CMS protocols, rats were tested for novelty-induced hypophagia (NIH) and forced swim test (FST).

Behavioral tests:

Novelty-Induced Hypophagia (NIH):

Rats were single housed and moved within their cage to a dimly illuminated room (50 lux under red light) for four consecutive days. After a 30 min acclimation period, they were presented with a highly palatable food (two cheese puffs made of corn flour, hydrogenated vegetable fat, cheese powder and salt). Following this training, animals were transferred to a novel cage (20 cm × 29 cm × 35.5 cm) and moved to a brightly lit room (500 lux, under white light). The latency to eat and total food consumption were recorded by an observer blinded to treatment (Fig. 17).

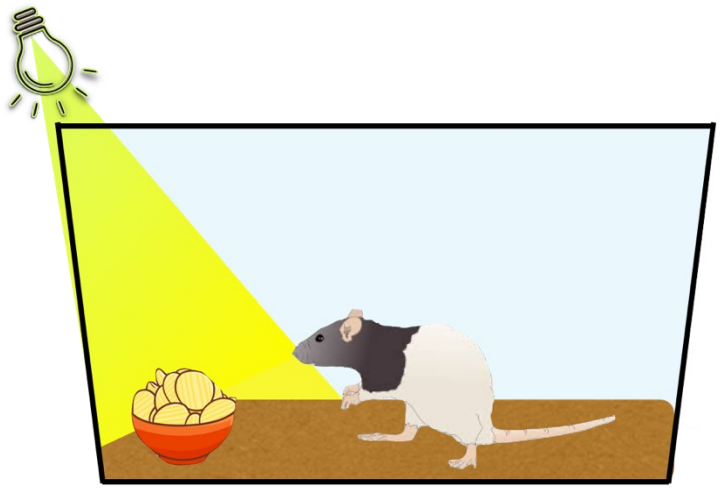


Figure 17. Schematic representation of NIH test.

Sucrose Preference Test (SPT):

Rats were initially tested for baseline sucrose consumption and preference. They were deprived of water for 15 h prior to the test, starting 1 h before the onset of the dark phase. Each animal was given access to one pre-weighed bottle containing a 2% sucrose solution in

tap water. One hour later, the bottle was removed and weighed again, and food and water were placed back in the cage. Then, sucrose consumption was assessed again every 3 to 4 days for the following 2 weeks. After stabilization of sucrose consumption, rats were given two bottles, containing a 2% sucrose solution (presented on either the left or the right side of the cage, in counterbalanced order) and water, respectively, for 4 h (Fig. 18). Sucrose preference was assessed as the ratio of sucrose solution/total liquid consumed by each rat.

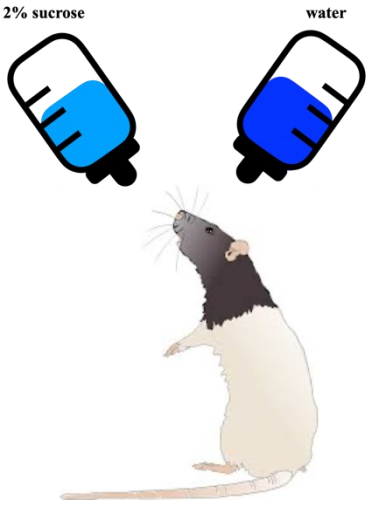


Figure 18. Schematic representation of SPT.

FORCED SWIM TEST (FST):

Rats were plunged individually into a vertical plexiglass cylinder (height 45.7 cm; diameter 30 cm) containing 30 cm of water maintained at 25°C (Fig. 19). After 10 min in the cylinder, they were removed and allowed to dry for 15 min in a heated enclosure (32 °C) before being returned to their individual cages. Environmental light was kept at 300 lux. Animals were video recorded, and the duration of immobility (s) and the latency to immobility (s) were measured.

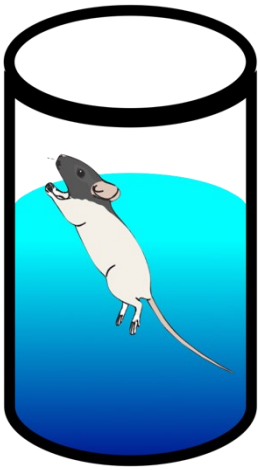


Figure 19. Schematic representation of FST.

IMMUNOBLOTTING:

Rats were sacrificed 20 minutes after FST, and brain areas were dissected, and flash frozen for western blot analysis. Samples were stored at -80°C until assayed. To analyze the expression levels of 5αR1 and 5αR2, tissues were weighted and diluted (10 µg/10 µl) in RIPA buffer containing 20mM Tris-HCl (pH 7.5), 1 50 mM NaCl, 1 mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, 1 µg/ml leupeptin and protease inhibitor cocktail. Small aliquots of the homogenate were used for protein determination using a modified Lowry protein assay method (DC protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Samples containing 15 µg of total proteins were run in duplicate onto 4-15% Criterion™ TGX Stain-free™ precast gels (Bio-Rad Laboratories) and transferred into nitrocellulose membranes (Bio-Rad Laboratories). Stain-free™ gel formulation includes a trihalo compound that, when exposed to ultraviolet (UV) irradiation, generates a covalent reaction with tryptophan residues of proteins and allows them to be visualized within the gel or after transfer to a blotting membrane. Following protein transfer, the membrane was detected by UV and blot image was collected for total protein. Primary antibodies against 5αR1 (#PA575919, Invitrogen, dilution 1:750) and 5αR2 (#MA537985, Invitrogen, dilution 1:1000) were incubated in TBS-T containing 3% (w/v) BSA buffer overnight at 4 °C. Next, blots were washed in TBS-T and then incubated in TBS-T containing goat anti-rabbit HRP-conjugated (#31462, Thermo Fisher Scientific; dilution 1:10000) or goat anti-mouse HRP-conjugated (#31430, Thermo Fisher Scientific; dilution 1:5000) secondary antibodies, for 90 minutes at room temperature. Chemiluminescence was detected with the ChemiDoc™ XRS+ Imaging System using the Clarity Western ECL substrate (Bio-Rad Laboratories). Bands were

quantified in arbitrary units and normalized using the software Image Lab (Bio-Rad Laboratories). Samples containing the same amounts of total proteins in each experimental group were run on the same immunoblots and then analyzed together. Membranes were stripped and re-probed with primary antibody anti-β-actin (mouse monoclonal #sc47778 Santa Cruz Biotechnology) to control for equal loading.

IMMUNOFLUORESCENCE (IF):

IF was used to verify the virus spread within the brain areas. Rats were euthanized with xylazine/ketamine (20/80 mg/kg^{-1}, IP), immediately after respiratory arrest rats were laid on their back and the thorax were carefully opened to avoid excessive bleeding. Then, carefully and quickly the rib cage was cut and the diaphragm removed for access to heart. A syringe filled with 1x PBS (0.145M NaCl, 0.0027M KCl, 0.0081M Na2HPO4, 0.0015M KH2PO4, pH 7.4) was insert into the left ventricle and the right atrium was cut open to allow the PBS slowly and constantly perfused into the heart and other organs. After most of blood had been flushed out, the brain was collected and placed into 4% paraformaldehyde (PFA) fixative (Thermo Fisher Scientific #J19943) at 4°C for 48h. After, brains were placed into 30% sucrose saline buffer (PBS) and left at 4°C for at least 48 hours. Slice of brain areas (35 µm) were then cut using a cryostat (Leica Biosystem CM1520) and placed on coated glass slides for microscopy. Slides were washed with Cyto-Q immunodiluent and blocking buffer (Innovex Bioscience #NB307) + 0.1 % Triton-X 100. Then, slides were blocked using Cyto-Q + 0.1% Triton-X100 + 0.2% BSA and overnight incubated with anti-GFP conjugated antibody (Rockland #600-101-215) at 4°C. Slides were washed again using Cyto-Q + 0.1% Triton-X 100 and then a liquid mountant with DAPI (Invitrogen ProLong™ Gold Antifade Mountant

with DAPI #P36931) was applied on slides. Images were taken using Zeiss LMS 700 (Carl Zeiss Microscopy, Germany) confocal microscopy and ZEIN BLACK Software (Carl Zeiss Microscopy, Germany).

STATISTICAL ANALYSIS:

All data are expressed as mean ± SEM and analyses were performed using GraphPad Prism 8 statistical package (GraphPad, San Diego, CA, USA). Behavioral and neurochemical data were analyzed via Student's T-Test (T-Test), One-way ANOVA, or Two-way ANOVA in order to determine if the means of the groups were significantly different. All *post-hoc* analyses were performed via Tukey's or Sidak tests. Significance was set at $p < 0.05$.

RESULTS

DEPRESSIVE-LIKE BEHAVIORS AND 5αR1 AND 5αR2 EXPRESSION LEVELS IN SOCIAL DEFEATED RATS:

Since social stress, such as SD, is a well validated model for depression, we evaluated depressive-like behaviors in Long-Evans male rats (*n*=12) after 35 days (the day when we detect a critical drop in sucrose preference) of SD. Rats were tested for NIH, and FST. Then the PFC, NAc, AMY and HIPPO were collected and analyzed for the expression levels of 5αR1 and 5αR2 by immunoblotting. Two-way ANOVA RM analyzes revealed a significant reduction in sucrose preference in SD group compared to the control group (Interaction: $F_{5,110}$=2.71, p=0.023 *; Time: $F_{5,110}$=2.59, p=0.029 *; SD exposure: $F_{1,22}$=2.48, p=0.12 n.s.; Subject: $F_{22,110}$=1.93, p=0.014 *. Sidak post-hoc analyses: day 1-7-14-21-28 n.s., day 35 p=0.006 **). Two-way RM ANOVA showed a significant reduction in food consumption in NIH test in SD group compared to the control group (Interaction: $F_{1,22}$=5.33, p=0.008 **; Time: $F_{1,22}$=17.98, p<0.0001 ****; SD exposure: $F_{1,22}$=7.48, p=0.01 *; Subject: $F_{22,22}$=4.68, p<0.0001 ****. Sidak post-hoc analyses: 10 min p=0.001 **). In FST, T-Test demonstrated a

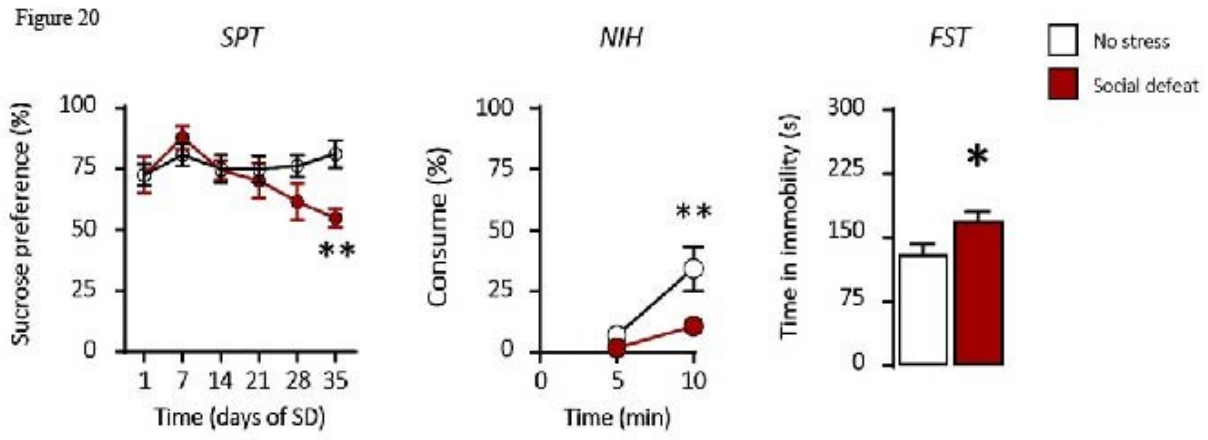


Figure 20. SPT, NIH and FTS in SD rats.

significant increase in time spent in immobility in the SD group compared to control group (T=2.60, p=0.01 *) (Fig. 20).

After the FST, rats were sacrificed, brain areas were harvested, and immunoblotting analyses performed. T-Test analyses revealed a significant reduction in the levels of expression of 5αR1 in HIPPO (T=4.20, p=0.001 **) and of 5αR2 in NAc (T=2.90, p=0.01 *) of the SD rats compared to the control group. No other significant differences were detected in the remaining areas for both enzymes (PFC 5αR1: T=1.16, p>0.05 n.s., PFC 5αR2: T=0.38, p>0.05 n.s.; NAc 5αR1: T=1.06, p>0.05 n.s.; AMY 5αR1: T=1.31, p>0.05 n.s.; AMY 5αR2: T=0.76, p>0.05 n.s.; HIPPO 5αR2: T=0.12, p>0.05 n.s.) (Fig. 21; Fig. 22).

Figure 21

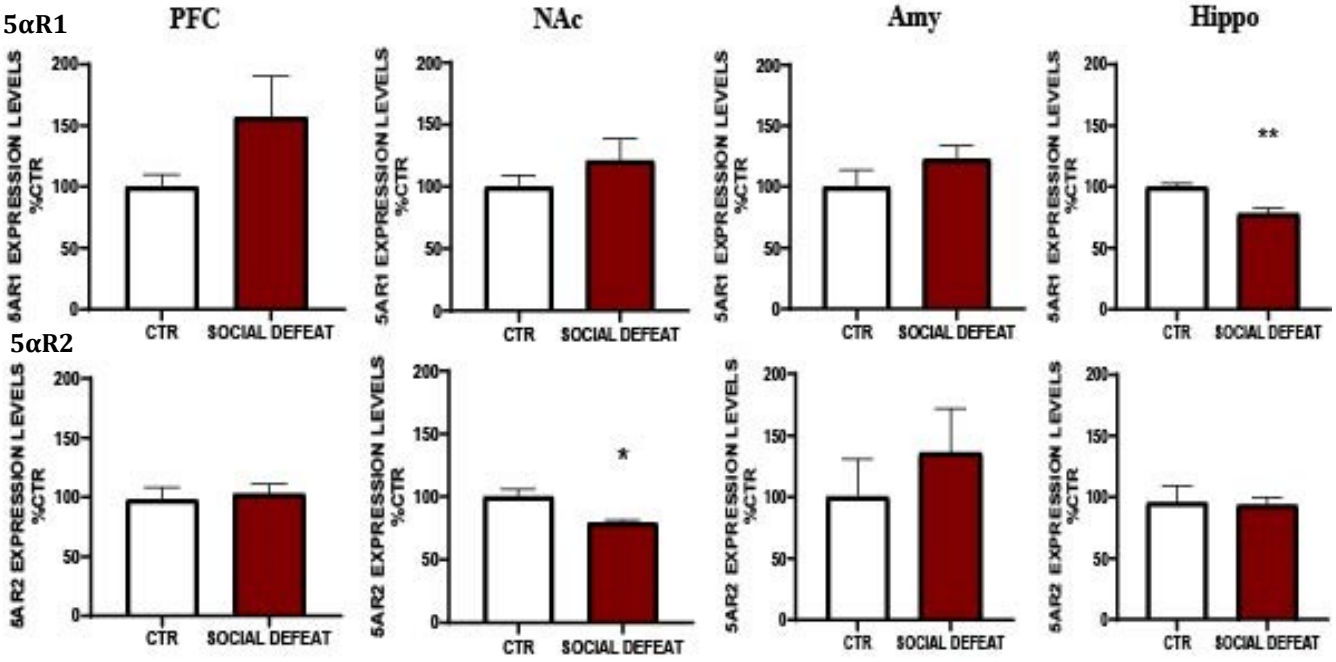


Figure 21. 5αR1 and 5αR2 expression levels in SD and CTR rats expressed as mean ± SEM.

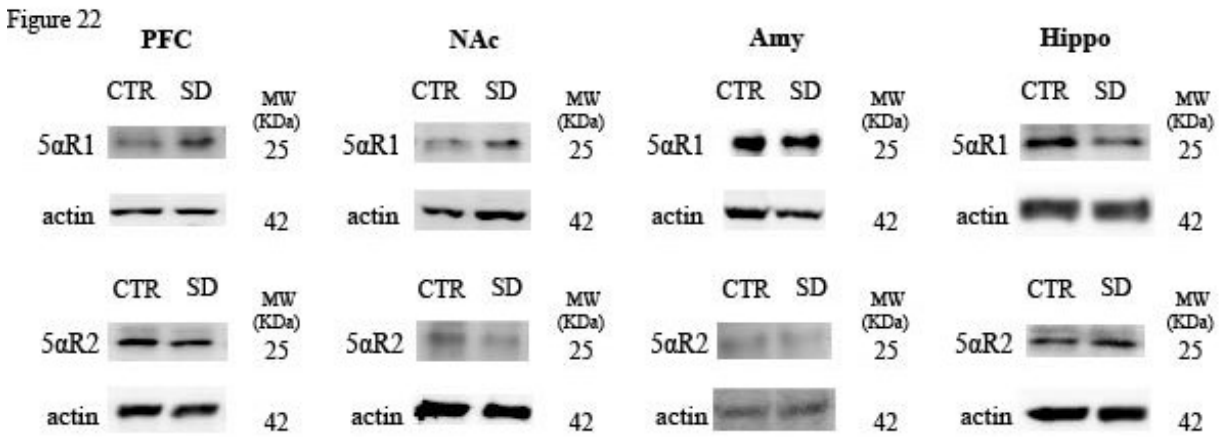


Figure 22. Representative blots of 5αR1 and 5αR2 in SD and CTR rats.

DEPRESSIVE-LIKE BEHAVIORS AND 5αR1 AND 5αR2 EXPRESSION LEVELS IN SOCIAL ISOLATED AND CHRONIC MILD STRESSED RATS:

Male and female rats (*n*=12) were divided into three experimental groups: group-housed (GH), single-housed (SH, only social isolated group), and chronic mild stressed rats (CMS, social isolated and stressed group). To better understand whether social isolation *per se* is sufficient to reduce the expression of 5αR enzymes or it needed to be combined with the exposure to a stressful environment, we divided the data into two analyses. We compared GH and SH rats, then SH and CMS groups. Since in the experiment that used Social Defeat as the stress protocol, we detected a critical drop in sucrose consumption after 35 days, we tested GH, SH, and CMS rats for SPT at the same time point. The analysis by T-Test did not show significant differences between GH and SH male and female rats (males: $T=0.75$, $p>0.05$ n.s.; females: $T=1.09$, $p>0.05$ n.s.). However, we could appreciate a significant reduction in

sucrose consumption in CMS male rats compared to SH (T=2.31, p=0.03 *), yet no differences were detected in females (T=1.15, p>0.05 n.s.) (Fig. 23).

Figure 23

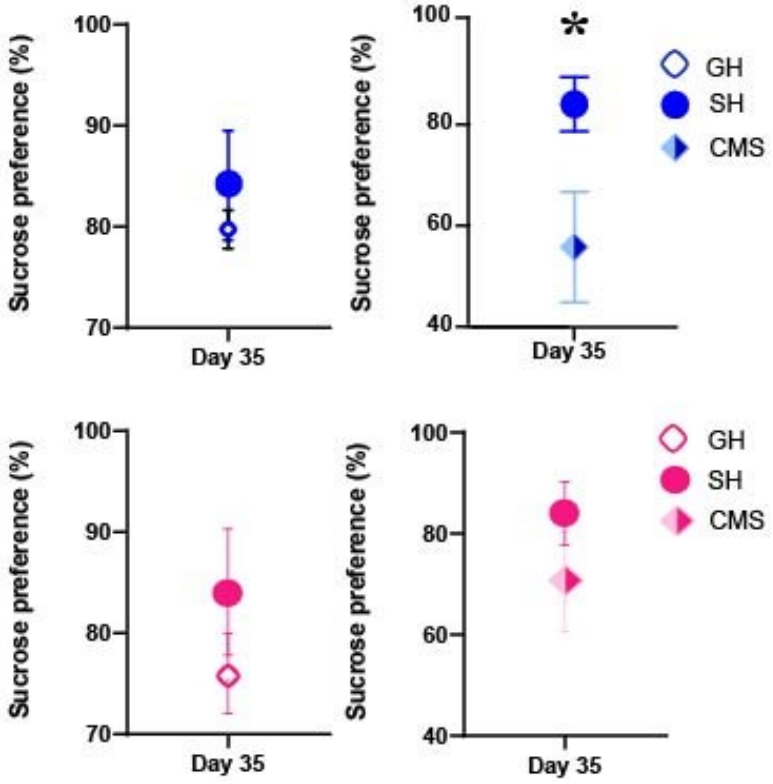


Figure 23. SPT in GH, SH, and CMS male (blue dots) and female (pink dots) rats.

Male and female rats were then tested for NIH. Two-Way RM ANOVA did not show significant differences between GH and SH male and female rats (males: Interaction: $F_{1,22}$=2.84, p>0.05 n.s.; Time: $F_{1,22}$=49.92, p<0.0001 ****; Isolation: $F_{1,22}$=2.66, p>0.05 n.s.; Subject: $F_{22,22}$=2.73, p=0.01*; females: Interaction: $F_{1,22}$=0.11, p>0.05 n.s.; Time: $F_{1,22}$=65.02, p<0.0001 ****; Isolation: $F_{1,22}$=1.67, p>0.05 n.s.; Subject: $F_{22,22}$=6.85, p<0.0001 ****). A significant reduction in food consumption were shown in CMS male rats compared to SH group (Interaction: $F_{1,22}$=4.50, p=0.04 *; Time: $F_{1,22}$=69.90, p<0.0001 ****; Isolation: $F_{1,22}$=20.82, p=0.0002 ***; Subject: $F_{22,22}$=3.38, p=0.003 **; Sidak post-hoc comparison 5 min p=0.009 **, 10 min p<0.0001 ****), yet no significant differences were shown in SH vs CMS female rats

Figure 24

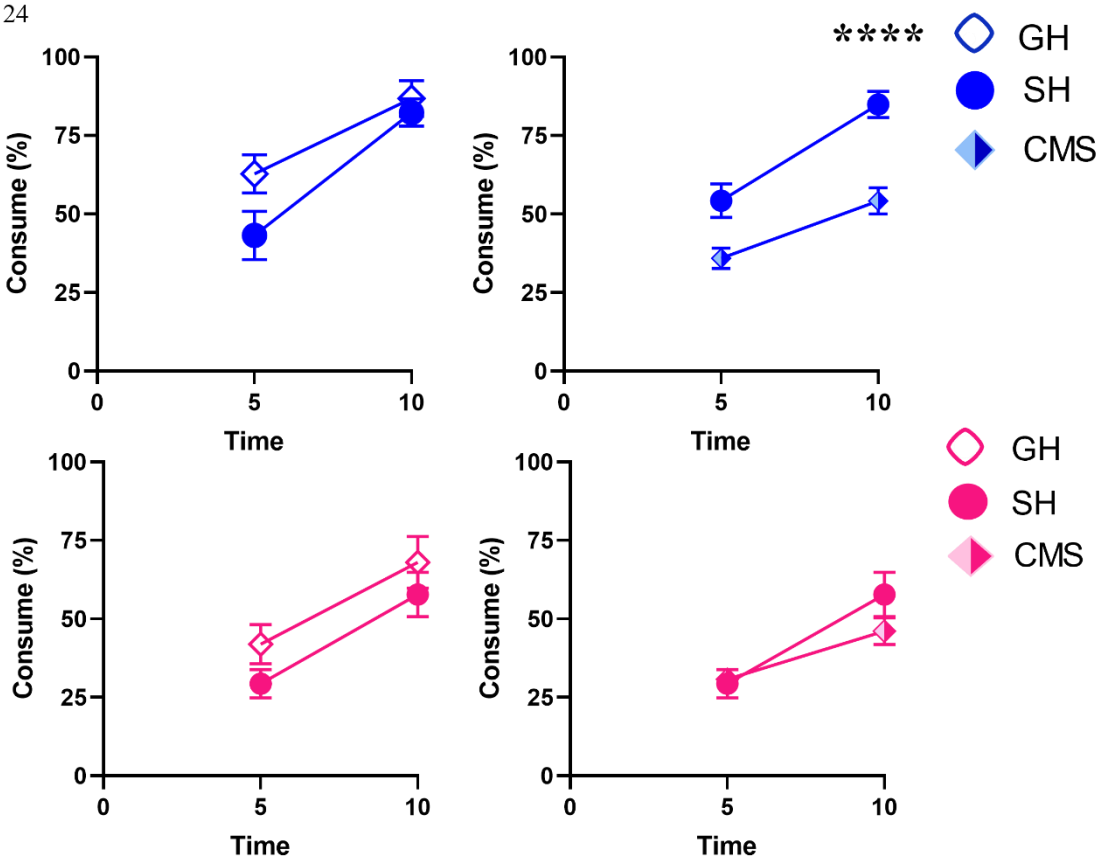


Figure 24. NIH in GH, SH, and CMS male (blue lines and dots) and female (pink lines and dots) rats.

(Interaction: $F_{1,22}$=7.76, p>0.05 n.s.; Time: $F_{1,22}$=53.31 p<0.0001 ****; Isolation: $F_{1,22}$=0.69, p>0.05 n.s.; Subject: $F_{22,22}$=4.22, p=0.0007 ***) (Fig. 24).

GH, SH, and CMS male and female rats then were tested in FST. T-Test analyses showed a significant increase in time spent in immobility in SH male rats compared to GH group

Figure 25

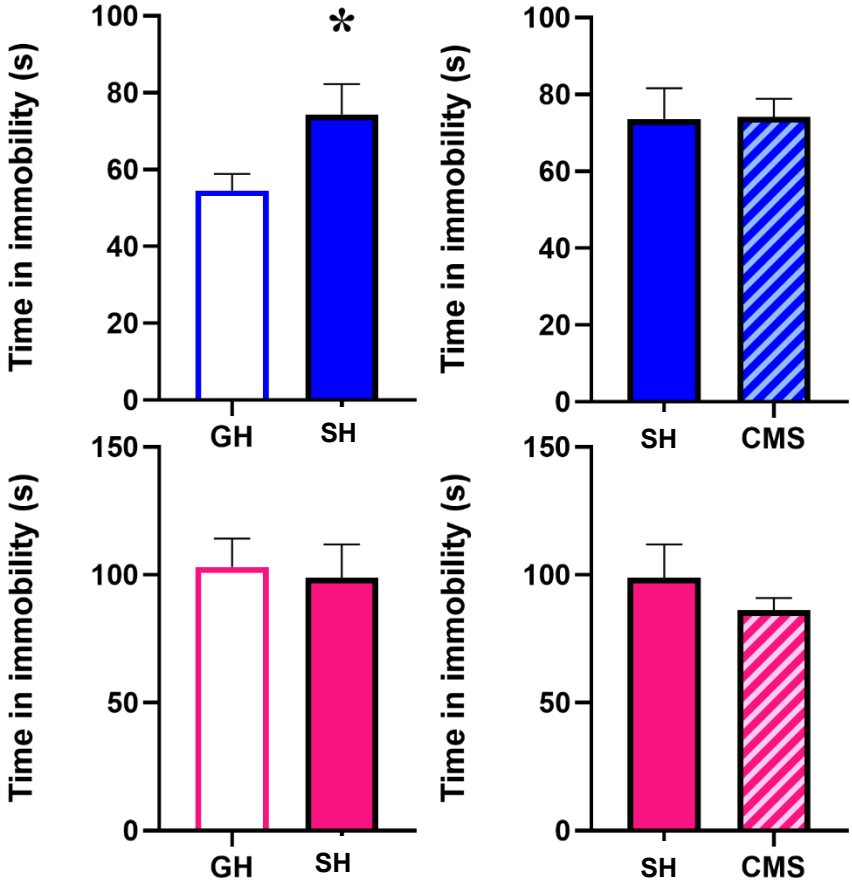


Figure 25. FST in GH, SH, and CMS male (blue) and female (pink) rats.

(T=2.19, p=0.04 *). No other significant differences were observed in the other groups of both sexes (males SH vs CMS T=0.06, p>0.05 n.s.; females: GH vs SH T=0.24, p>0.05 n.s.; SH vs CMS T=0.92, p>0.05 n.s.) (Fig. 25).

After FST, rats were sacrificed, brain areas were harvested, and immunoblotting analyses performed. In the PFC one-way ANOVA analyses revealed a significant reduction of 5αR1 and 5αR2 expression levels in CMS male rats compared to SH and GH groups (5αR1: $F_{2,33}$=5.66, p= 0.007 **, Tukey's post-hoc comparison CMS vs GH p= 0.01 *, CMS vs SH p=0.02 #. 5αR2: $F_{2,33}$=4.42, p= 0.01*, Tukey's post-hoc comparison CMS vs GH p= 0.04 *, CMS vs SH p=0.03 #); and in female rats a reduction of 5αR2 expression levels in CMS compared to SH and GH groups ($F_{2,33}$=7.40, p= 0.002 **, Tukey's post-hoc comparison CMS vs GH p= 0.002 **, CMS vs SH p=0.02 #). The 5αR2 expression levels were also reduced in HIPPO of CMS male rats compared to the SH group ($F_{2,33}$=4.82, p= 0.01 *, Tukey's post-hoc comparison CMS vs SH p=0.01 *). No other significant differences were detected in the remaining areas in both sexes

Figure 26 A

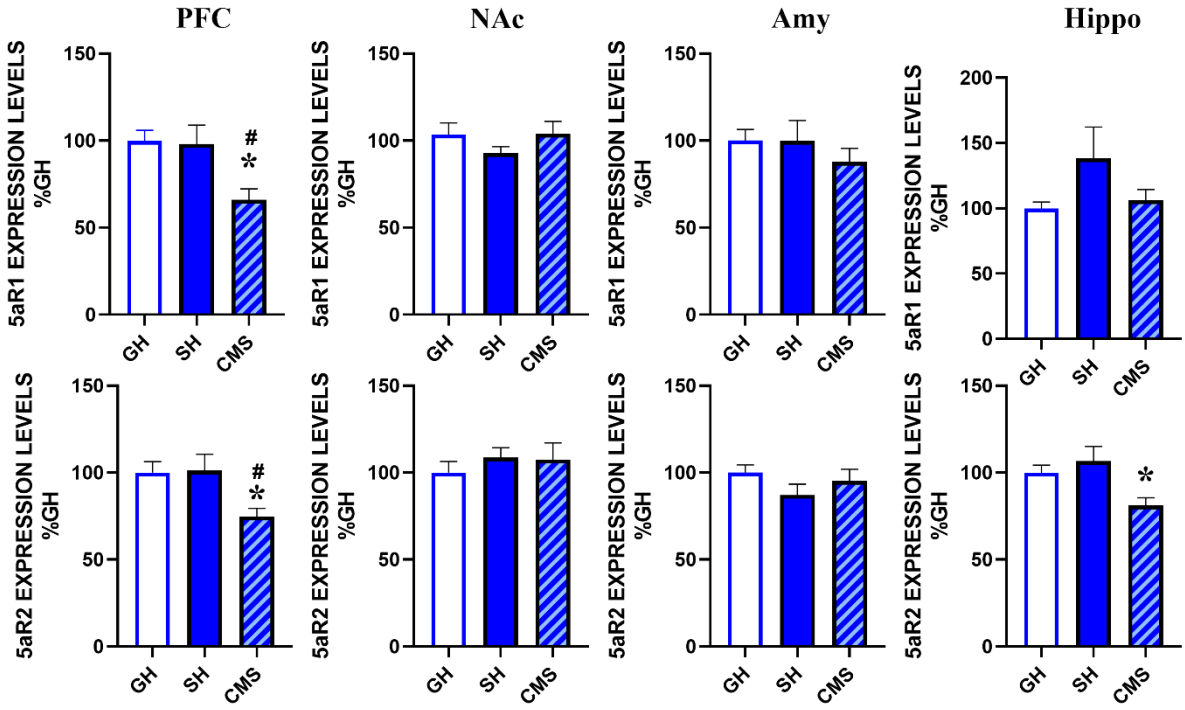


Figure 26 A. 5αR1 and 5αR2 expression levels expressed as mean ± SEM in male rats.

(PFC 5αR1 female: $F_{2,33}$=0.003, p>0.05 n.s. HIPPO 5αR1 males: $F_{2,33}$=1.90, p>0.05 n.s. HIPPO females 5αR1: $F_{2,33}$=0.69, p>0.05 n.s.; 5αR2: $F_{2,33}$=1.32, p>0.05 n.s. NAc males 5αR1: $F_{2,33}$=1.08, p>0.05 n.s.; 5αR2: $F_{2,33}$=0.39, p>0.05 n.s. NAc females 5αR1: $F_{2,33}$=0.10, p>0.05

n.s.; 5αR2: $F_{2,33}$=0.40, p>0.05 n.s. AMY males 5αR1: $F_{2,33}$=0.61, p>0.05 n.s.; 5αR2: $F_{2,33}$=1.26, p>0.05 n.s. AMY females 5αR1: $F_{2,33}$=0.31, p>0.05 n.s.; 5αR2: $F_{2,33}$=0.26, p>0.05 n.s.) (Fig. 26A-B, Fig. 27A-B).

Figure 26 B

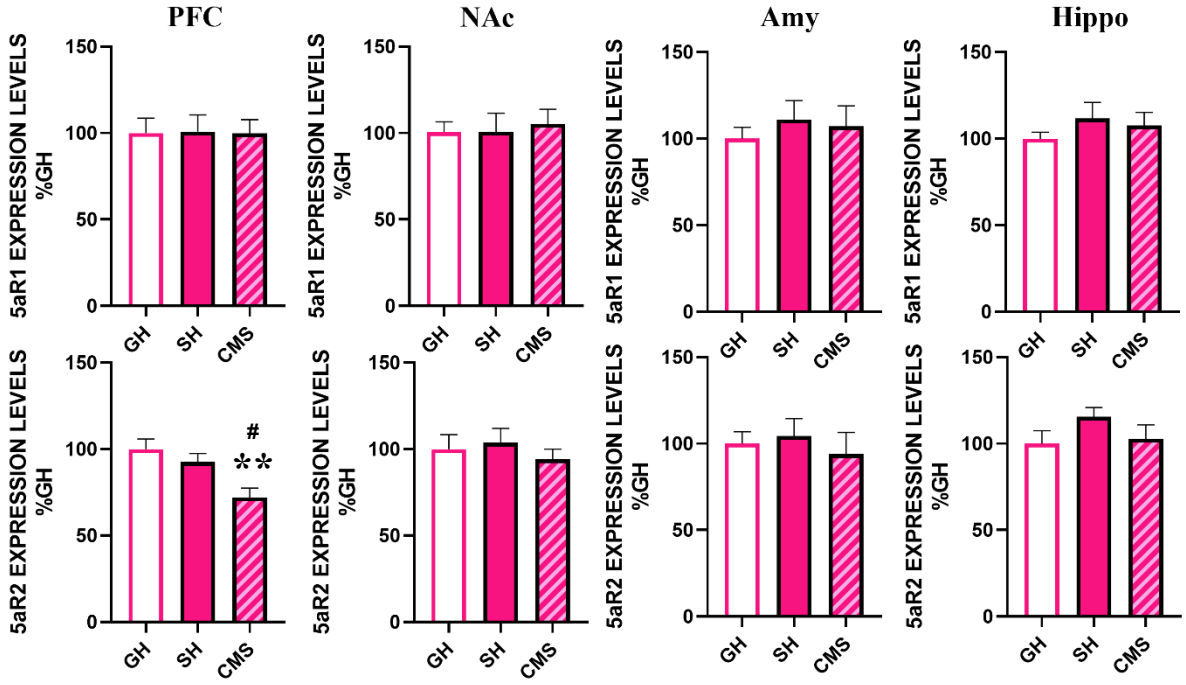


Figure 26 B. 5αR1 and 5αR2 expression levels expressed as mean ± SEM in female rats.

Figure 27A

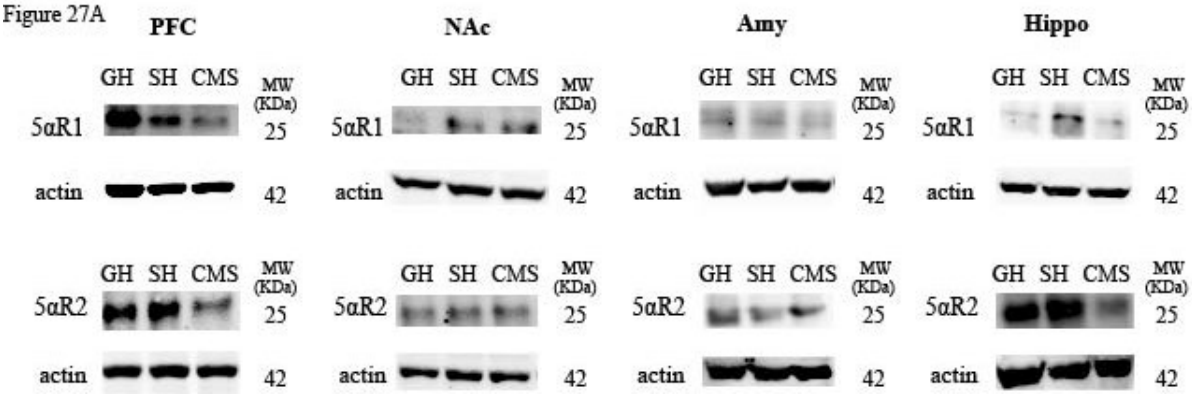


Figure 27 A. Representative images of immunoblots of 5αR1 and 5αR2 expression levels in male rats.

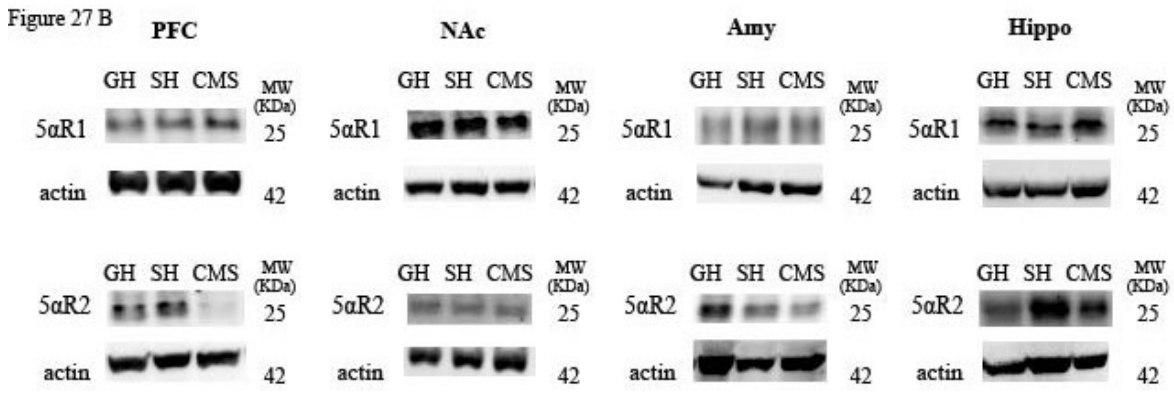


Figure 27 B. Representative images of immunoblots of 5αR1 and 5αR2 expression levels in female rats.

DEPRESSIVE-LIKE BEHAVIORS IN MALE RATS WITH REDUCED EXPRESSION OF 5αR1 OR 5αR2 IN THE PFC:

Human studies of 5αR2 expression levels suggested that this enzyme was implicated in the pathophysiology of MDD. Also, in our animal models of depression induced with social stress, and chronic unpredictable mild stress, I observed that levels of 5αR2 were reduced in both the PFC and NAc. Thus, in order to assess whether decreased expression of this enzyme resulted in a depressive phenotype, we used 5αR1 and 5αR2 KD rats to determine whether these enzymes contribute to the onset of depressive-like behaviors. For these experiments we used male rats (n=10).

In rats with KD of 5αR2 in the PFC, two-way RM ANOVA analyses revealed a significant reduction in sucrose consumption compared to the control group (Interaction: $F_{7,126}$=4.97, p<0.0001 ****; Time: $F_{7,126}$=0.22, p>0.05 n.s.; KD: $F_{1,18}$=5.60, p=0.02 *; Subject: $F_{18,126}$=13.42, p<0.0001 ****. Sidak's post-hoc comparison h 72 p=0.03 *, h 96 p=0.04 *, h 144 p=0.04 *, h 168 p=0.02 *).

No significant differences were observed between rats with KD 5αR1 in the PFC and the control group (Interaction: $F_{7,126}$=0.66, p>0.05 n.s.; Time: $F_{7,126}$=0.24, p>0.05 n.s.; KD: $F_{1,18}$=0.004, p>0.05 n.s.; Subject: $F_{18,126}$=19.46, p<0.0001 ****) (Fig. 28). Two-way RM ANOVA showed no significant differences in NIH test for both groups of KD rats (5αR1:

Figure 28

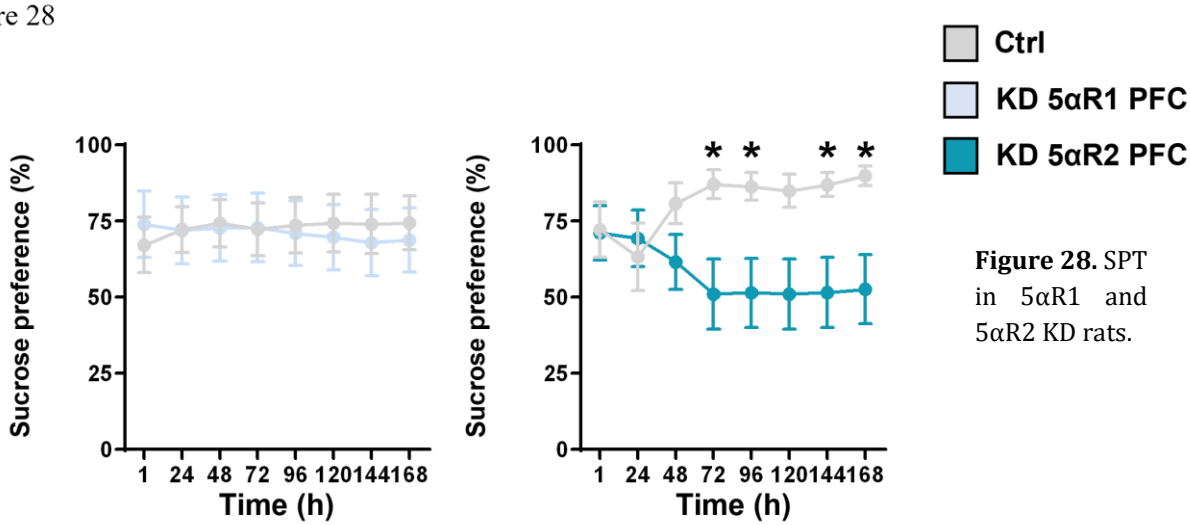


Figure 28. SPT in 5αR1 and 5αR2 KD rats.

Interaction $F_{1,18}$=0.48, p>0.05 n.s.; Time: $F_{1,18}$=35.73, p<0.0001 ****; KD: $F_{1,18}$=1.19, p>0.05 n.s.; Subject $F_{18,18}$=6.24, p=0.0002 ***. 5αR2: Interaction $F_{1,18}$=0.64, p>0.05 n.s.; Time: $F_{1,18}$=28.26, p<0.0001 ****; KD: $F_{1,18}$=0.05, p>0.05 n.s.; Subject: $F_{18,18}$=10.16, p<0.0001 ****) (Fig. 29).

Figure 29

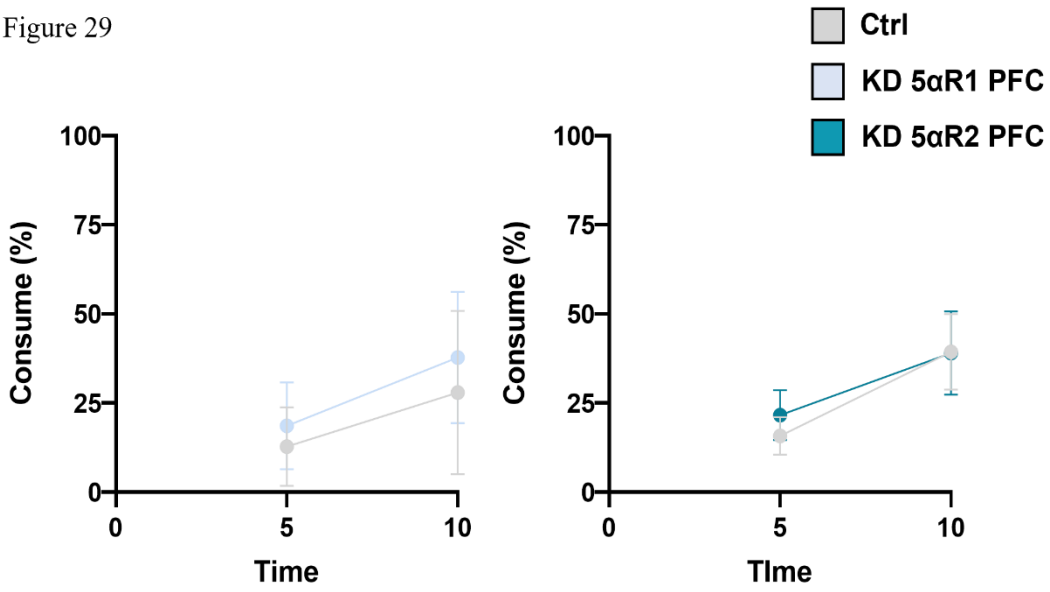


Figure 29. NIH in 5αR1 and 5αR2 KD rats.

In the FST one-way ANOVA revealed a significant increase in immobility time in KD 5αR2 PFC rats compared to KD 5αR1 PFC, and control groups ($F_{2,21}$=6.96, p=0.003 **; Sidak's post-hoc comparison: KD 5αR2 PFC vs CTRL p=0.006**, KD 5αR2 PFC vs KD 5αR1 PFC p=0.01 *) (Fig. 30).

Figure 30

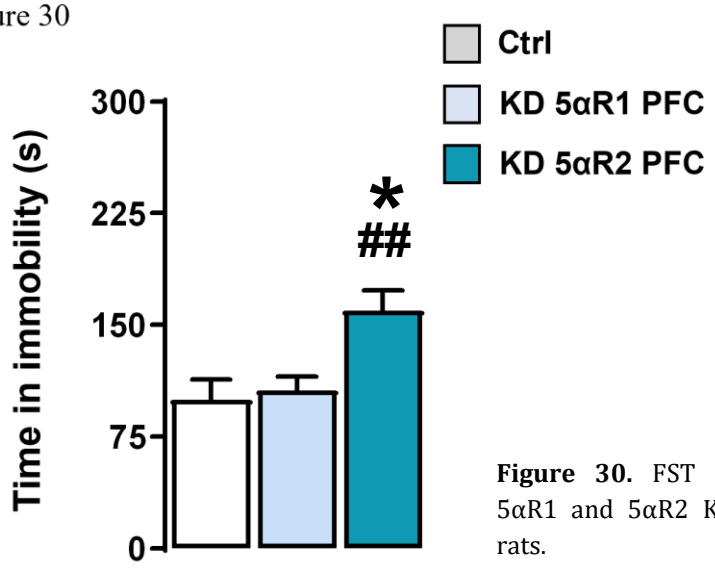


Figure 30. FST in 5αR1 and 5αR2 KD rats.

DEPRESSIVE-LIKE BEHAVIORS IN MALE RATS WITH REDUCED EXPRESSION OF 5αR1 OR 5αR2 IN THE NAC:

Based on our previous results in human samples and stress models of depression, in order to assess whether lower expression of 5αR2 in NAc resulted in depressive phenotypes, I knocked-down this enzyme in this area of male rats (*n*=10). I also used 5αR1 KD rats to determine whether this enzyme contributed to the onset of depressive-like behaviors. In rats with KD of 5αR1 in the NAc, two-way RM ANOVA revealed no significant differences compared to the control group for sucrose consumption (Interaction: $F_{7,126}$=0.18, p>0.05 n.s.; Time: $F_{7,126}$=1.82, p>0.05 n.s.; KD: $F_{1,18}$=0.85, p>0.05 n.s.; Subject: $F_{18,126}$=18.21, p<0.0001 ****). Whereas rats with KD of 5αR2 in the NAc showed a decrease in sucrose consumption compared to the control group, significantly different at 120h and 144h (Interaction: $F_{7,126}$=2.63, p=0.001 *; Time: $F_{7,126}$=0.65, p>0.05 n.s.; KD: $F_{1,18}$=5.35, p=0.03 *; Subject: $F_{18,126}$=5.91, p<0.0001 ****. Sidak post-hoc comparison H120 p=0.01 *, h 144 p=0.03*) (Fig.31).

Figure 31

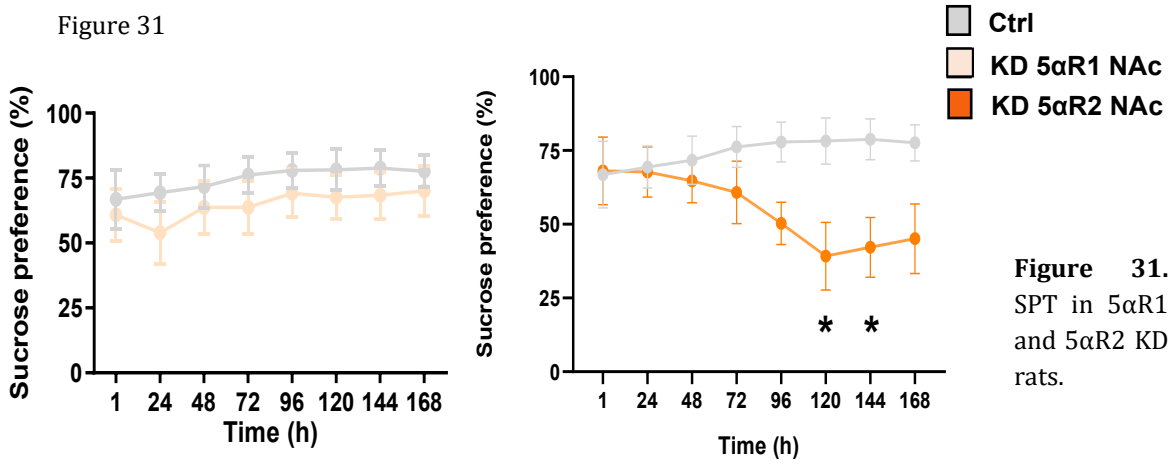


Figure 31. SPT in 5αR1 and 5αR2 KD rats.

In the NIH test, two-way RM ANOVA showed no significant differences between KD groups and control groups at 5 minutes, yet at 10 minutes a significant reduction in food consumption was demonstrated in 5αR2 KD rats compared to the control group (5αR1:

Interaction: $F_{1,18}$=0.62, p>0.05 n.s.; Time: $F_{1,18}$=48.80, p<0.0001 ****.; KD: $F_{1,18}$=0.12, p>0.05 n.s.; Subject: $F_{18,18}$=10.12, p<0.0001 ****. 5αR2: Interaction: $F_{1,18}$=24.41, p=0.0001 ***; Time: $F_{1,18}$=32.48, p<0.0001 ****; KD: $F_{1,18}$=4.17, p>0.05 n.s.; Subject: $F_{18,18}$=15.67, p<0.0001 ****. Sidak post-hoc comparison 10 min p=005 **) (Fig. 32).

Figure 32

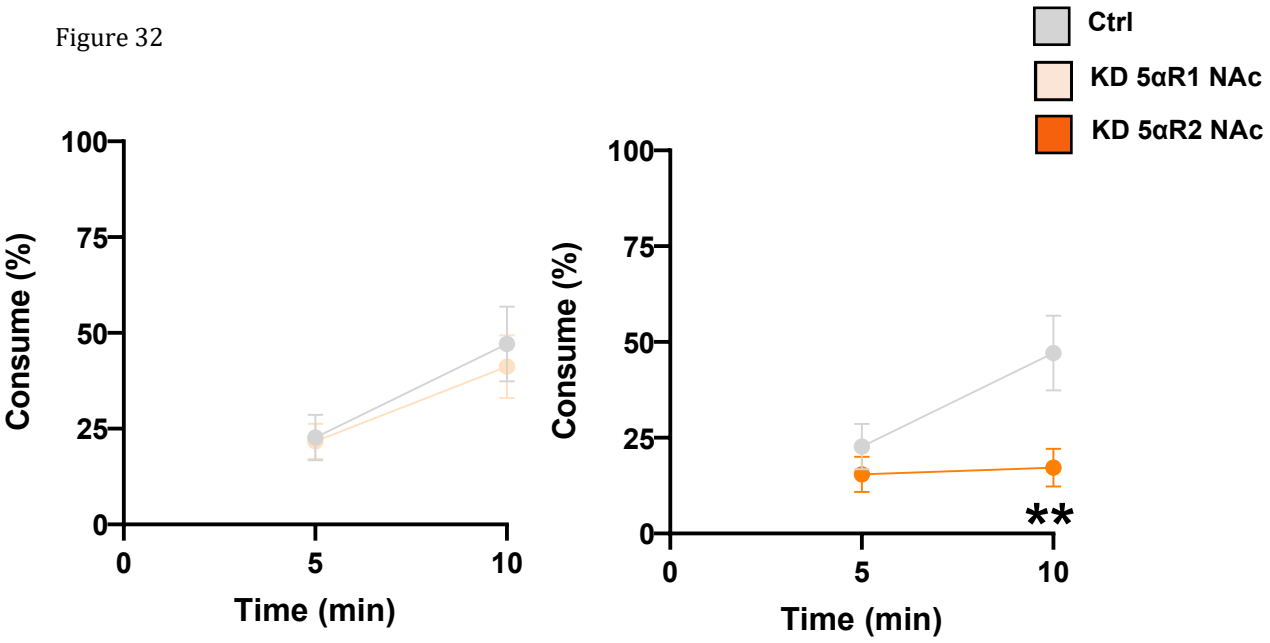


Figure 32. NIH in in 5αR1 and 5αR2 KD rats.

Figure 33

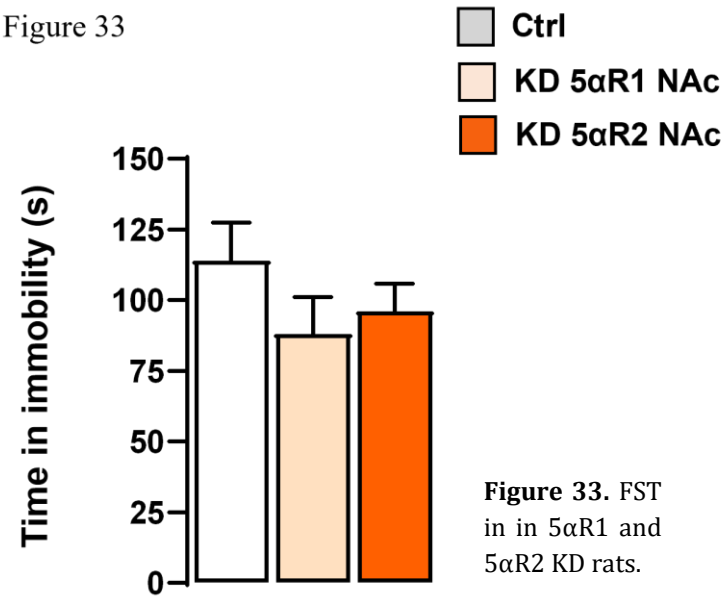


Figure 33. FST in in 5αR1 and 5αR2 KD rats.

Analysis with One-way ANOVA revealed no differences between the different groups in the FST ($F_{2,21}$=0.26, p>0.05 n.s.) (Fig. 33).

DEPRESSIVE-LIKE BEHAVIORS IN FEMALE RATS WITH REDUCED EXPRESSION OF 5αR2 IN THE PFC:

Since the levels of expression of 5αR2 are reduced in stressed female rats, I investigated the role of this enzyme with KD procedures in female rats (*n*=8). All tests were performed during the estrous cycle. Two-way RM ANOVA showed no significant differences in sucrose consumption between the KD and the control group (Interaction: $F_{7,84}$=1.41, p>0.05 n.s.; Time: $F_{7,84}$=1.26, p>0.05 n.s.; KD: $F_{1,12}$=0.51, p>0.05 n.s.; Subject: $F_{12,84}$=10.83, p<0.0001 ****) (Fig. 34). Two-way RM ANOVA did not reveal any differences in the NIH test between the

Figure 34

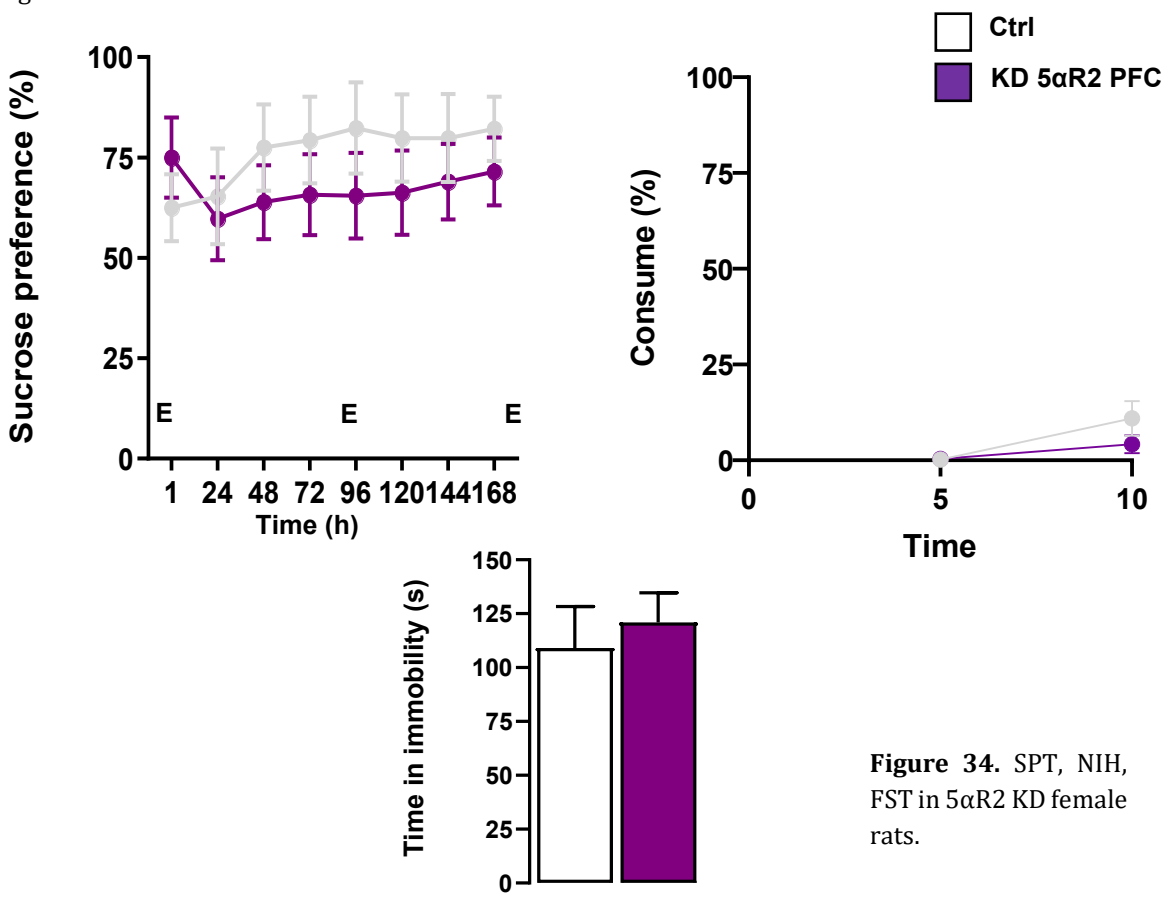


Figure 34. SPT, NIH, FST in 5αR2 KD female rats.

groups as well (Interaction: $F_{1,11}$=2.31, p>0.05 n.s.; Time: $F_{1,11}$=10.36, p=0.008 **; KD: $F_{1,11}$=1.97, p>0.05 n.s.; Subject: $F_{11,11}$=1.07, p>0.05 n.s.) (Fig. 35). In the FST, T-Test revealed no significant differences between groups (T=0.49, p>0.05 n.s.) (Fig. 34).

Depressive-like behaviors in female rats with reduced expression of 5αR2 in the NAc:

To assess the role of 5αR2 in the NAc of female rats, I evaluated depressive-like behaviors in 5αR2 KD and control rats (*n*=8). Two-way RM ANOVA and T-Test of behavioral tests did not show differences between groups in the SPT, NIH, and FST (SPT: Interaction: $F_{7,84}$=2.60, p=0.01 *; Time: $F_{7,84}$=1.45, p>0.05 n.s.; KD: $F_{1,12}$=0.40, p>0.05 n.s.; Subject: $F_{12,84}$=85.69,

Figure 35

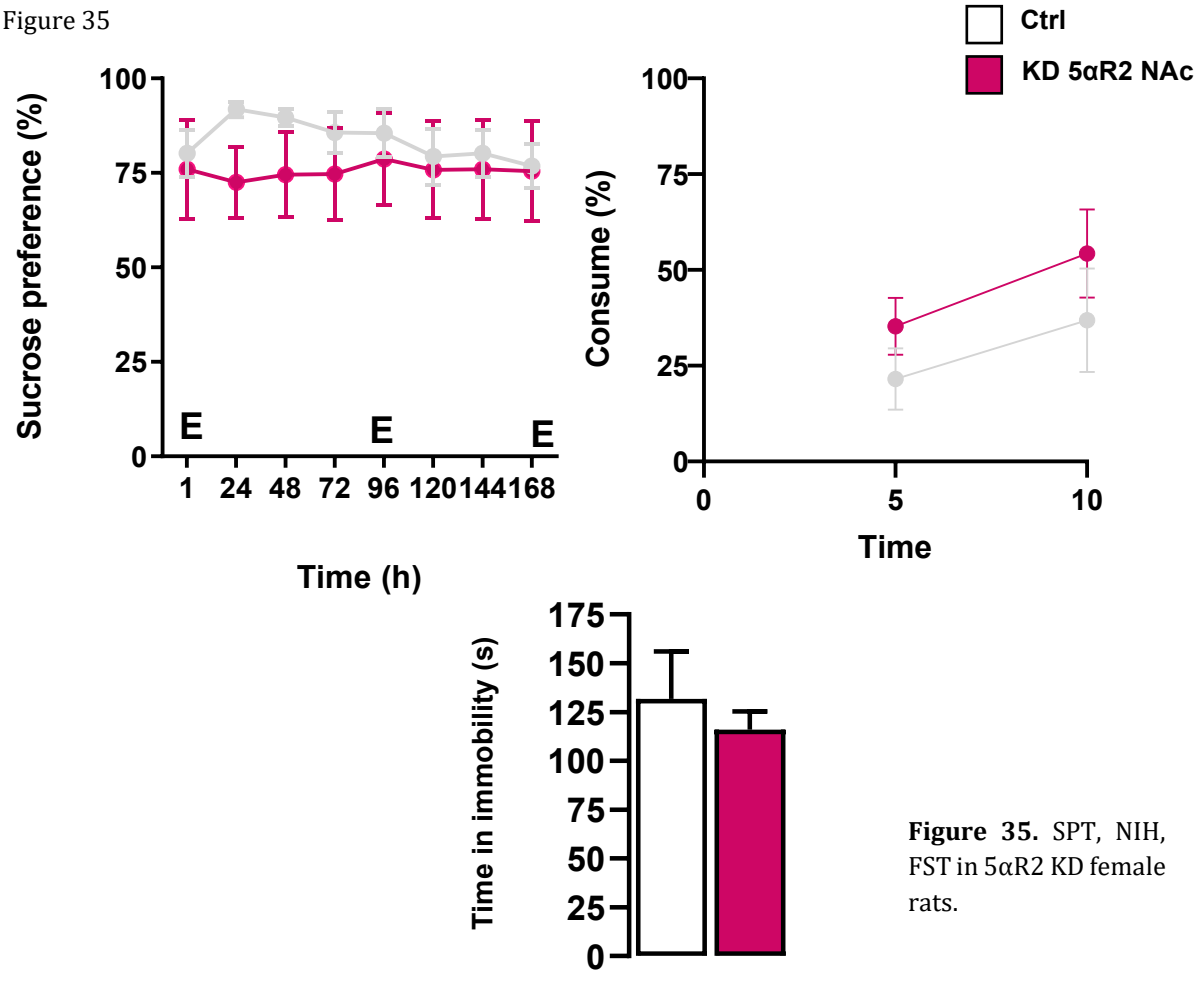


Figure 35. SPT, NIH, FST in 5αR2 KD female rats.

p<0.0001 ****; Sidak post-hoc comparison not significant. NIH: Interaction: $F_{1,11}$=0.14, p>0.05 n.s.; Time: $F_{1,11}$=12.22, p=0.005 **; KD: $F_{1,11}$=1.93, p>0.05 n.s.; Subject: $F_{11,11}$=8.47, p=0.0007 ****. FST: T= 0.49, p>0.05 ns) (Fig. 35).

VALIDATION OF 5αR1 AND 5αR2 KD ON THE PFC AND NAC OF MALE RATS:

To further validate our KD model, at the end of behavioral tests I measured the expression levels of 5αR1 and 5αR2 in the PFC and NAc of KD and control male rats (Fig. 36).

T-Test analyses confirmed a reduction in levels of 5αR1 (PFC: T=4.24, p=0.01*; NAc: T=3.09, p=0.03 *) and 5αR2 (PFC: T=2.85, p=0.04*; NAc T=3.88, p=0.01 *) in KD rats compared to control group in both areas.

Figure 36

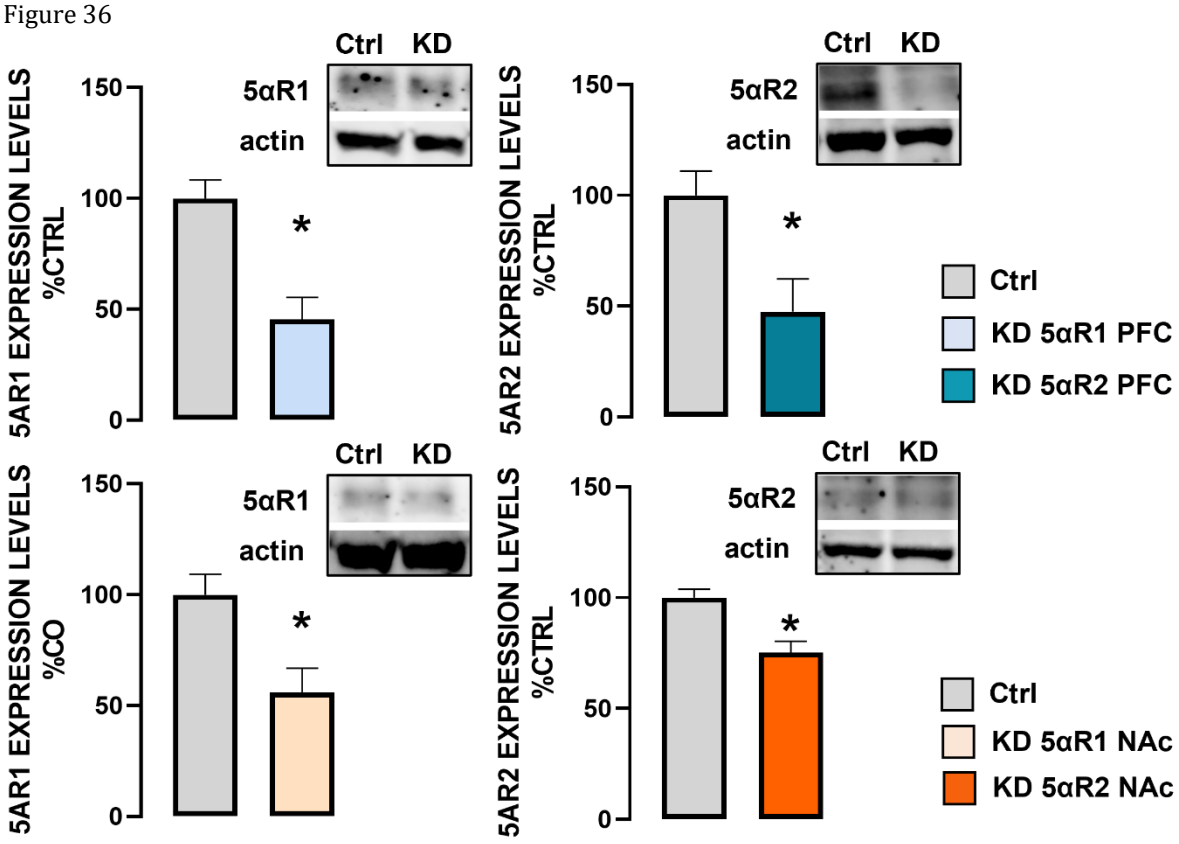


Figure 36. 5αR1 and 5αR2 expression levels in KD and CTRL male rats.

I also investigated the spread of the viral vector via immunofluorescence (Fig. 37). Confocal analysis of PFC and NAc slices confirmed that the virus spread was mostly confined to these two areas.

Figure 37

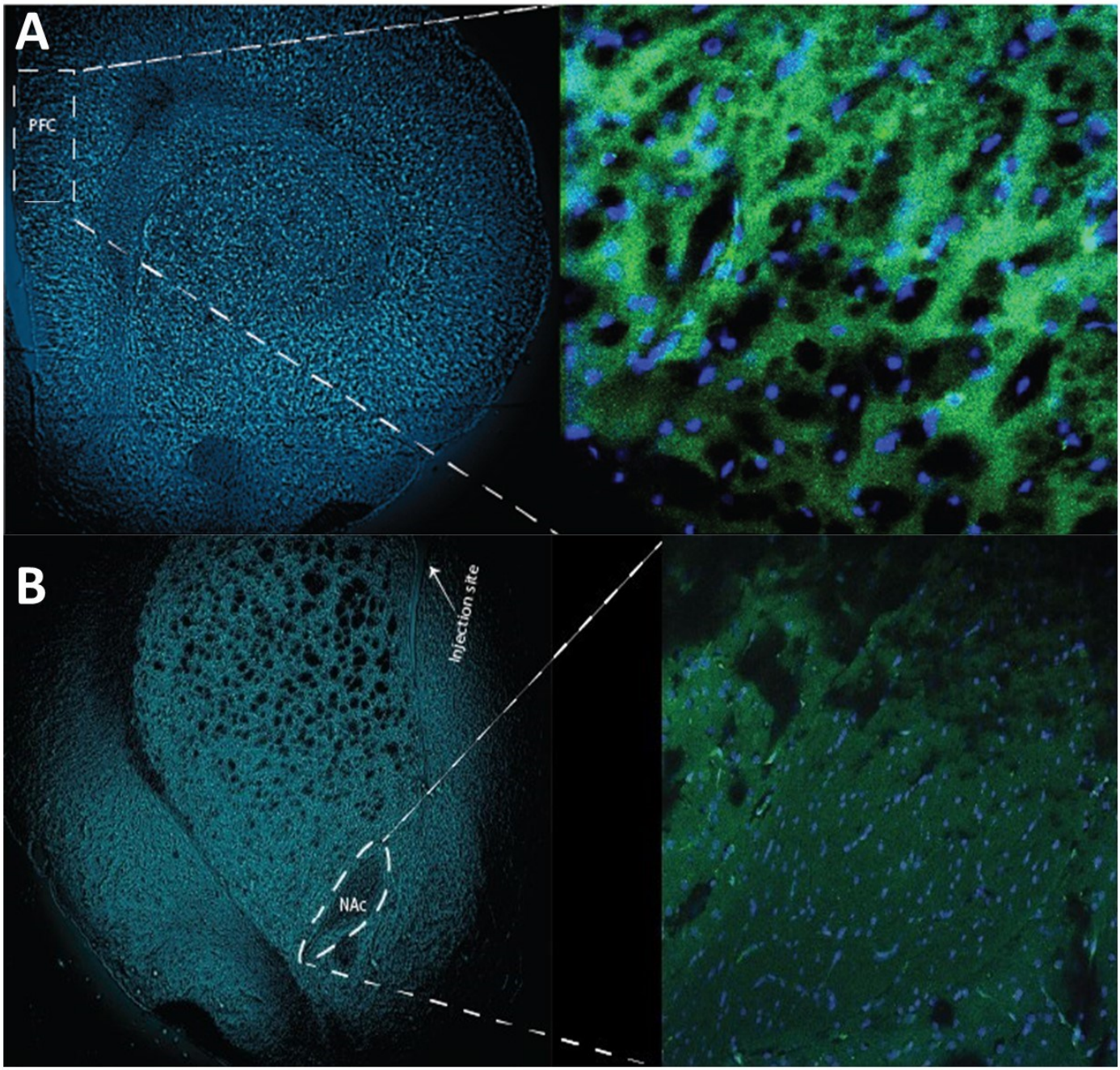


Figure 37. A) Slide representing PFC (2x) on left, on the right magnification of the PFC area (20x): green color indicates GFP spread and blue color indicates nuclei. B) Slide representing NAc (2x) on left, on the right magnification of the NAc area (20x): green color indicates GFP spread and blue color indicates nuclei.

DICUSSION

In this part of the study, I addressed the role of 5αR enzymes in the pathophysiology of MDD in animal models.

Both of stress models of MDD that we analyzed (CMS and SD) revealed a clear depressive-like phenotype, with significant differences between the CMS or SD groups compared to their respectively control groups in three of the most common tests used to assess depressive-like behavior (SPT, NIH, FST). SPT and NIH evaluate anhedonia and reward-driven behaviors. NIH also evaluates anxiety, a common component of MDD. FST measures coping strategies to an inescapable stress, and it aims to reproduce what happens in humans forced to live a under particular unavoidable stressful situations across the lifespan.

During the CMS experiments we also evaluated whether social isolation *per se* was sufficient to induce a depressive phenotype in rats. Our results showed no differences between the control groups (group-housed) and SH group in the SPT and NIH tests. Since the CMS group was composed of rat single-housed, SH group was chosen as the control group of CMS rats. In this way both groups have been maintained in the same experimental conditions. The immobility time in the FST in the SH group compared to the GH group was significantly different, which confirms that social isolation is a well validated method to induce a depressive-like phenotype in rodents. Yet no significant differences were found in FST between SH and CMS rats, probably because social isolation already has an impact on the reactivity to adverse environment and other mild stressors have no additional effects.

We also investigated whether SD and CMS influenced the levels of expression of 5αR1 and 5αR2 enzymes. Western blot analyses of these two enzymes revealed a significant reduction in the PFC, NAc and HIPPO. In particular, SD reduced the expression levels of 5αR2 in the NAc and 5αR1 in the HIPPO, and CMS reduced 5αR1 and 5αR2 expression in the PFC, and 5αR2 in the HIPPO of male rats. Female rats exposed to CMS showed a reduction of 5αR2 levels only in the PFC. As discussed above, the PFC, NAc, and HIPPO are three of the major areas implicated in the onset of MDD. Decreases in the levels of expression of 5αRs enzymes could change the levels of neurosteroids in these areas, which can ultimately affect neural activation or maintenance of connection pathways in the models of depressive behaviors used in this study. These data are in accordance with results obtained in brain samples of subjects with diagnosis of MDD, where 5αR2 levels were reduced in cortical areas (such as OFC and AAC) and NAc. Thus, these results support the hypothesis that lower levels of 5αR2 in the PFC and NAc may play a crucial role in the pathophysiology of MDD.

To further demonstrate that the reduced expression of 5αRs is linked to the induction of a depressive-like phenotype, I developed KD rats for 5αR1 or 5αR2 in the PFC and NAc. I confirmed that the KD groups had lower levels of specific 5αR enzyme via western blot and IF analyses. While in male rats reduced levels of 5αR2 in the PFC seem to be essential for the development of some depressive-like behaviors (see the behavioral response in the FST of 5αR2 KD male rats), the reduction in the levels of 5αR2 in the NAc seems to play a critical role in different depressive-like behaviors, such as the decreased reward-driven responses, and anxiety, as demonstrated in the SPT and NIH tests. This result could be related to the fact that the NAc is primarily involved in the cognitive processing of motivation, reward, aversion, and salience. On the other hand, the reduced expression of 5αR1 in the NAc or PFC

do not seem to be related to the development of a depressive-like phenotype. In fact, KD rats for 5αR1 did not show differences in their responses in the SPT, NIH, and FST compared to the control groups. Not only these results are aligned with our previous results in human MDD subjects, but they further address the critical role of 5αR2, but not 5αR1, in the onset of depression. The lower expression of 5αR2 in the PFC likely causes lower levels of neurosteroids. Since neurosteroids play an important role in neuronal excitability (Reddy, 2010), the lack of 5αR2 can lead to an inhibitory effect and consequently a disruption of connectivity between the PFC and related areas. This can explain what has been reported in human MDD subjects where connectivity networks are shown to be disrupted in the OFC (Zhou et al., 2020) and an overall inactivation has been reported in the ACC and OFC (Pizzagalli & Roberts, 2021). However, we still do not know which specific neurosteroids synthesis is affected in each different region by the reduced expression of 5αR2 and the specific mechanism that leads to a depressive-like state it is not yet demonstrated, thus inferences remain speculative.

Another crucial point that should be highlighted is the different scenario which emerges from the results obtained in female rats. In both stressed and KD female rats, behavioral tests and neurochemical analyses did not show differences compared to the control groups. The only exception is the reduction in 5αR2 levels in CMS female rats, that underlies again the fundamental role that this enzyme plays in the response to stressful situations which can lead to the onset of stress-related disorders. Since the levels of neurosteroids are affected by physiological process such as those related to ovarian cycle, we tested females rats only in the estrus phase. Nevertheless, we should repeat the same behavioral experiments in metestrus phase to assess whether 5αRs levels are influenced by the cyclic hormonal

changes. Also, we should study female rats with 5αR1 KD in the PFC or NAc to further evaluated whether or not the decreased expression of this enzyme plays a role in the onset of depressive behaviors, at variance with what I observed in male rats. As a matter of fact, female control rats have a higher value of immobility time at baseline in the FST compared to male control rats. For example, the female GH group spent 100 seconds in immobility in FST while the male GH group spent about 50 seconds. The same situation was reproduced in control female and male groups of 5αR2 KD rats. This represents for certain a crucial point. An explanation why we can't be able to appreciate any differences in female rats between control groups and stressed or KD groups could be related to the fact that since females have lower baseline levels of 5αR, it is difficult to determine further decreases. Since MDD is more common in female than in male, it could be speculated that women have a lower level of 5αR2 compared to men. Regardless, studies of gender differences in levels of 5αRs should be done in order to assess the existence of possible differences.

Summarizing, the results of my experiments demonstrated that the decreased expression of 5αR2 is crucial to induce a depressive-like phenotype in rats and point to plausible gender differences in the expression levels of this enzyme. In future studies I should assess whether overexpression of 5αR2 is sufficient to ease depressive-like behaviors and restore reactivity to aversive and rewarding stimuli. Moreover, since 5αR2 is responsible for the conversion of various neurosteroids, it will be relevant to investigate the levels of neurosteroids in the PFC via lipidomic analyses to further explore which of these compounds could be mainly linked to MDD.

CHAPTER 6
CONCLUSIONS

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

To summarize, the results of my thesis project showed that in brain samples from patients with diagnosis of two major psychiatric disorders (MDD and ASB) where 5αRs could play a pivotal role in pathophysiology, there were no changes in SRD5A1 and SRD5A2 transcriptomics, compared to the respective control groups, although these enzymes are involved in the regulation both of impulsivity and depressed mood.

On the contrary, protein levels of 5αR2 appear to be reduced in MDD patients, but not in ASB patients, in particular brain areas such as the OFC, AAC, and NAc. Moreover, it is worthy to highlight that in male patients lower levels of 5αR2 showed a negative correlation with the severity of depressive symptoms according to the Hamilton Rating Scale for Depression. To verify whether the reduced expression of 5αR2 was also present in animal models of depression, I exposed Long-Evans rats to social stress, social isolation, or chronic mild stress and then tested them for depressive like behaviors and measured 5αRs levels in some critical areas involved in MDD (including the PFC and NAc). Interestingly, along with the development of depressive phenotypes, these rats displayed lower levels of expression of 5αR2, mostly in the PFC region. As in human samples, lower 5αR2 levels were observed in female rats, which indicates again the possibly involvement of hormonal changes between the sexes. At this point, in order to test whether 5αR2 played a key role in depressive-like phenotypes, I induced a reduction in the expression of this enzyme via gene knock-down in

rats. Male rats showed clear depressive-like responses while female rats showed a blunted phenotype. These results are probably related to the lower baseline levels of 5αR2 in female rats that, even after exposure to repeated stressful stimuli, do not further significantly decrease, showing a ceiling effect. Also, it is plausible that male and female rats have different baseline levels of neurosteroids, and a dysregulation in neurosteroid synthesis could result in different behavioral responses between the sexes.

These results also indicate that the regions clearly associated with depressive-like behaviors and decreased 5αR2 levels are the cortical regions and the NAc. As said before, the PFC is implicated in the onset of depression and primarily responsible for cognitive/affective symptoms, whereas the NAc is mostly involved in reward-driven behaviors. Since neurosteroids (especially AP) play a major role in activation of $GABA_A$Rs, this process could lead to an hypoactivation of cortical areas and ultimately to a disruption in the PFC connectivity. Same could be true for the NAc. Lower activity or expression of 5αR2 induced by exposure to stressful stimuli may also lead to antidopaminergic effects (Bortolato et al., 2008, 2022; Godar, Cadeddu, et al., 2019; L. Li et al., 2018; Quessy et al., 2021) in the mesocortocolimbic dopaminergic pathway which connects cortical areas and the NAc. We can speculate then, that a combination of dysregulation of GABAergic and dopaminergic systems may lead to the onset, or the exacerbation, of depressive symptoms in humans and in rodent models of depression. We can hence hypothesize that low expression of 5αR2 is correlated with low mood typically present in most depressive patients. Then, increased levels of 5αR2 could not only improve mood, but could be implicated in a state of mind characterized by excitement, high energy, and euphoria typically observed in patients displaying manic episode or with diagnosis of mania. In fact, in preliminary studies

performed in human samples of bipolar patients with or without manic phase, I observed higher levels of 5αR2 in subjects with diagnosis of manic episodes (data not shown). This data confirms and further supports the importance of this enzyme in balancing mood phases and maintaining a stable mood.

Further issues that in the future should also be addressed are whether the enzymatic activity of 5αR2 is modified along with its expression, and whether a reduction in activity is sufficient to determine the manifestation of depressive phenotypes. Of course, lower 5αR2 expression levels can be translated into a lower enzymatic activity compared to the baseline, but still, it is crucial to establish which specific substrate and product of synthesis (if any) is more affected by the reduced enzymatic activity. Preliminary analyses conducted in 5αR1 and 5αR2 KD Long-Evans rats in the PFC showed a significant decrease in the levels of AP and isoallopregnanolone (IsoAP) in the 5αR2 KD group compared to the control group (data not shown). This is particularly relevant because low AP levels are known to be critical in depression and higher AP levels have been reported after treatment with SSRIs (Almeida et al., 2020), and antidepressant effects of AP are also well known (Khisti et al., 2000; Rodríguez-Landa et al., 2007). Moreover, in MDD patients changes in the levels of neurosteroids, such as DHT and DHEA, were reported (McHenry et al., 2014; Weber et al., 2000) and their depressogenic role could be associated with different expression or activity of 5αR2. In order to experimentally ascertain the role of AP, we should reinstate the levels of AP in 5αR2 KD rats and test whether this is necessary and sufficient to ease depressive-like behaviors.

Despite the limitations of the present results (e.g., I should validate whether 5αR2 levels are different between sexes, determine the enzymatic activity, further investigate which neurosteroid is primarily involved in MDD and in animal models of depressive symptoms), 5αR2 can be seen as a potential therapeutical target for the treatment of depression. At the same time, if it will be demonstrated that peripheral changes of 5αR2 are also correlated with MDD, levels of this enzyme could be used as a disease index allowing a diagnosis of MDD before the overt onset of symptoms and offering more chances for a successful treatment.

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