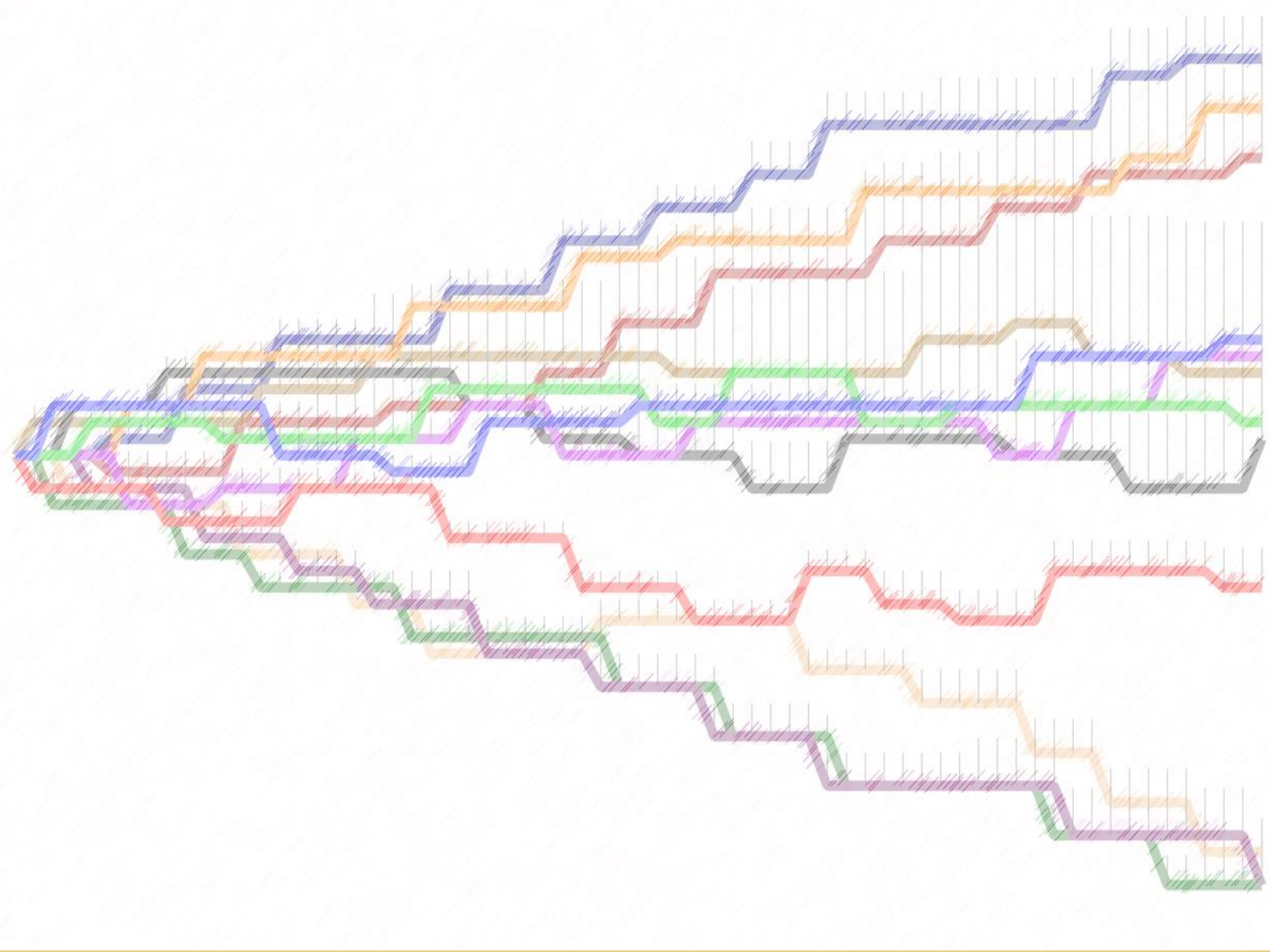

Renato José Melo Sousa

Dissection of hierarchy formation in mice: behavioral and molecular correlates of dominance

Dissertação de mestrado em Biologia Celular e Molecular com especialização em Neurobiologia

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UNIVERSIDADE DE COIMBRA

Dissection of hierarchy formation in mice: behavioral and molecular correlates of dominance

Dissecção da formação de hierarquias sociais em murganhos: correlações comportamentais e moleculares de dominância

Dissertação de mestrado em Biologia Celular e Molecular com especialização em Neurobiologia, orientada por João Peça e Ana Luísa Carvalho e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

Renato José Melo Sousa

Coimbra, 2016

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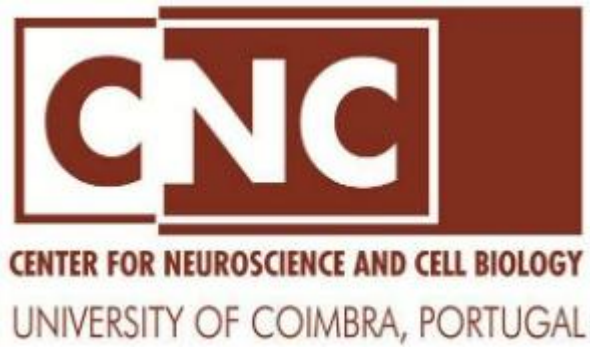

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“We can complain because rose bushes have thorns,

or rejoice because thorn bushes have roses.”

Abraham Lincoln

“Why does the eye see a thing more clearly in dreams

than the imagination when awake?”

Leonardo da Vinci

“Invincibility lies in the defense;

the possibility of victory in the attack.”

Sun Tzu

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Resumo

Animais de espécies sociais interagem entre si para desenvolverem relações de dominância que são fundamentais para a estratificação social do grupo, e que levam à formação de uma hierarquia social. Uma vez estabelecidas, as hierarquias de dominância impõem ao grupo um conjunto de normas sociais aos seus membros, como por exemplo o acesso a recursos alimentares, locais de repouso e a parceiros sexuais. No entanto, sabe-se muito pouco sobre as correlações comportamentais e genéticas que poderão prever se um animal irá possuir uma probabilidade aumentada ou diminuída para desenvolver um fenótipo dominante ou submisso no seu grupo social.

Neste trabalho, pretendemos caracterizar a formação de hierarquias sociais em murganhos, usando uma metodologia de torneiro "Round-Robin" no "tube test". Apesar das hierarquias naturais serem confinadas dentro de grupos e são, portanto, fortemente suportadas por fatores relacionados com o grupo, algumas vezes são observadas relações de dominância entre grupo distintos. O método "Round-Robin" permitiu-nos observar uma melhor distinção entre comportamentos dominantes e submissos, e também avaliar a capacidade intrínseca dos animais para adquirirem dominância fora da sua caixa de origem. Observamos que, nestas situações, a formação da hierarquia no "Round-Robin" reflete a hierarquia dentro de cada grupo individual e que é mais influenciada por dinâmicas temporais e fatores intrínsecos ao animal do que por eventos estocásticos.

No decorrer da nossa investigação não identificamos características comportamentais evidentes que fossem preditivas de classe hierárquica. Contudo, expor os animais ao "tube test" promoveu alterações no desenvolvimento normal de comportamentos de forro social e depressivo. Mais importante, demonstramos pela primeira vez que os níveis de expressão do receptor tipo 1 do neuropeptídeo Y (Npy1r) no córtex medial pré-frontal (mPFC) estão inversamente correlacionados com dominância. Deduzimos que o comportamento submisso poderá estar ligado a um défice de controlo excitatório cortical sobre regiões subcorticais mediado pelo Npy1r. Também verificamos uma tendência para uma expressão aumentada do marcador cFos ("immediate-early gene") no mPFC de indivíduos submissos. Não obstante, este último resultado requer futura caracterização para compreendemos que tipo de atividade neuronal poderá estar a ser refletida pela expressão de cFos.

Palavras-chave: Hierarquia social, torneio "Round-Robin" comportamento animal, dominância, Npy1r.

Abstract

Animals from social species interact with each other to develop dominance relationships that are fundamental for social stratification of the group and that lead to the formation of a social hierarchy. Once established, dominance hierarchies impart the group with social norms for its members, such as access to food resources, nesting places and sexual partners. Nevertheless, very little is known on the genetic and behavioral correlates that may predict if an animal will have an increased or decreased probability towards developing a dominant or submissive phenotype in its social group.

In this work, we aimed at further characterizing the formation of social hierarchies in mice, using a Round-Robin Tournament methodology in the tube test. Although natural hierarchies are confined within groups and are, therefore, strongly supported by group-related factors, occasional intergroup dominance relationships are observed. The Round-Robin method allowed us to observe a better distinction between dominant and submissive behaviors, and also to assess the intrinsic capability of animals to acquire dominance outside of their original home cage. We observed that, in these situations, hierarchy formation in the Round-Robin reflects the hierarchy within each individual group and that it is influenced more by temporal dynamics and animal intrinsic factors than by stochastic events.

In the course of our investigation we did not identify overt behavioral traits that could be predictive of hierarchical rank. However, subjecting animals to tube test did promote alterations in the normal development of social- and depressive-related behaviors. More importantly, we show for the first time that the expression levels of the type 1 neuropeptide Y receptor (Npy1r) in the medial prefrontal cortex (mPFC) are inversely correlated with dominance. We hypothesize that submissive behavior may be linked to an Npy1r-mediated impairment of cortical excitatory control over subcortical regions. We also find a trend towards increased expression of the immediate-early gene cFos in the mPFC of subordinate individuals. Nevertheless, this later result requires further characterization to understand which type of neuronal activity may be reflected by such cFos expression.

Keywords: Social hierarchy, Round-Robin Tournament, animal behavior, dominance, Npy1r.

Chapter I | Introduction

Social hierarchy

Hierarchy can be defined as a system in which members are ranked according to their relative status or rank. In nature, this stratification is mostly established by aggressive-submissive encounters among members of the same community[1], and the main consequence of stratification is restricting acceptable behaviors, allocating roles and imposing a collection of social norm[1–7]. Additionally, in psychological terms, dominance has also been defined as a mental state in which someone feels under or in control of others[2].

Aggression strongly characterizes social hierarchies during unstable phases, such as initial hierarchy formation or whenever there is the need to reestablish it (removal and addition of an individual, death of a member, trespassing social norms)[1]. Triangular loops and circular components of dominance orders are common during these phases, but they tend to linearize and lead to more stable hierarchies **(Figure 1)** without the previous aggressiveness. This results in higher efficiency of the group and this stabilization may be described as social inertia[1,6].

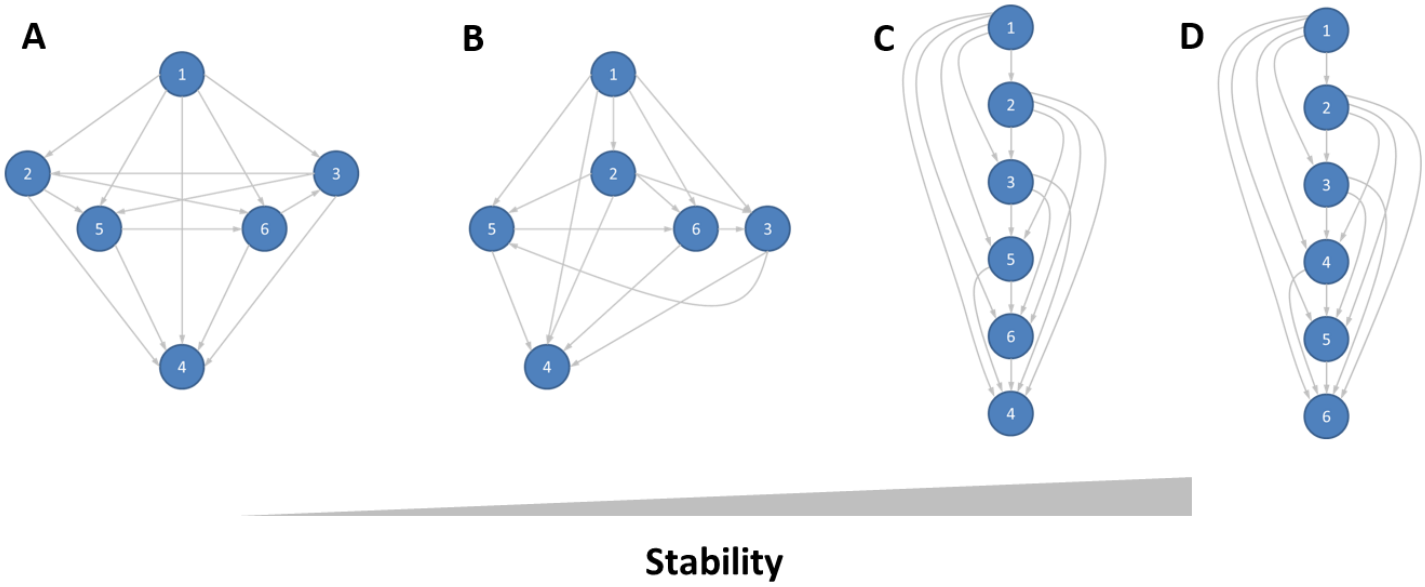


Figure 1 – Transitivity in hierarchy is directly related to stability. (A and B) Circular loops characterize unstable hierarchies in early phases. **(C and D)** Hierarchy linearization over time confers stability; arrows represent directionality of dominance and numbered circles represent different individuals. Adapted from E. O. Wilson, (2000).

The first well documented study regarding social organization in vertebrates was performed by the Norwegian zoologist and psychologist Thorleif Schjelderup-Ebbe in 1921[1,6]. Thorleif observed an arrangement within a group of domestic fowls where some individuals (the ones with the higher status) had priority in accessing food, choosing resting places and sexual partners. He gave this hierarchical distribution of primacies the term “pecking order”, proposing that such organization improved group dynamics and fitness by reducing the number and intensity of conflicts[6,8].

In addition to aggressiveness, individual *characteristics* such as: size, age, gender, kinship and personality, are potential factors that influence hierarchy formation across various animal societies. In most primate societies, older primates tend to occupy top ranks and the descendants of high-ranking mothers often

belong to the same rank as their mothers. However, males might move further up in the hierarchy, whereas females usually do not[1]. In fact, most mammalian societies are patriarchal and males usually occupy dominant roles over females. Moreover, animals that repeatedly win dominance encounters are more likely to succeed in subsequent matches; this influences who will acquire high or low positions in the hierarchy[1]. This observation is colloquially referred to as the *winner effect* [9,10].

Importantly, timid members are associated with subordination[1,11] and several studies have revealed evidence that different behavioral traits may also influence and confine dominance expression to certain levels. Experiments on Flinders Sensitive Line (FSL) rats, a well characterized animal model of depressive-like behaviors, point towards low dominance acquisition in this specific strain[12–14]. Similarly, others have characterized submissive behavior in rat and mouse as suitable for depression-like behavior models[15,16]. Furthermore, social dominance is asymmetrically linked to both high and low-social behavior displayed by *OPRM1 A112G* and *Shank3* animal models, respectively[17,18]. Moreover, both *Shank3* mutations mouse models also expressed anxiety-like behaviors[17], whereas highly-anxious rats were shown to display low-levels of social dominance[19].

Notwithstanding, extrinsic factors such as *serendipity and stochastic* events also affect hierarchical relationships amongst individuals. For example, an encounter between a subordinate individual and a fatigued high-ranked opponent exemplifies how hierarchical rank is dependent on a combination of intrinsic and extrinsic factors for each dyadic encounter[1]. Moreover, characteristics inherent to the group itself also influence local hierarchy. For instance, in a flock of fowls containing about ten animals, the hierarchy is usually linear and stable, which confers high group efficiency, while flocks containing a higher number of individuals have more circular loops and shift at a considerable higher rate, which is characteristic of unstable hierarchies[6,20]. Therefore, hierarchy formation results from an interplay between intrinsic and extrinsic factors.

Once settled, social tiering allocates each individual its dominance rank, norms and role in society[7], and the precise perception of these elements, with consequent adjustment of their behavior, is crucial for the proper functioning of the community and survival of the group under hazardous or challenging conditions[2].

Social hierarchy in humans and non-human animals

Human sociability manifests in several contexts comprising different groups and thus allows an individual the possibility to occupy different ranks across different social contexts. Therefore, humans tend to live social hierarchy in a multidimensional way, and attribute greater value to the one in which they rank the highest[5]. However, this does not imply that humans may not experience linearity across each different hierarchy.

Although it has been recently suggested that humans display two types of dominance personalities - *social dominance* and *aggressive dominance*; the latter reflecting the use of aggression, flattery, threat and

deceit to persuade the others[21] -, humans mainly use cognitive factors such as intelligence and reasoning to persuade others of their status[2]. Physical factors, such as body size, are more related to the perception of dominance in early young ages (i.e. around the age of ten months old)[22].

In animal societies, dominant individuals display higher fitness as a consequence of their perks within the community and facilitated breeding. This presents future generations of the species with genetic features of dominant individuals, thus social stratification in non-human animal societies can be considered as a process of natural selection[1]. In most animal societies, individuals live together within the same group and do not often exchange social interactions with members outside of that group (with exception of territorial conflicts and/or departure of some individuals)[1]. These constraints led Robert M. Sapolsky (2005) to claim that most non-human animal hierarchies are shaped in a linear and unidimensional way[5].

In these groups, social stratification and the process of its stabilization are accomplished mainly through physical strength and aggressiveness (including fighting and threatening)[2,5,23]. As such, a high incidence of stress correlates with social hierarchy rank, with the most subordinate individuals subjected to higher levels of stress[23]. However, in some circumstances, high rank individuals may in turn be those who experience the most stress **(Table 1)**[5]. Considering these dissimilarities between humans and other animals, it would be of particular difficulty to extrapolate studies with non-human animal models to human reality. Nevertheless, the socioeconomic status in human societies, defined by occupational position, income and instructional education[8], does indeed resemble the concept of social hierarchy of non-human animal groups.

Table 1 – Effect of societal characteristics on stress incidence along rankings. * Rank-related tendency. Adapted from Robert M. Sapolsky, (2005).

Societal characteristic	Individuals experiencing the most stress
Dominance style and means of maintaining despotic dominance	
Despotic hierarchy maintained through frequent physical reassertion of dominance	High-ranking
Despotic hierarchy maintained through intimidation	Low-ranking
Egalitarian hierarchy	*
Style of breeding system	
Cooperative	High-ranking
Competitive	*
Stability of ranks	
Unstable	High-ranking
Highly stable	Low-ranking
Availability of coping outlets for subordinates	
High availability	*
Low availability	Low-ranking
Ease with which subordinates avoid dominant individuals	
Easy avoidance	*
Difficult avoidance	Low-ranking
Availability of alternative strategies to overt competition	
Present	*
Lacking	Low-ranking
Personality	
Dominants perceive neutral interactions as challenging; subordinates take advantage of coping strategies	High-ranking
Dominants are adept at exerting social control and highly affiliative; subordinates are poor at exploiting opportunities for coping and support	Low-ranking

Social hierarchy and its influence on health

Dominant-subordinate relationships imply retaliations, both in a physical and direct manner, or in the case of humans, in a psychosocial fashion[5,24]. Incidence of health problems is directly correlated with stress prevalence across social ranks with the activation of the hypothalamus-pituitary-adrenal (HPA) axis and sympathetic nervous system as the main underlying causes[24]. This aspect affects a range of physiological systems comprising immune, reproductive, cardiovascular and neurological pathologies **(Figure 2)**[5,24,25].

Therefore, it is not surprising that socioeconomic status has been taken as the strongest single predictor of human health in western societies[5,8,26]. Thus, unveiling the neurobiological underpinnings of social hierarchy formation and maintenance is of particular relevance towards human health. However, "belonging" to a given socioeconomic rank *per se* does not seem to be what predicts the underlying cause for the incidence of health problems, but rather the "perception" of belonging to a specific rank. In other words, "feeling" submissive may explain why "being" submissive is predictive of high health problem incidence[5]. Nevertheless, little is known on the intrinsic and exogenous factors that function as social determinants of social ranking.

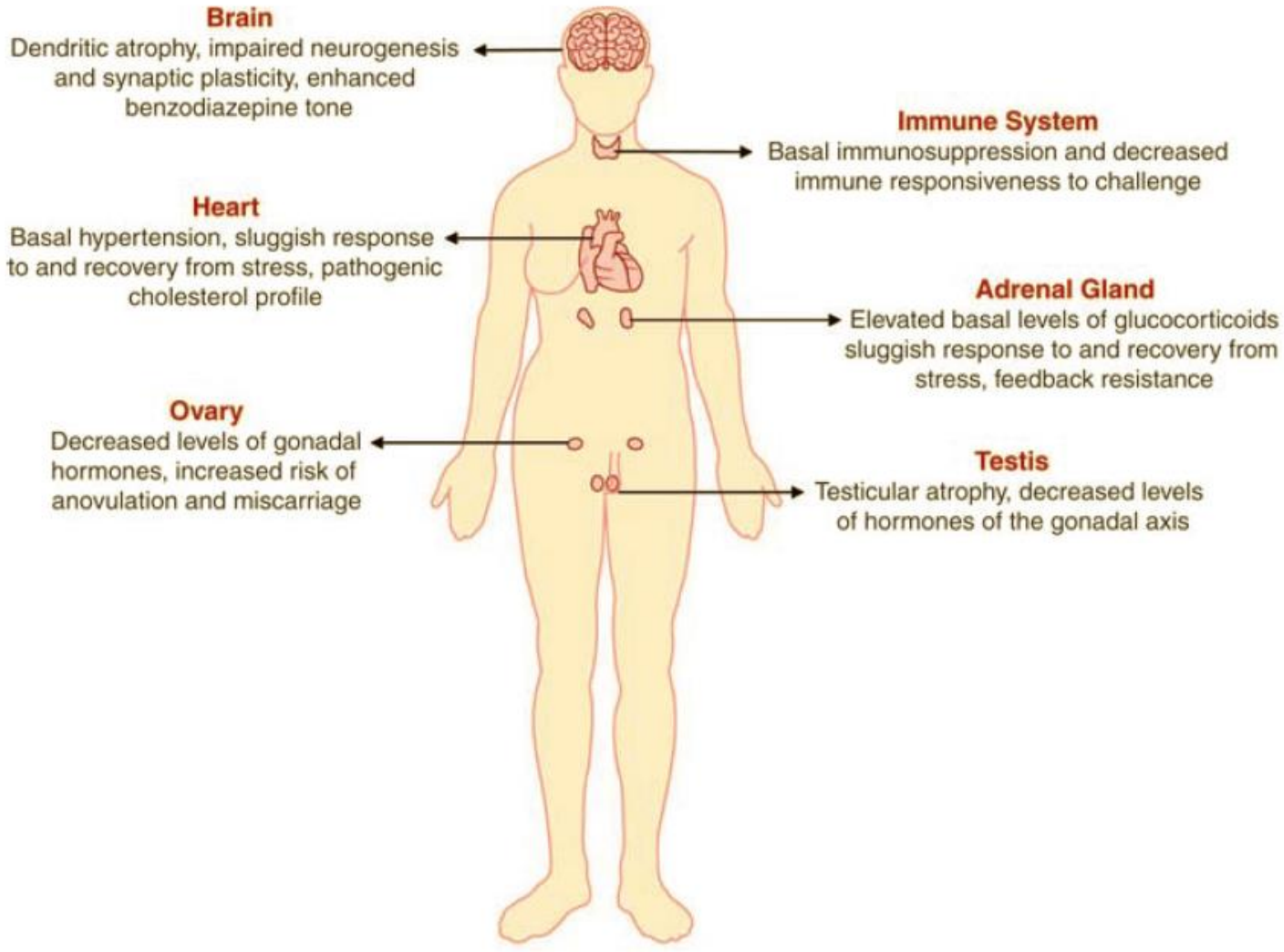


Figure 2 –Physiological consequences of a stressful social rank. Adapted from Robert M. Sapolsky, (2005).

Role of the medial prefrontal cortex

Zink *et al.* (2008) reported that when human participants face superior players (relatively to their own rank) in a social paradigm representing an unstable hierarchy, the mPFC was exclusively recruited[27]. By representing an unstable hierarchy, this paradigm allowed the participants upward and downward transitions across the hierarchy, mimicking what happens during hierarchy formation. An upward shift was attainable in the case of participants that had won against superior players and interestingly, the mPFC was significantly activated only during these circumstances. However, the mPFC was not activated neither during the non-social context of the same test paradigm, nor during a paradigm representing a stable hierarchy[27]. These results, together with the fact that the activity of this brain region is more directed to possible upward transitions on the hierarchy, suggest that the mPFC might play a prominent role in social hierarchy transitions.

On the contrary, activity on the ventromedial prefrontal cortex (vmPFC) was shown to correlate with inference of both social and non-social cues. In this study, human volunteers had to learn and associate value to an array of galaxies (non-social cue) and human faces (social cue) during a bid game. The confidence rate during ranking of the cues and monetary amount wanted to invest were measured[28]. Moreover, patients with lesions on this region were unexpectedly still able to properly attribute dominance to social dominance cues, although they appeared to use different strategies. These patients appeared to be insensible to some characteristics of the stimuli, such as gender and age (strong variables that report on dominance), rather than being completely incapable of performing social judgments. This led the authors to conclude that, more than an impairment in judging social cues (e.g. dominance cues), these patients are likely more insensitive to the social value of the cue itself[29]. Thus, the role of the vmPFC still remains largely unclear. Nonetheless, this raises the notion that within the mPFC, different subregions may underlie different behavioral roles in social hierarchy contexts and that local circuitry within brain regions should also be considered.

Naked mole-rats display a very distinct pattern of vocalizations when engaging with dominant and/or subordinate individuals. However, after a bilateral lesion in the mPFC, these animals vocalized similarly towards dominants and subordinates, leading to the conclusion that they were no longer able to recognize and distinguish hierarchical ranks[30]. In a similar study, R. Robert Holson (1986) observed that rats with a mesial prefrontal cortical lesion showed increased *timidity* accompanied by a lower social rank compared to the controls[11].

Social hierarchies comprise a detailed collection of social norms in accordance with the different social tiers, which influence the behavior of each individual within each tier[7]. Since these norms tend to vary across different cultures[31,32], it may be speculated that the perception of social hierarchical cues might be conducted differently in terms of neural activity and that the behavior within each tier can also be different. In support of this view, data from J. B. Freeman *et al.* (2009) showed that Japanese and American individuals recruit high mPFC activity when facing a subordinate and dominant cue, respectively[33]. This indicates that the activity of the mPFC seems to be related with the process of perceiving the cue itself, rather than with translating its

value. Taken together, these results clearly support the involvement of the mPFC as a brain region encoding social hierarchy through the perception of cues and hierarchy formation.

Using mice and more invasive laboratory procedures, Fei Wang *et al.* (2011) studied the role of the mPFC in coding social hierarchy[34]. In rodents, the mPFC can be divided from ventral to dorsal in four areas: the infralimbic (IL), the prelimbic (PL), the anterior cingulate (ACG) and the medial agranular (AGm) regions[35]. When testing layer V pyramidal neurons in the prelimbic area of the mPFC, they found that higher-ranking mice exhibited an increase in the amplitude of miniature excitatory post-synaptic currents (mEPSC). Given the fact that synaptic strength strongly influences the amplitude of the ionic/receptor current in this sort of procedures, and that the amount of synaptic AMPA receptors (AMPAR) strongly determines this strength, the authors aimed at manipulating social hierarchy by acting on the AMPAR-mediated synaptic strength. After stabilization of the hierarchy, a transition from lower to higher ranks was achievable by increasing the AMPA/NMDA receptors *ratio*. This study showed that adjustable molecular mechanisms in specific brain regions may influence behaviors responsible for hierarchical ranking.

The mPFC mediates and participates in the processes of both hierarchy perception and formation, raising a hypothesis were it might play a role as a central regulator **(Figure 3A)**. Several upstream brain regions carry information relative to the social ranking of others may converge in the mPFC (hierarchy perception) where, depending on those inputs, a response mediated by subcortical downstream regions **(Figure 3B)** responsible for the dominance behavior itself is triggered (behavioral activation)[8].

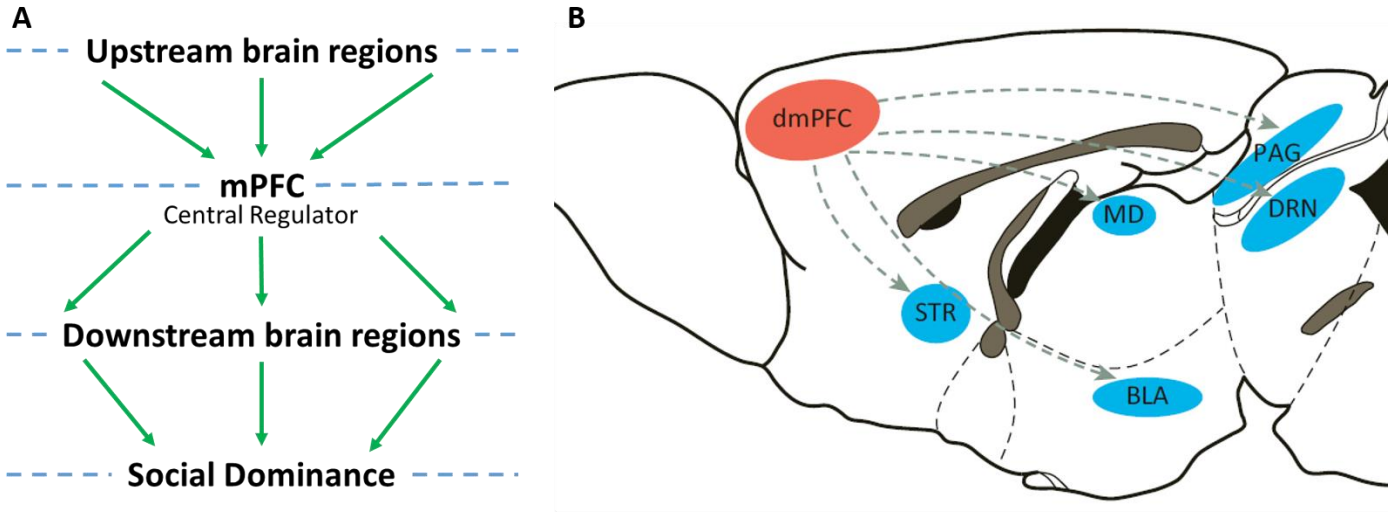


Figure 3 – Putative medial prefrontal cortex role as a central regulator for several subcortical brain regions responsible for dominance behaviors. dmPFC, dorsomedial prefrontal cortex; STR, striatum; BLA, basolateral amygdala; MD, mediodorsal nuclei of thalamus; PAG, periaqueductal grey; DRN, dorsal raphe nucleus. Adapted from Fei Wang *et al.,* (2014)

Cellular correlates of social dominance

Edward O. Wilson in *Sociobiology: The New Synthesis* (2000), uses a twist on one of Samuel Butler's aphorisms – "*A hen is only an egg's way of making another egg."* – to make: "*the organism is only DNA's way of making more DNA."* [1]. DNA determines molecular mechanisms that influence cellular dynamics, which in turn may confine brain functional connectivity and neuronal activity underlying organism's physiological processes responsible for behavior. Therefore, genetic load will most likely influence neuronal processing responsible for dominance gradient/social stratification.

Eduardo Dias-Ferreira *et al* (2009) observed that rats, subjected to a chronic unpredictable stress, display deficits in goal-directed behavior and atrophy of both prelimbic cortical layer 2/3 cells and dorsomedial striatal neurons[36]. Their protocol consists partially in a social defeat behavioral assay, which can be considered as forced aggressive-submissive encounter between animals. In another study, a psychosocial stress-related subordination methodology was used to assess similar cellular parameters. Dominance dyads of unfamiliar male tree-shrews were generated by inducing territorial conflict, followed by 28 days of unaggressive psychosocial stress. Subordinates were characterized by reduced apical branching and dendritic length in hippocampal CA3 pyramidal neurons as compared to controls **(Figure 4A)**[37]. The authors hypothesized that this effect might be due to an increased excitatory amino-acid release and therefore, used phenytoin - known to negatively interfere with excitatory amino-acid release - to try to counteract these observations. Phenytoin successfully prevented a decrease in apical branch points and apical length, suggesting that such cellular results are due to an increased excitatory amino acid release from mossy fibers.

Activation of the HPA axis appears to be inherent to social stratification, and thus, most of the dominance-related cellular findings may be due to biological processes for stress responses. Indeed, social rank in *Salmo irideus* negatively correlates with adenohypophysial cell synthetic activity and therefore, with adrenocorticotropic hormone (ACTH) production[38].

Yevgenia Kozorovitskiy and Elizabeth Gould (2004) used the visible burrow system (VBS) and verified that neurogenesis is also influenced by dominance hierarchies. In the dentate gyrus, dominant individuals had an increased number of BrdU-labeled cells when compared to subordinates **(Figure 4B)** and controls, and no differences were detected between controls and subordinates. At the same time, similar numbers of Ki-67 and Histone-H3 positive cells were observed in all conditions, suggesting that increased neurogenesis in dominants is probably due to an increased cell survival rather than proliferation[39].

Together, these studies suggest that cellular factors such as neuronal arborization and cell survival may be hallmarks of social hierarchies related to stress response. Nevertheless, it is not yet clear if the cellular alterations are a consequence or a cause towards of social dominance.

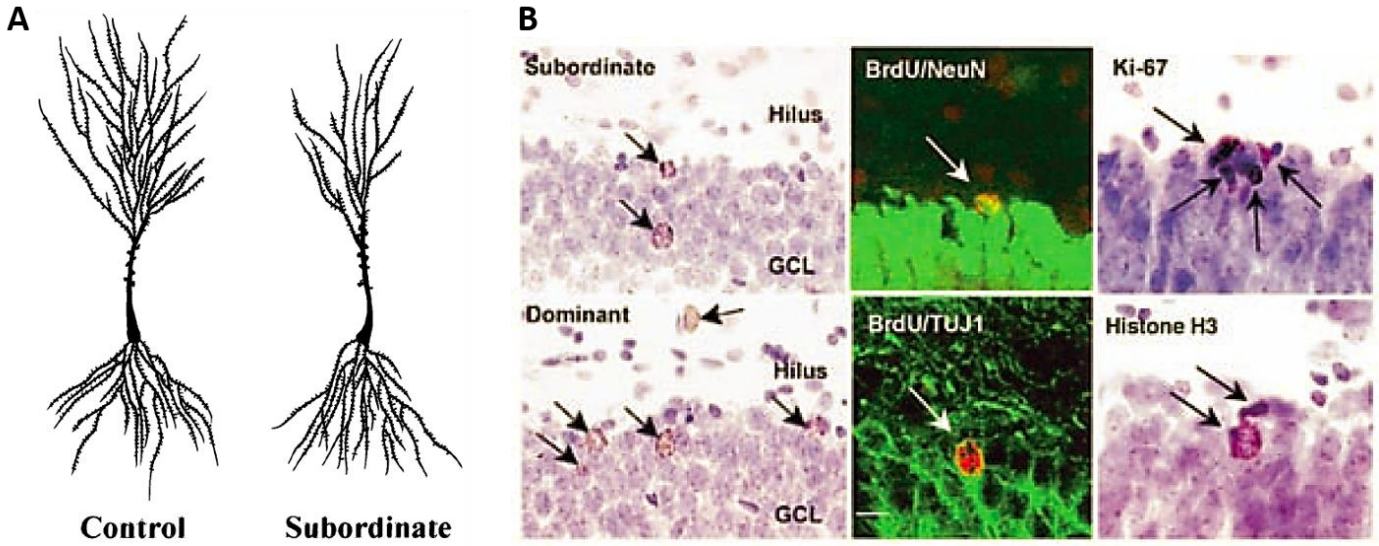


Figure 4 – Dominance-related cellular alterations. (A) representation of Golgi-impregnated staining in CA3 pyramidal neurons of dominants and subordinate tree-shrews; **(B)** New cells in the dentate gyrus of (top left) subordinate and (bottom left) dominant rats; double labeling of BrdU (top middle) with a neuronal marker NeuN and (bottom middle) TUJ1, marker for mature and immature neurons; (top right) Ki-67 and (bottom right) phosphorylated histone H3, cell proliferation endogenous markers; Scale bar 10μm; Adapted from **(A)** Ana María Magariños *et al*, (1996) and **(B)** Yevgenia Kozorovitskiy and Elizabeth Gould, (2004).

Genetics of social dominance and subordination

Several parameters, intrinsic to individuals (e.g. size, aggressiveness, personality) may strongly dictate which social rank they might attain[1]. Certain genetic features determined at birth will necessarily be responsible in determining factors important towards dominant or subordinate behavior. Therefore, searching for genetic determinants of dominance behavior which may strongly impact health[5,24–26] and influence natural selection[1,6], is of particular relevance and interest, as they might reveal new insights to the neurobiology of hierarchy encoding.

The work of Lynne U. Sneddon and colleagues (2011) hypothesized that a distinctive pattern of brain gene expression could characterize each rank in a community and be related to behavioral phenotypes. They considered three different ranks (dominant, subdominant and subordinate) and used microarray methodology in whole brain samples from rainbow trout, to cluster two groups of genes **(Figure 5)**. In subdominants (S), cluster 1 was upregulated and cluster 2 downregulated, relatively to both dominants (D) and subordinates (U). Expression differences between D and U were not as salient as those verified with S, with exception for ependymin and phosphoglycerate kinase. Ependymin positively correlates with its protein levels across hierarchical *strata*: 4.5-fold larger expression levels in S than D and 2-fold higher levels in D than U (S > D > U) **(Figure 5)**. During hierarchical manipulation experiment, ependymin expression levels changed largely towards a decrease[40].

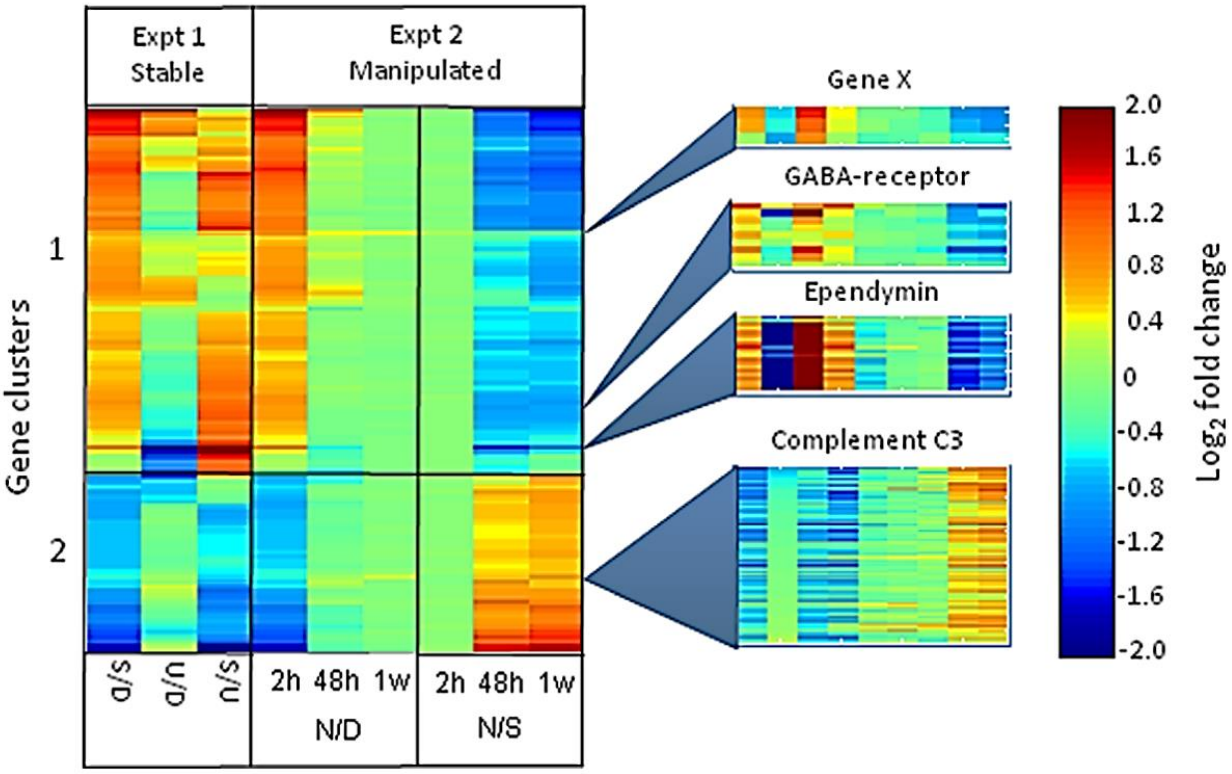


Figure 5 – Gene expression pattern in stable and manipulated rainbow trout hierarchies. D, dominants; S, subdominants; U, subordinates; N, new dominants; Adapted from Lynne U. Sneddon *et al*, (2011).

In order to infer on the behavioral consequences of ependymin inactivation the authors used intracerebroventricular injection of anti-ependymin serum, both in subdominant (AntiS) and dominant (AntiD) animals. When administered to subdominants an increased frequency of aggression was seen, whereas in dominants it led to a decrease. In parallel, a higher percentage of food consumption was verified in AntiS, with no significant effect observed in AntiD[40]. These results seem to suggest that there is a pattern of genetic expression that correlates with social ranking. Importantly, ependymin appears to repress aggressive behavior and competitive ability in subdominants, at least until a certain level of expression, as verified by the further decrease in attack frequency by dominants injected with antiserum.

Moreover, ependymin expression has been shown to be enhanced by stress[41,42], and the fact that several genes from the expression profile of subdominants also belong to stress response cascades, further suggests a link between stress and social hierarchies[40]. In another study, the genetic expression patterns in cortex of adult rats submitted to the resident-intruder stress paradigm was assessed[16]. Subordinates appear to express significant higher levels of proteins related to stress response cascade, as it is the case of heat shock protein 27 (Hsp27), ribosome-associated membrane protein 4 / stress-associated endoplasmic reticulum protein 1 (SREP1/RAMP4) and interleukin-18 (IL-18), together with cytoskeleton proteins like β3-tubulin and α-tubulin[16,43]. Given the stressful nature of the paradigm, such observations may represent a coping response to stress. As discussed above, cellular morphological alterations may result due to stressful situations, and therefore, alterations in cytoskeleton proteins expression converge with the remaining results as stress response-related observations.

Additionally, high prevalence of a trait in selectively bred populations can be used in a way to measure the underlying genetic component of a trait[44]. Jandira Masur and Marco A. C. Benedito (1974) used inbreeding in rats to genetically select and generate dominant- and submissive-prone populations[45]. A similar approach was used in an outbred mouse strain (Sabra mice), leading to a strengthening of the traits across generations, as verified by a continuous increase in the number of dominance-submissive relationships and earlier display of dominance and subordination[46]. This progressively stronger manifestation of dominance-submissive relationships raises the hypothesis that there is indeed possible heritable dominance-related genetic determinants. A further study used these animals to perform a microarray analysis in order to find those candidate genes responsible for the observed behaviors. Subordinates were associated with significant upregulated transcriptional levels of synapsin II variant b (Syn IIb) in hippocampal and striatal samples, but not in the prefrontal cortex and cerebellum, when comparing dominant and control animals[47]. In addition, statistically significant elevated levels of Syn IIb in the hippocampus of postnatal day 1 submissive pups, further support the heritability of this type of behavioral traits[47]. However, as a consequence of the complex variety of afferent projections to the striatum **(Figure 6)**[48], elucidating what could be the consequence of its increased syn IIb expression is particularly challenging.

Our laboratory (Franco *et al*, unpublished data) and others[49,50] have shown that early life adversity induces submissive behavior in mice later in life. We took advantage of this to generate a strong submissive phenotype in mice to then collect mPFC samples from control and stress animals for RNA sequencing. As a result, we found a total of 180 genes significantly altered in the mPFC of the subordinate/stressed animals. Genes that would code for membrane receptors and that would simultaneously influence behavior were the main focus of our selection for downstream analysis. From all genes in all GoMiner classes depicted in the **Figure 7**, 9% were involved in receptor activity and other 9% in behavior, with 8 genes in common between both categories **(Figure 7A and 7B)**. Type 1a dopamine receptor D1 (Drd1a) and dopamine receptor D5 (Drd5) stood out due to previous observations that striatum dopamine receptors levels are deeply influenced by social dominance and their possible effect on dominance-related reward[51–53]. Nonetheless, our experiment provided possible candidates that might be related and underpin the expression of dominance on those animals.

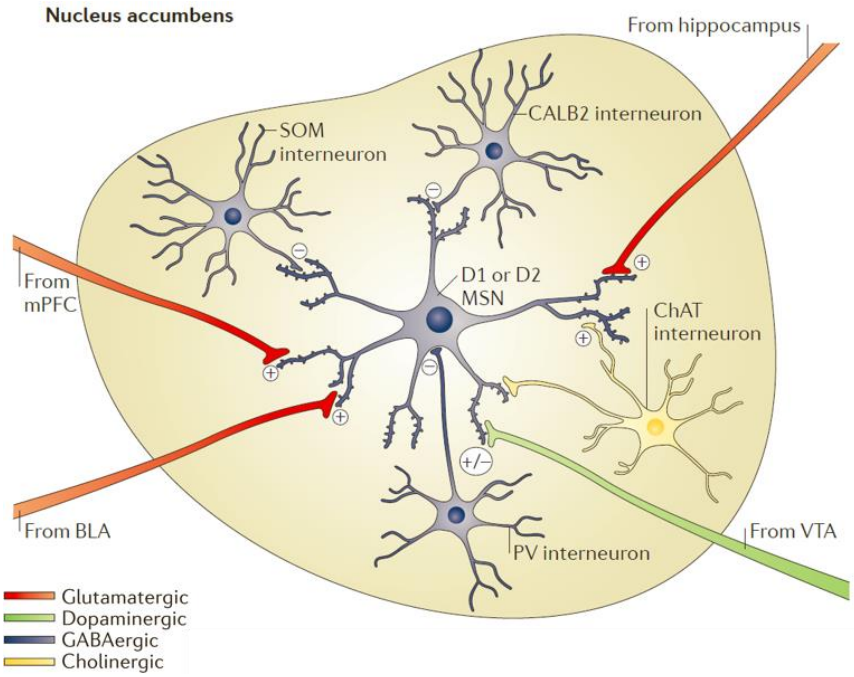


Figure 6 – Neuronal inputs to the nucleus accumbens in the striatum. BLA, basolateral amygdala; mPFC, medial prefrontal cortex; VTA, ventral tegmental area; ChAT, choline acetyltransferase; PV, parvalbumin; SOM, somatostatin; CALB2, calbidin; MSN, medium spiny neuron; D1, dopamine receptor 1; D2, dopamine receptor 2; Adapted from Scott J. Russo and Eric J. Nestler *et al*, (2013).

Other line of evidence, reviewed by Joan Y. Chiao (2010), highlights the short and long allele polymorphisms (S allele and L allele respectively) in the serotonin transporter gene (5-HTTLPR) which correlates with categories of hierarchy organization. The S allele expresses considerably less mRNA and protein levels for the serotonin transporter, presumably resulting in sustained higher concentration of serotonin at the synapse of individuals carrying this polymorphism. Human individuals bearing this allele are more prone to develop conditions associated with an increased negative emotion and affection. Interestingly, primate communities with strict societal rules are often polymorphic for this trait, exhibiting at least one S allele. On the other hand, more tolerant primate societies are commonly monomorphic for the L allele. Consistently, human cultures more prone towards social stratification, as opposed to those favoring *egalitarianism*, have higher incidence of the S allele polymorphism in the population[54].

Furthermore, a mouse model for the human single nucleotide polymorphism (SNP) in the μ-opioid receptor gene (*OPMR1 A118G)* show increased social dominance[18]. Additionally, both autism spectrum disorders (ASDs)-linked and schizophrenia-linked mutations in the *Shank3* gene, generated mouse models with increased dominance expression as well[17].

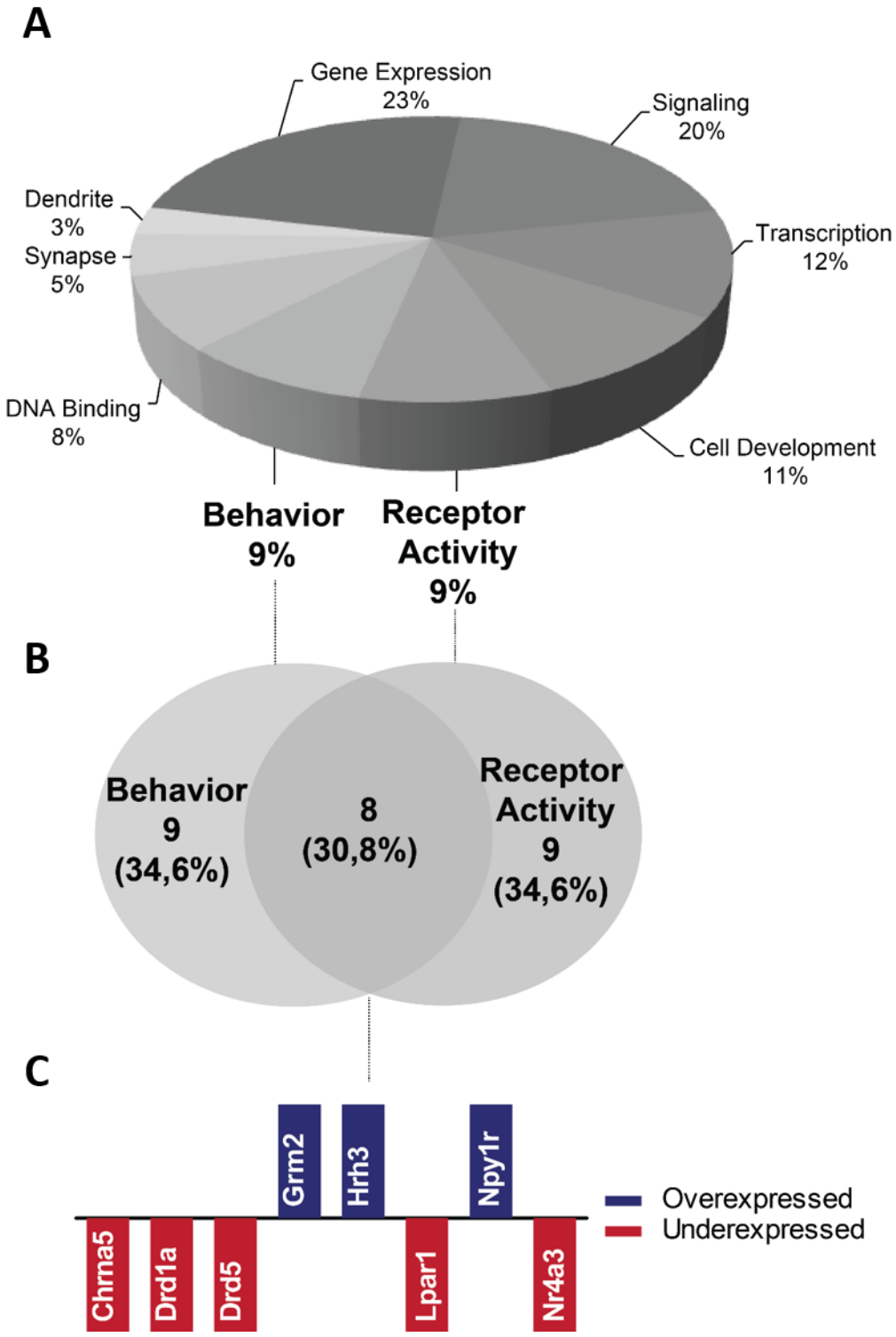


Figure 7 – RNA sequencing results organized by ontological classes. (A) Ontological classification of the 180 genes with altered expression. **(B)** Behavior and Receptor Activity classes have 8 genes in common, of which **(C)** 5 are underexpressed and 3 over expressed. Chrna5, cholinergic receptor nicotinic alpha 5 subunit; Drd1a, type 1a dopamine receptor; Drd5, dopamine receptor D5; Grm2, glutamate receptor, metabotropic 2; Hrh3, histamine receptor H3; Lpar1, lysophosphatidic acid receptor 1; Npy1r, type 1 neuropeptide Y receptor; Nr4a3, nuclear receptor subfamily 4, group A, member 3.

Role of neuropeptides in social hierarchy

Oxytocin (OT) and vasopressin (AVP) are neuropeptides synthesized by magnocellular neurons located in the paraventricular (PVN) and supraoptic hypothalamic nuclei, which project to the posterior pituitary to release those substances into peripheral blood circulation **(Figure 8B)**. Additionally, parvocellular neurons situated in the PVN send axonal projections towards several brain regions - as it is the case of amygdala, hippocampus, striatum, suprachiasmatic nucleus, bed nucleus of *stria terminalis* and brainstem – where OT and AVP can influence neurotransmission **(Figure 8A)**. Furthermore, these peptides also retrogradely signal to the hypothalamus (from PVN and supraoptic nucleus) **(Figure 8B)**[55]. This peptides play several roles in behavior, such as social exploration, attachment, social cognition and recognition, aggression, anxiety and both fear condition and extinction[55]. Hierarchy formation, perception and consequent maintenance requires social behaviors, such as those produced by the OT and AVP systems. Moreover, several brain regions involved in these systems are also recruited during hierarchy-related behavioral paradigms (e.g. amygdala, striatum, hippocampus and the brainstem [PAG and DRN]). Due to the release of these peptides to nearby regions, the hypothalamus is affected and therefore these systems may be also involved in aggression-related behavior[56,57], which is particularly important for dominance. Studies have consistently attributed to OT a role in enhancing motivation to participate in social interactions[55], which is possibly related to the brainstem innervation with parvocellular axonal projections and action at the DRN. This is supported by evidence that DRN serotonergic neurons in mice express OTR[55]. In this view, the putative motivational defect in subordinates may be a consequence of an impoverished oxytocinergic function.

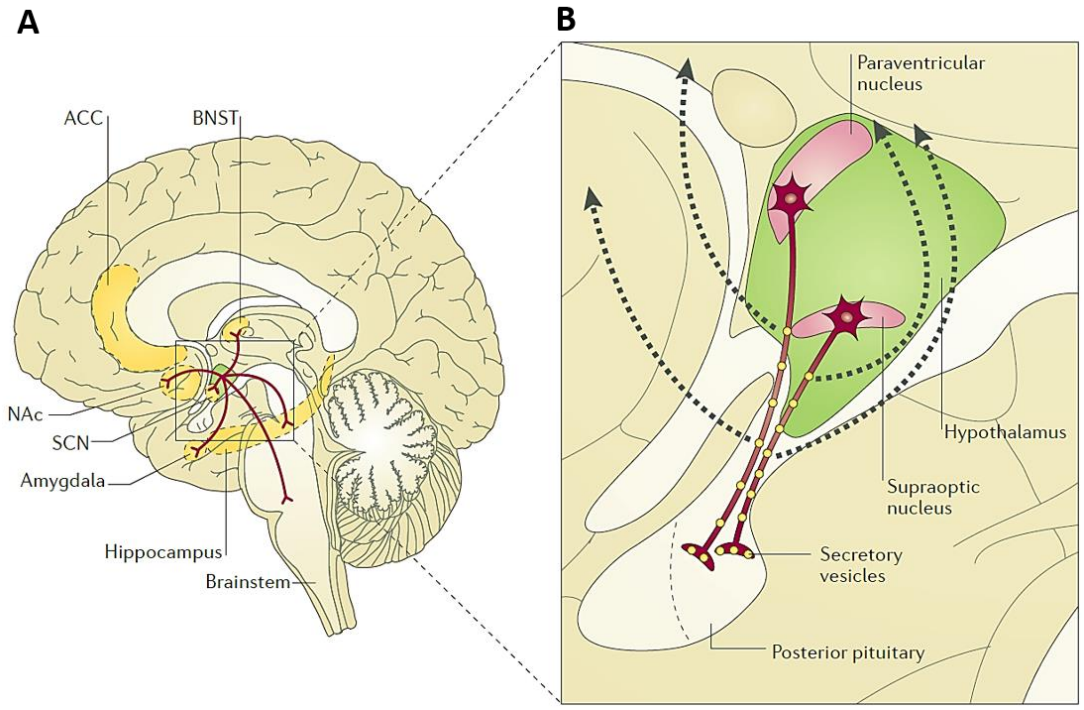


Figure 8 – The oxytocinergic and vasopressinergic systems. (A) axonal projections' targets of parvocellular neurons in the paraventricular nucleus; **(B)** paraventricular and supraoptic nuclei output to the posterior pituitary and (doted arrows) retrograde release of oxytocin and vasopressin; SCN, suprachiasmatic nucleus; NAc, nucleus accumbens; ACC, anterior cingulate cortex; BNST, bed nucleus of the stria terminalis; Adapted from Andreas Meyer-Lindenberg *et al*, (2011).

AVP, but not OT, has a particular role in regulating the HPA axis and ACTH release from the anterior pituitary[55], and thus, might be also linked to the hierarchy-related stress incidence. Dominance hierarchies generate a considerable amount of stress across the hierarchical *gradient*, which may confound results from different approaches in identifying cause and consequence on hierarchy-related neuronal activity and dominance behavior. Indeed, Marjan Timer *et al*. (2011) verified that stress facilitates the formation of dominance hierarchies through an oxytocin-mediated mechanisms[58]. Stress-paired subordinate rats expressed significantly less OT receptors (OTR) in the medial amygdala (MeA) when compared to stress-paired dominants, as well as subordinates and dominants that were not submitted to stress. Infusion of a selective OT antagonist in the MeA of non-stressed subordinates further corroborated the hypothesis, since hierarchy was maintained even 1 week later, but not in non-stressed subordinates administered with a vehicle solution[58]. Thus, stress appears to potentiate the subordinate status by decreasing OTR levels in the MeA, resulting in a long-term establishment of social hierarchy. The MeA is implicated in the circuitry responsible for modulation of aggressive behavior[57], which is of particular relevance for establishment of dominance relationships. Thus, decreased sensitivity to OT in the MeA might be leading to an inadequate aggressive response in subordinate rats. Also, recent imaging studies attributed a significant role to the amygdala in hierarchical learning[59]. Early life stress events seem to be also associated with an impaired oxytocinergic action in buffering stress after in life[55], and at the same time, it may be causative of strong submissive phenotype (Franco *et al*, unpublished data)[49,50]. Similarly, the OT-mediated long-term hierarchical stabilization was mediated towards a potentiation of submissiveness. Therefore, stress, and more precisely early life stress, could be decreasing the sensitivity of the oxytocinergic system by reducing the availability of OTR, resulting in a potentiation of the submissive behavior after in life (possibly due to impairment in buffering stress), as characteristic of animals submitted to this procedure.

Differently from Marjan Timer *et al* (2011), low OT immunoreactivity was observed in the paraventricular nucleus of dominant mandarin voles in comparison with subordinates[60]. The location and nature of this labelling results suggest that dominants could have a partially reduced OT production, therefore it is possible that in this study, dominant animals have an overall decreased oxytocinergic action. The association between dominant behavior and low OT action could be made if considering the relevance of aggression in hierarchy establishment, since OT knockout mice exhibit increased aggression[60]. Therefore, dominant mandarin voles would manifest an enhanced aggressiveness as a consequence of low OT availability. However, considering the theory where subordinate rats with low levels of OTR in the MeA would have an impaired aggressive behavior, together with the putative increased aggressiveness in dominant mandarin voles with reduced OT-immunoreactivity in the PVN, is contradictory. Furthermore, it is unclear whether the observed reduction in OT-immunoreactivity in the PVN is in fact functionally relevant, since there are no differences in the supraoptic nucleus (other region for OT synthesis) when considering social rank. Therefore, stressing the role of OT in social hierarchy-related neuronal encoding and validating these hypotheses is particularly difficult, unless further studies are conducted to fully elucidate how OT functions. Even though the dual role of OT should be addressed, it remains a possibility that OT may trigger different functions on dominance behavior in a region-specific manner.

Dominant mandarin voles were also characterized by higher amounts of AVP-immunoreactive neurons in the PVN, supraoptic nuclei and both lateral and anterior hypothalamus[60]. Moreover, it was shown in another study that AVP 1b receptor knockout mice display significant impairments in aggressive behavior[61]. These results together with the anterior hypothalamus role in regulating aggressive behavior[57,60], suggest that the increased AVP immunoreactivity in dominant animals might underlie their ability to win aggressive-submissive encounters (important for hierarchy establishment). However, absence of manifestation of aggression *per se* does not necessarily imply that an individual is more prone to win agonistic encounters. Thus, AVP-related aggression might be deceptive, because AVP activity seems to relate with anxiogenesis[55], which strongly influences dominance behavior[19]. On the other hand, OT action in serotonergic neurons produces an anxiolytic-like effect.

Another neuropeptide very abundant in the brain is the Neuropeptide Y (NPY)[62,63]. This molecule is differentially expressed throughout the brain. Cortical and limbic regions together with the hypothalamus represent the areas where NPY reactivity is most prevalent[62]. In the rodent hypothalamus, more precisely in the arcuate nucleus (ARC), simultaneous activity of this peptide with agouti-related peptide (AGRP) has been strongly associated with an orexigenic response (triggering of feeding behavior), whereas pro-opiomelanocortin (POMC) expressing neurons, also present in this nucleus, generate an opposite effect (anorexigenic response)[64–67].

Dominance hierarchies deeply shape the access to food in animal societies and also, access to restrict food sources can trigger the development of dominance relationships[1]. Under this view, the results obtained from our RNA sequencing experiment seem promising since they demonstrate that early life stressed subordinate mice have an increased expression of the Npy1r in the mPFC. Radiolabelling assays have indeed shown that the frontal cortex in rats is particularly enriched with this receptor[68]. Thus, given the role of mPFC in social hierarchy, its brain function might indeed overlap in terms of hierarchy neuronal encoding and control over feeding behaviors. Supporting this hypothesis, a study performed in rainbow trout the authors showed that subordinate individuals had higher levels of NPY mRNA expression in the preoptic area compared to dominants. The results were further complemented with the expression levels of the corticotropin-releasing factor (CRF). Subordinate fish expressed significantly more CRF mRNA in the preoptic area than dominants and expression levels of these two molecules correlated positively[69].

These observations might suggest that incidence of stress across social hierarchies is not proportional and that individuals belonging to tiers with chronic high stress incidence may develop mechanisms through which submissive behavior is maintained and enforced. The NPYergic activity can then be one type of brain activity that participates on those processes. The preoptic area in rainbow trout has been associated with feeding behavior[70], whereas in goldfish there is evidence of its involvement in sexual behavior[71] - both dominance-related behaviors.

Notwithstanding, the directionality of this supposed overlap should be considered. Is hypothalamic activity, related to feeding (and sexual) behavior that exerts some control over the mPFC and thus relates

this type of behavior with dominance, or the opposite? Evidences in the literature support that it is unlikely the hypothalamus that exerts direct afferent control over the mPFC to trigger and control dominance-related behaviors, due to lack of connections from the hypothalamus[72]. Moreover, a study performed by Christian Broberger *et al* (1998) demonstrated that in the rat frontal cortex the NPY-positive terminals were negative for AGRP[73], supporting the idea that projections related to feeding behavior do not reach the mPFC. Nevertheless, indirect connections[74–76] may still influence cortical activity. In fact, the mPFC is widely classified as region of top-down executive control over subcortical regions[74,75,77]. Therefore, the brain function underlying this putative behavioral overlap between dominance and feeding behavior might be related to cortical top-down control over the hypothalamus.

Specific objectives

Our work aims to address the following main objectives:

1) Is it possible to form intercage hierarchies in mice? If yes, is this a stable hierarchy reflecting animal intrinsic properties or is it supported by stochastic events?
2) Does ranking in intergroup hierarchy reflect ranking within each group, or are they independent?
3) What strategies may animals adopt inside the tube test?
4) Does behavior in the tube test involve learning?
5) Do some behavioral traits predispose individuals to a certain hierarchical rank?
6) Does the formation of intergroup hierarchies induce any alterations in behavioral traits?
7) Does social stratification induce any changes in expression of candidate genes and do they correlate with intergroup ranking?

Chapter II | Materials and Methods

Animals

Male C57/BL6 mice, 3-5 months old (Charles River) were used for all behavioral experiments. Animals were housed at the vivarium of the Faculty of Medicine at the University of Coimbra, in groups of 4 per cage with food and water provided *ad libitum,* and maintained in a 12 hour light/dark cycle in temperature- and humidity-controlled rooms. When arriving, animals acclimatize to the vivarium and daily routines for at least one week after which they were handled twice a week. Animal identification was performed by subcutaneous injection of green and/or black dyes in the paws. All experiments were carried with the approval of the animal ethics committee of the Center for Neuroscience and Cell Biology, University of Coimbra (ORBEA), the approval of the Portuguese DGAV and in accordance with EU directives in regards to animal use in research.

Behavioral tests

All experiments were performed in the light cycle (08h00 to 20h00) and animals were allowed to acclimatize to testing room environment for at least 1 hour prior to testing.

Elevated plus maze

The elevated plus maze arena was of white acrylic, with arms measuring 30 cm in length, 6 cm wide and with closed arms walls 15 cm tall. The maze was elevated 50 cm above the ground. Indirect illumination intensity of the open arms was set to approximately 150 lux (white LED light). At the start of the trial animals were placed at the center of the maze facing a closed arm and were allowed to explore the arena for 10 minutes. The relative position of the animals was tracked using EthoVision XT 11.0 (Noldus). Analyzed parameters include, latency to enter open arms, time spent in open arms and time spent in closed arms.

Three-chamber social test

This behavior test was performed as described elsewhere[1]. Briefly, the behavioral arena was from Stoelting and consisted of a 60 x 43 x 22 cm transparent acrylic box divided in three chambers. Lateral chambers communicate with the middle chamber through openings with 5 cm width and 8 cm height. The middle chamber was modified in order to have a smaller available area (15 cm x 20 cm). Wire cages (Galaxy Cup, Spectrum Diversified Designs) were used to hold social stimulus mice. Plastic cups were placed on top of the wire cages so test animals could not move or climb the wire cages. Illumination was maintained at 30 - 40 lux intensity. Stimulus mice were age-matched male BALB/c mice previously trained to remain inside the wire cage.

Each test subject was introduced to the middle chamber and allowed to explore all three chambers during 20 minutes to acclimatize to the arena. The animal was then held in the middle chamber and a stimulus subject (Stranger 1) placed within a wire cage in one of the lateral chambers, and an empty wire cage was positioned in the opposite lateral chamber. A first social interaction session started for 10 minutes by allowing the test animal to explore the arena. A second 10-minute session started with the introduction of a novel stimulus animal to the previously empty wire chamber (Stranger 2). The arena and wire cages were cleaned out between test subjects.

The relative position of the animals was tracked using EthoVision XT 11.0 (Noldus). To evaluate sociability, time spent in each chamber, as well as time spent closely investigating (within 5 cm) the wire cages, within the first 5 minutes of the session, was quantified.

Forced swimming test

Forced swimming test was conducted in a 2 L glass beaker (14 cm diameter and 19 cm height) filled with 1,5 L of water at 18-20 ^{o}C. Test subjects were placed inside the beaker and let to swim/float for 6 minutes. Animals were then removed and placed on cage lids, covered with absorbent paper and dried before returning to the home-cage.

Camera recordings were analyzed and quantified offline using The Observer XT 12.0 (Noldus). Quantification was performed blinded to animal identification. Depressive-like behavior was assessed as changes in latency to stop swimming/struggling and total time spent immobile.

Tube test

Tube test was performed in a transparent plexiglass tube, 33 cm long with an inner diameter of 3 cm. Acrylic ramps permitted the animals to easily access and retreat back from the tube. Testing started by introducing two different subjects to the edges of the tube. Testing ended as soon as one of the subjects had all paws outside of the tube for at least 4 seconds.

In a Round-Robin Tournament, tube test schedule was designed to allow every subject to run against all the others (total population of 12 mice; cage and non-cage mates) in a subject-specific bracket of 5 - 10 trials. Introduction to the tube test entrance was randomized and balanced. All animals were weighed before and after each Round-Robin test. Animals rested 24 - 48 hours between trials.

Camera recordings were performed in side view and video-taping was scored offline using The Observer XT 12 (Noldus). Dominance relationships were generated by determining the subject who leaves the tube as the subordinate in each particular dyad.

Tube test behavioral decoding

The Observer XT 12.0 (Noldus) was used to quantify different types of behaviors and parameters displayed during a tube test trial. Observations begun at the time-frame immediately after tail-release of subjects and ended 4 seconds after an animal left the tube completely. A coding scheme was defined and attributed to subject dependent behaviors (e. g. grooming, pushing and retreats) for further analysis. Distance measurements were performed with MB-Ruler software. Distance observations were standardized to one side of the tube.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. Significance was settled for p value < 0.05. Further details of the analyses performed are described together with the results (Chapter III).

Tissue collection

For tissue collection, animals were sacrificed by decapitation after brief anesthesia with isoflurane (Abbott Laboratories, Illinois, USA). Brain was dissected and immersed in ice-cold saline. After short wash, brain slices were obtained using stainless steel blades (BIC, Clichy, France) at 1 mm interval in an adult mouse brain slicer matrix (Zivic Instruments, Pittsburgh, USA). Hole punches (2 mm diameter) were used to acquire brain tissue from regions of interest. Immediately after, tissue was preserved at - 80 ºC for further processing.

RNA extraction

RNA extraction was performed using the AllPrep DNA/RNA/miRNA Universal kit and according to instructions of the supplier (Qiagen).

Quantitative real-time PCR

Complementary DNA (cDNA) was synthesized from 250 μg of total extracted RNA by using the NZY First-Strand cDNA Synthesis kit (NZYTech), following the instructions of the supplier. For quantitative real-time PCR (qRT-PCR), 4 μL of cDNA (1:5 dilution), plus 5 μL of iQ SYBR Green Supermix (Bio-Rad) and specific commercially available primers for candidate genes at 2,5 μM, were mixed to a volume of 10 μL per reaction. The iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) was used to scan and measure the

signal produced during each elongation step during the PCR reaction. Negative and positive controls were run for all assays. As negative controls, non-template control (reaction lacking the cDNA template) and a non-transcriptase control (reaction lacking the transcriptase) were used to detect unspecific amplification products and presence of contamination elements. Each reaction was run in triplicate. Reactions were then normalized to the internal control - hypoxanthine-guanine phosphoribosyltransferase gene (Hprt) was used as internal control, as it was shown not to vary with the behavioral manipulations performed. Final analysis was performed using PfafII methodology[78,79].

Postsynaptic densities isolation

The isolation of postsynaptic densities was performed as described elsewhere[80]. Briefly, tissue (isolate as described above) was pooled and manually homogenized by a glass-teflon homogenizer (30 - 40 strokes) in 1 mL of HEPES-A solution (HEPES (Fisher) 4 mM; Sucrose (Fisher) 0,32 M; pH 7,6) and transferred to 1,5 mL eppendorf tubes. Next the resulting homogenate was centrifuged (Heraeus Fresco 21, Thermo Scientific) at 700 g for 15 minutes at 4 ºC to obtain the nuclear (pellet) and brain lysate (BL; supernatant) fractions. This step was repeated to yield the washed BL fraction. At this point, 100 µL of BL fraction was collected and stored at - 80 ºC after adding 200 µL of HEPES-A, 43 µL of SDS 20% and 90 µL of 9M urea. Next, the resulting supernatant was submitted to an 18 000g spin (Heraeus Fresco 21, Thermo Scientific) for 15 minutes at 4 ºC to generate the crude synaptosomal portion (pellet). The synaptosomal pellet was resuspended in 1 mL HEPES-A buffer and centrifuged as above. The resulting washed crude synaptosomal pellet was then resuspended in a total of 3 mL HEPES-B (HEPES 4 mM; pH 7,4) and moved to a homogenizer and a total of 10 strokes were applied. The homogenized material was placed in 15 mL falcon tubes to rotate in the cold chamber (4 ºC) during 1 hour for osmotic release of materials from synaptosomes.

The following centrifugation steps were performed in UltraClear Beckman Coulter Centrifuge Tubes. When small volumes were used, tubes were pre-filled (to avoid tube collapse) with 9 mL with HEPES-B and then centrifuged at 25 000 g (Optima XE-100 Ultracentrifuge, Beckman Coulter) in a swinging bucket rotor (SW 41 Ti, Beckman Coulter) for 20 minutes at 4 ºC. All ultra-centrifugation steps were performed at 4ºC for 20 minutes. The resulting pellet comprises a lysed synaptosomal membrane fraction which was resuspended in 500 µL of HEPES-C (HEPES 50mM; EDTA (Fisher) 2mM; pH 7,6) plus 26,3 µL of Triton X-100 10%. From here, 100 µL of sample were recovered together with 9 µL of SDS 20% and stored at -80ºC. All content was transferred to 1,5 mL eppendorf tube and left rotating in the a circular rotator for 15 minutes. Subsequently, sample volume was transferred to ultracentrifuge tubes and volume adjusted to approximately 9 mL with HEPES-B. Ultracentrifugation was carried out at 32 000 g. Pelleted postsynaptic densities were resuspended in 100 µL of HEPES-C and diluted to 500 µL with HEPES-C. A volume of 26,3 µL Triton X-100 10% was added to the samples and placed in a circular rotator for 15 minutes. Final centrifugation was carried out at 200 000 g to obtain the final and purified/isolated postsynaptic densities portion. Samples were resuspended

in 100 µL of HEPES-C plus 14,5 µL of SDS 20 % and 45 µL 9 M urea. All steps were performed on ice and/or at 4 ºC. CLAP was added to all HEPES buffers (1:1000 dilution *ratio*) immediately before starting the protocol. With exception of 9 M urea and SDS 20 %, all solutions were used at ice-cold temperatures.

Protein quantification

Protein quantification was performed accordingly to Pierce BCA Protein Assay kit (Thermo, MA, USA) protocol.

Gel electrophoresis (SDS-PAGE)

Protein samples were loaded on 12% polyacrylamide resolving gel (1.5 M Tris pH 8.8 (Fisher, PA, USA), 40 % acrylamide (Fisher), 20 % SDS (Fisher), 10 % APS (Acros, NJ, USA) and TEMED (NZYtech, Lisbon, Portugal)) and a 4% polyacrylamide stacking gel (0.625 M Tris pH 6.5, 40 % acrylamide, 20 % SDS. 10 % APS and TEMED), immersed in 1x running buffer (5x running buffer – 15 g of Tris base (Fisher); 72 g of glycine (Fisher); 5 g of SDS (Fisher)) to allow separation by size of denatured proteins. Running was set to a fixed voltage,100 V, and time of running was adapted depending on the desired size resolution between target proteins. Loaded samples were diluted into 4x Laemli Buffer (Biorad) containing β-mercaptoethanol and left overnight at 4 ºC.

Western blot

Size-separated proteins were transferred to methanol activated PVDF membrane (GE Healthcare, NJ, USA), by electroblotting in a Mini-transfer blot system (Biorad, CA, USA) at 100 V during 120 minutes at 4 ºC. Transfer cassette was immersed in a 1x transfer buffer (5x transfer buffer – 0,025 M Tris base; 0,192 M glycine) with 5% of methanol. After transfer, membranes were briefly washed in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl pH 7,6) containing 0,1 % (v/v) Tween-20 (TBS-T), followed by 1-hour incubation with 5% low-fat milk solution (5 % (w/v) low-fat powdered milk in TBS-T). Afterwards, membranes were placed in 5mL of the 5% milk solution with the diluted antibodies for the targeted protein(s) **(Table 2)** and incubated overnight at 4 ºC. Next, membranes were washed in TBS-T and incubated for 2 hours with secondary antibodies **(Table 3)**. After wash steps, Pierce ECL Western Blotting Substrate (Thermo Scientific, MA, USA) was used to reveal the membrane. Membrane scanning was performed using the ChemiDoc Touch ImagingSystem (Biorad, CA, USA).

Immunohistochemistry

For immunohistochemistry experiments, animals under isoflurane anesthesia were rapidly dissected to expose the heart. Next, 20 mL of ice-cold 1x phosphate-buffered saline (PBS) (10x PBS - 87,6 g of NaCl (Acros); 32,5 g of Na2HPO4.7H2O (Fisher); 4 g of KH2PO4 (Fisher) was perfused through the left ventricle. A cut in the atrium was done in order to allow the blood to exit circulation and eventually sacrifice the anesthetized animal by blood loss. Afterwards, 40 mL of fresh ice-cold 4% paraformaldehyde (PFA) (4% PFA - 4 g of PFA (Fisher); in 1x PBS) was perfused to fix the tissue. Dissection and removal of the brain was carried out and brain samples were then immersed in ice-cold 4 % PFA solution and left overnight at 4ºC. Samples were changed to a 4 % PFA solution containing 30 % sucrose (30 g sucrose (Fisher); 100 mL of 4 % PFA) at 4 ºC, to further fix and preserve the tissue through an osmotic exchange. Brain samples were then sliced in the vibratome (Leica, Wetzlar, Germany) to generate 30 um thick slices. These were then repeatedly washed in 1 mL 1x PBS and incubated with blocking buffer (0,5 mL of 5 % goat serum (Thermo); 0,2 g of bovine serum albumin (BSA) (NZYTech); 20 uL of 0,2 % Triton-X 100 (Acros) at room temperature for 1 hour. Afterwards, slices were incubated with blocking buffer containing the primary antibody against cFos **(Table 1)** for 48 hours at 4 ºC. Additionally, after three wash steps, slices were incubated in blocking buffer with the secondary antibody **(Table 2)** overnight at 4 ºC and mounted with 4',6-diamidino-2-phenylindole (Dapi)-containing mounting media (Sigma). Imaging was performed in the Fluorescence Confocal microscope LSM 710 (Zeiss, Oberkochen, Germany).

Antibodies

Table 2 – Primary antibodies utilized in this project.

Primary antibodies	Application (dilution)	Source
PSD-95	WB (1:1 000)	NeuroMab (California, USA)
β-tubulin	WB (1:200 000)	Sigma (Sintra, Portugal)
Synaptophysin	WB (1:100 000)	Abcam (Cambridge, UK)
Npy1r	WB (1:500)	Abnova (Taipei, Taiwan)
cFos	Immunohistochemistry (1:500)	Abcam (Cambridge, UK)

Table 3 – Secondary antibodies utilized in this project.

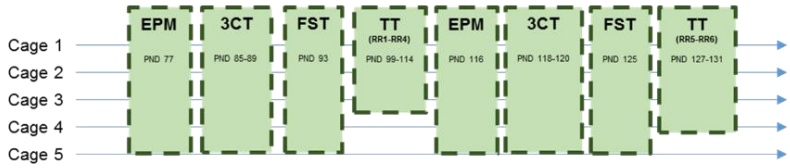
Secondary antibodies	Application (dilution)	Source
Horse radish peroxidase conjugated donkey – Anti-mouse	WB (1:5 000)	Jackson Laboratories (Baltimore Pike, USA)
Horse radish peroxidase conjugated donkey – Anti-rabbit	WB (1:5 000)	Jackson Laboratories (Baltimore Pike, USA)
Alexa Fluor 568 conjugated goat - Anti-rabbit	Immunohistochemistry (1:1000)	Life Technologies (California, USA)

Chapter III | Results

Round-Robin Tournament enables the formation of a meta-hierarchy

To assess if mice belonging to different social groups are able to establish hierarchies (here referred as *meta-hierarchies*) we tested if intercage ranking are maintained/stable over time. We also asked if there is a wider stratification of social ranks, and if we could discriminate and identify the strongest dominance phenotypes in the tube test for further testing. Towards this, we designed a pseudo-random Round-Robin Tournament scheme **(Figure 9B)** taking into consideration the following: each individual undergoes one trial at every group of 6 dyads, left/right entrances are balanced, group order was rearranged over time to confer a higher degree of randomness and avoid exacerbation and repetition in the order of encounters. .A total population of 12 C57/BL6 mice were used, given a total of 66 possible dyadic encounters in *all vs all* organization. Tournaments were performed according to the experimental timeline **(Figure 9A)**.

A



B

Round-Robin Tournament

Trial #	Animal Left	Animal Right
1	1.2	2.3
2	3.3	1.1
3	3.4	2.2
4	3.1	1.3
5	2.4	3.2
6	1.4	2.1
7	2.3	1.1
8	1.2	2.2
9	2.4	3.3
10	2.1	3.1
11	1.3	1.4
12	3.2	3.4
13	2.3	3.3
14	2.4	1.1
15	3.1	1.2
16	2.1	3.2
17	1.3	2.2
18	1.4	3.4
19	2.3	3.1
20	3.2	1.1
21	2.1	3.3
22	3.4	1.3
23	1.2	2.4
24	1.4	2.2
25	1.1	3.1
26	3.3	3.4
27	1.3	2.3
28	2.2	3.2
29	1.4	2.4
30	1.2	2.1
31	3.1	3.4
32	3.2	3.3
33	1.1	2.2
34	1.3	2.4
35	2.1	2.3
36	1.2	1.4
37	3.1	3.2
38	3.3	1.3
39	2.2	2.4
40	1.4	2.3
41	1.1	2.1
42	3.4	1.2
43	2.4	3.1
44	2.2	3.3
45	1.3	2.1
46	3.2	1.2
47	1.1	1.4
48	2.3	3.4
49	2.4	2.1
50	1.2	1.3
51	3.1	3.3
52	3.2	1.4
53	2.2	2.3
54	3.4	1.1
55	3.3	1.2
56	3.1	1.4
57	2.3	3.2
58	2.4	3.4
59	2.1	2.2
60	1.1	1.3
61	3.3	1.4
62	2.3	2.4
63	3.4	2.1
64	1.1	1.2
65	3.2	1.3
66	2.2	3.1

Figure 9 – Schematic plan for the behavioral experiments. **(A)** Experimental timeline, depicting the behavioral experiments performed and temporal organization. Approach-avoidance anxiety, sociability and depressive-like behavior were assessed in the depicted order, before and after 4 Round-Robin Tournaments. **(B)** Round-Robin Tournament trials map was design in a pseudo-random fashion for a total population of 12 mice, in groups of 4 animals. At every six trials groups, each animal undergoes a single trial, which may distance from the following in a subject-specific bracket of 5 to 10 trials. Entrance in the tube is balanced for every animal. EPM, elevated plus maze; 3CT, Three-chamber social test; FST, forced swimming test; TT, tube test; RR, Round-Robin Tournament; PND, post-natal days.

Stratification of individuals is achievable when following this trial map in the tube test assay **(Figure 10A)**. Social ranks closer to the *X* axis display some degree of overlapping in rank, whereas most dominant and subordinate individuals exhibited a clearer segregation from the remaining population. These animals presented a sequentially tendency to win and lose trials in the course of the tournament. On the other hand, cage hierarchies of 4 elements **(Figure 10C to 10E)** are not characterized by overlapping elements in comparison with meta-hierarchies, and appear to present greater degree of stability. Nonetheless, periods of more stability are present in both types of hierarchies, respectively represented as the standard error of the mean (SEM) and rank transitivity. Every subject above the *x* axis is considered as dominant and below as subordinate. Moreover, it is also possible to observe that individuals belonging to the same cage are unevenly disturbed across the meta-hierarchy and present a bias to some degree **(Figure 10A)**. Every cage presents at least one dominant and subordinate individual in the meta-hierarchy, however, subjects of some cages are mostly dominant (cage 1), others more submissive (cage 3) and others occupy the majority of intermediate ranks (cage 2). However, when comparing only individuals of the same cage, ranking at the meta-hierarchy is predictive of their ranking in the home-cage **(Figure 10B)**.

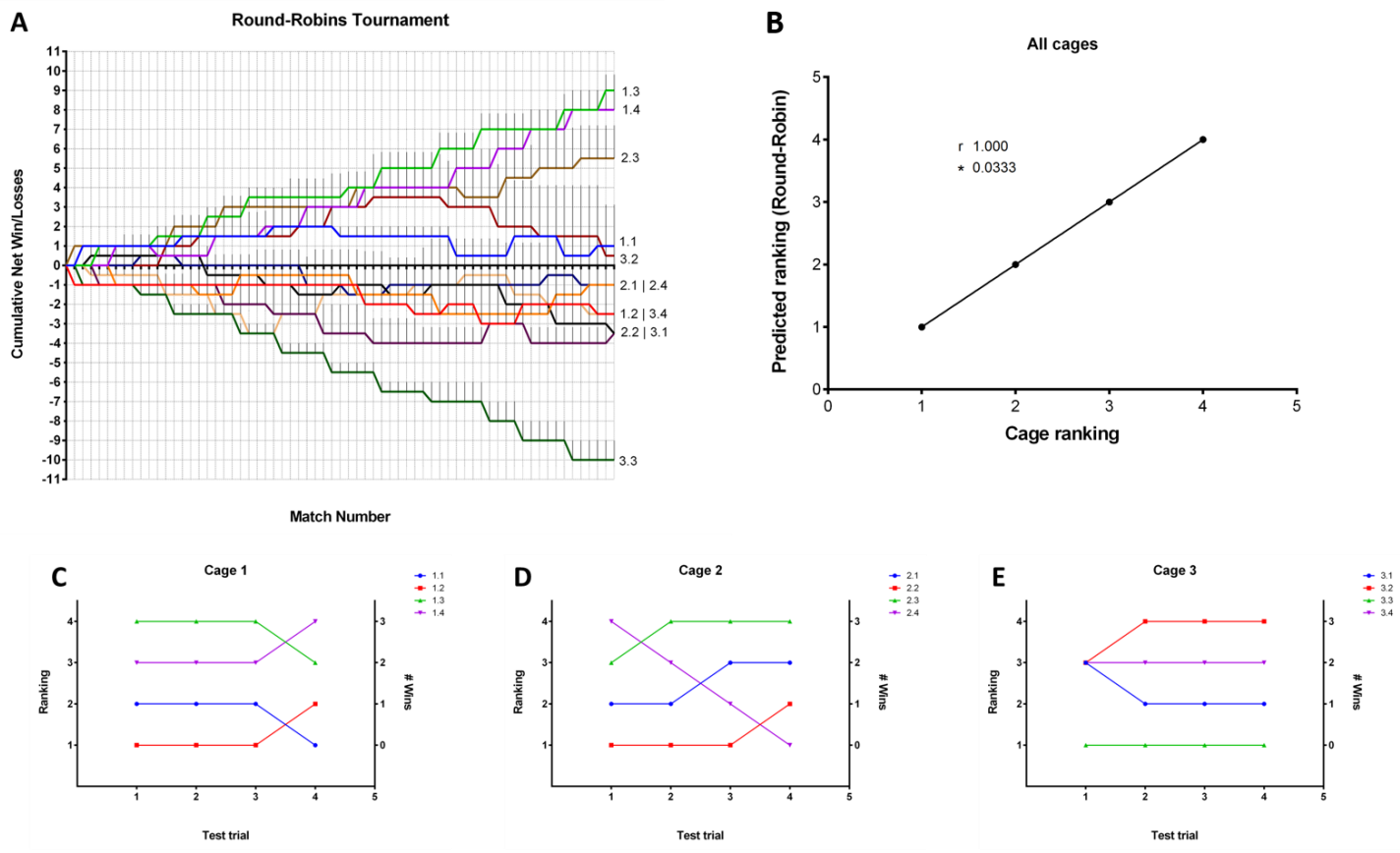


Figure 10 – Meta-hierarchies are predictive of subjects ranking within each individual cage. (A) Example Round-Robin Tournament, this scheme allows formation of meta-hierarchies and stratify animals according to performance in the tube test. Average rank classification of 4 independent tournaments is depicted. Vertical dashed line represents a dyadic encounter and animals of that dyad (represented by color lines) move upwards or downwards at the intersection with the dashed line. To each win or loss is respectively attributed a relative value of +1 and -1. Subjects above the *x* axis are considered dominant and those below as subordinate. **(B)** Ranking in the Round-Robin Tournament predicts ranking in each cage. Mean percentage of wins along 4 tournaments was used to determine the averaged ranking in each cage. **(C to E)** Hierarchy in each cage over 4 trials is characterized by stable and dynamic phases, allowing ranking changes over time. Data in **(A)** is presented as the relative social rank mean ± SEM over 4 tournaments. SEM is representative of Spearman correlation, P value * < 0.05.

Temporal variables in the tube test are dependent on the dominance relationship within dyads

In order to better understand how dominance acquisition is performed in the context of the tube test, we measured temporal variables according to cage of origin and dominance relationship of each dyad. We found that trials in which dominants lose, take longer to resolve when compare to trials where subordinates lose, independently of the opponent **(Figure 11A)**. These observation is maintained along tournaments, however, encounters gradually become shorter in duration **(Figure 11B)**.

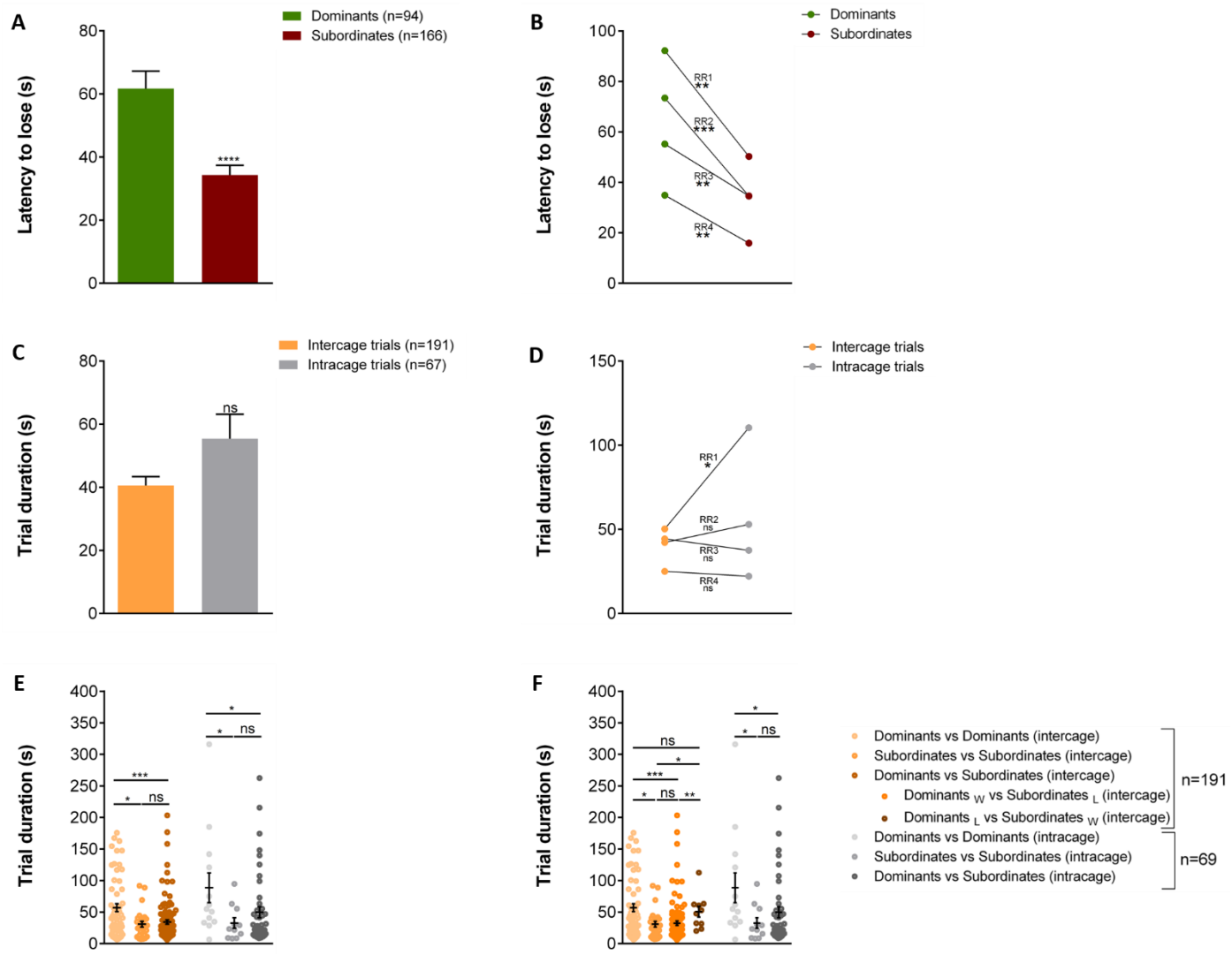


Figure 11 – Trial duration is dependent on experience and dominance rank of the subjects. (A) Every trial duration over 4 tournaments in which a dominant or a subordinate lose and **(B)** average of each tournament. Dominant animals take longer to lose a trial when compared to subordinates, but both lose faster over time. **(C)** All intercage and intracage trials duration from 4 tournaments and **(D)** averages along tournaments. No significant differences are observed in trial duration between dyads comprising individuals of the same cage or different cages, over 4 Round-Robin Tournaments. First tournament is exceptional as it differs between conditions. **(E and F)** Trials duration of all events from 4 tournaments are disposed according to type. **(E)** Homologous trials between dominants last longer than the remaining conditions, whether they are intercage or intracage. **(F)** In heterologous dominant-subordinate trials, when dominant mice win, trials take less time to resolve. Subordinate loss is faster than the remaining conditions. Intracage trials are not characterized by dominants losing dyads. For all analysis dominance was attributed according to ranking in each respective tournament and intracage rankings were allocated regarding only cage mates. W, winner; L, loser. All data is depicted as the mean ± SEM. Mann-Whitney t-test was used in all analysis, p value ns (not significant) > 0.05, * < 0.05, ** < 0.01, *** < 0.001.

Moreover, subjects from different cages (intercage) or the same cage (intracage) of origin, when faced together in the tube test, show no overall differences in trial duration **(Figure 11C)**, with exception of the first tournament **(Figure 11D)**. Along 4 Round-Robin Tournaments, intracage trials take significantly longer to resolve in comparison to intercage encounters in the first tournament, but afterwards there are no differences from intercage trials.

Next, we arranged trials according to the dominance relation between subjects **(Figure 11E)**, we found that homologous trials between dominants (e.g. Dominants vs Dominants), last longer than the remaining conditions. Also, heterologous events between dominants and subordinates (e.g. Dominants vs Subordinates) were not significantly different than trials entirely composed of subordinates (e.g. Subordinates vs Subordinates). This happened equally both in intercage and intracage trials. To retrieve further information about trial duration, we unfolded the heterologous events in two: those where dominants won the encounter ($Dominants_W$ vs $Subordinates_L$) and those where subordinates won ($Dominants_L$ vs $Subordinates_W$) **(Figure 11F)**. In intracage trials this was not possible due to inexistence of trials where dominants lost. We observed that trial duration is always faster when a subordinate individual loses, independently of its opponent.

Following the same type of analysis, we next checked whether duration to first contact in the tube test could be shaped upon dominance relationships of each encounter and provenance of the individuals regarding their home cage. Similarly to trial duration, time to first contact in the tube test in not different between intercage and intracage dyads **(Figure 12A)**. This observation is maintained along Round-Robins, still, the first tournament presents trials with increased duration to first contact **(Figure 12B)**. However, after segregation of trials in accordance to type of dominance relationship, we observe that intracage trials there is no differences between conditions, whereas in intercage, homologous trials comprising subordinates (Subordinates vs Subordinates), present an increased duration to first contact / to engage in social interaction **(Figure 12C)**. Unfolding heterologous trials does not change the observations made previously, however, analysis between $Dominants_L$ vs $Subordinates_W$ with the remaining conditions is inconclusive **(Figure 12D)**. Therefore, without considering the outcome of heterologous trials, duration to first contact is generally increased in intercage dyads of two subordinates.

Furthermore, we had hypothesized that subjects similar in rank would have more difficulty in establishing a dominance relationship than those with more disparate rankings, and that this could influence temporal variables within the tube test. Hence, we correlated discrepancy in social rank between subjects in a dyadic dominance interaction with both trial duration and time to first contact in the tube **(Figure 13A and 13B)**. Results show a negative correlation between social rank discrepancy and trial duration **(Figure 13A)**, but no significant association with duration to first contact **(Figure 13B)**.

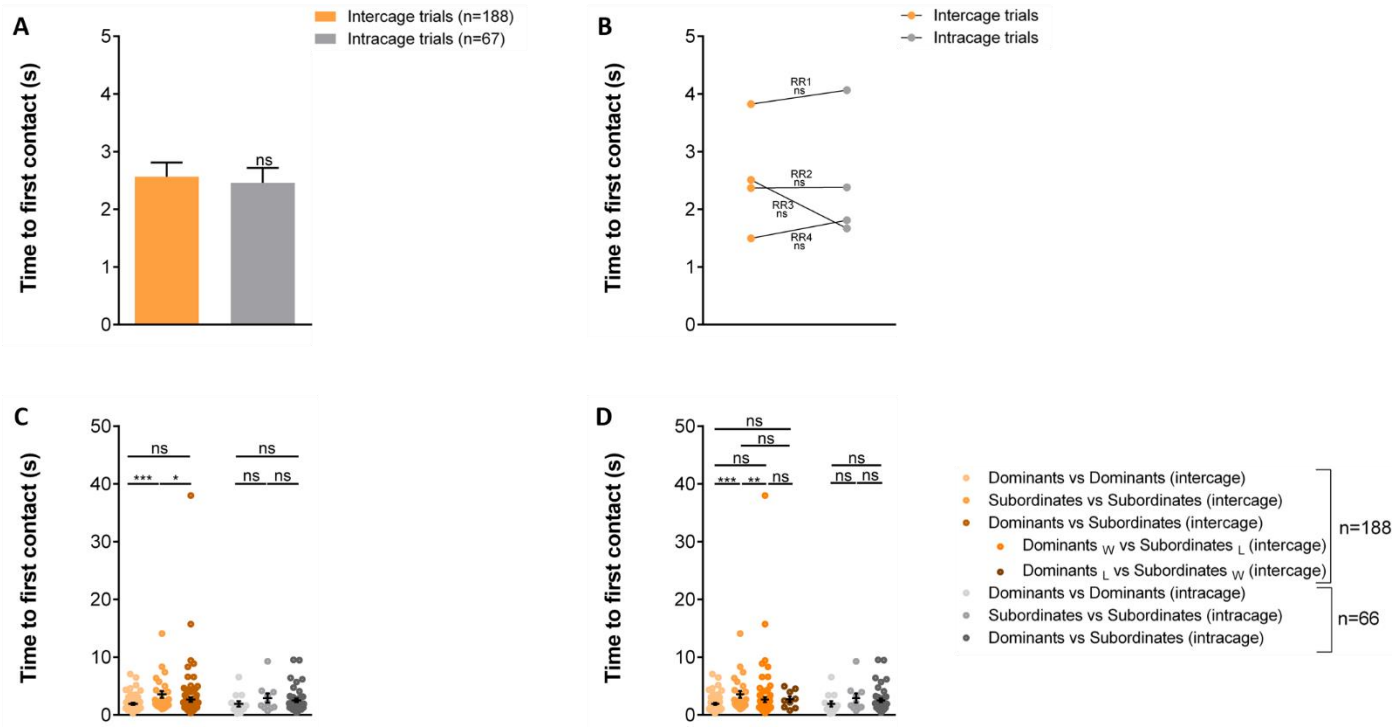


Figure 12 – Time to first contact is dependent on dominance rank of the subjects. (A) Time to first contact in intercage and intracage trials from all 4 tournaments and **(B)** averages along tournaments. No significant differences are observed in time to first contact between dyads comprising individuals of the same cage or different cages, over 4 Round-Robin Tournaments. During the first tournament, both conditions take longer than the following tournaments. **(C and D)** Time to first contact in all events from 4 tournaments are disposed according to type. **(C)** In homologous intercage trials between subordinates, time to first contact within the tube is significantly increased when compared to the remaining conditions, whereas in intracage trials no differences are observed. **(D)** These observations do not change after segregation of intercage heterologous dominant-subordinate trials, and time to first contact does not significantly differs depending on the winner or loser of those trials. Only when a trial is entirely composed of subordinate elements from different cages, time to first contact is increased. Intracage trials are not characterized by dominants losing dyads. For all analysis dominance was attributed according to ranking in each respective tournament and intracage rankings were allocated regarding only cage mates. W, winner; L, loser. All data is depicted as the mean ± SEM. Mann-Whitney t-test was used in all analysis, p value ns > 0.05, * < 0.05, ** < 0.01, *** < 0.001.

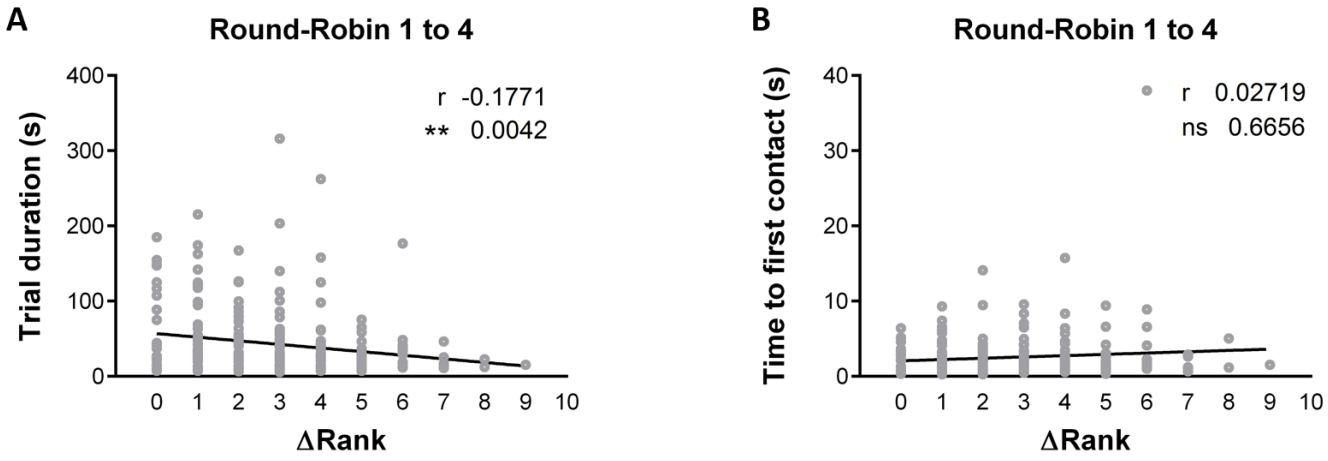


Figure 13 – Discrepancy in rank predicts trial duration, but not time to first contact. (A and B) Correlations between trial duration and time to first contact from 4 tournaments and differences in social rank of subjects. **(A)** Increasing differences in social rank of subjects in a given dyad decreases the duration of that event. Duration of a trial is negatively correlated with rank discrepancy of subjects undergoing it, whereas **(B)** time to first contact does not. For analysis dominance was attributed according to ranking in each respective tournament. Spearman correlation, p value ** < 0.01.

To evaluate if the localization of the first contact could be important for trial outcome, and also if it represents some sort of advantage or disadvantage that could be adopted by individuals during encounters, we identified the location of the first contact-point within the tube test for each individual and related it with *winning* and *losing*. We observe a similar pattern in both graphics; no cluster of contact-points was generated towards any side of the tube in association with *winning* or *losing* a tube test dyad **(Figure 14A and 14B)**. For instances, upon *winning*, intermediate ranks show a tendency to advance more in the tube (right shift on the pattern), however the same also happens at a similar extend when they lose. This demonstrates that location of the first contact does not appear to have an impact in the final outcome of a trial, and that this variable might only depend on the individual.

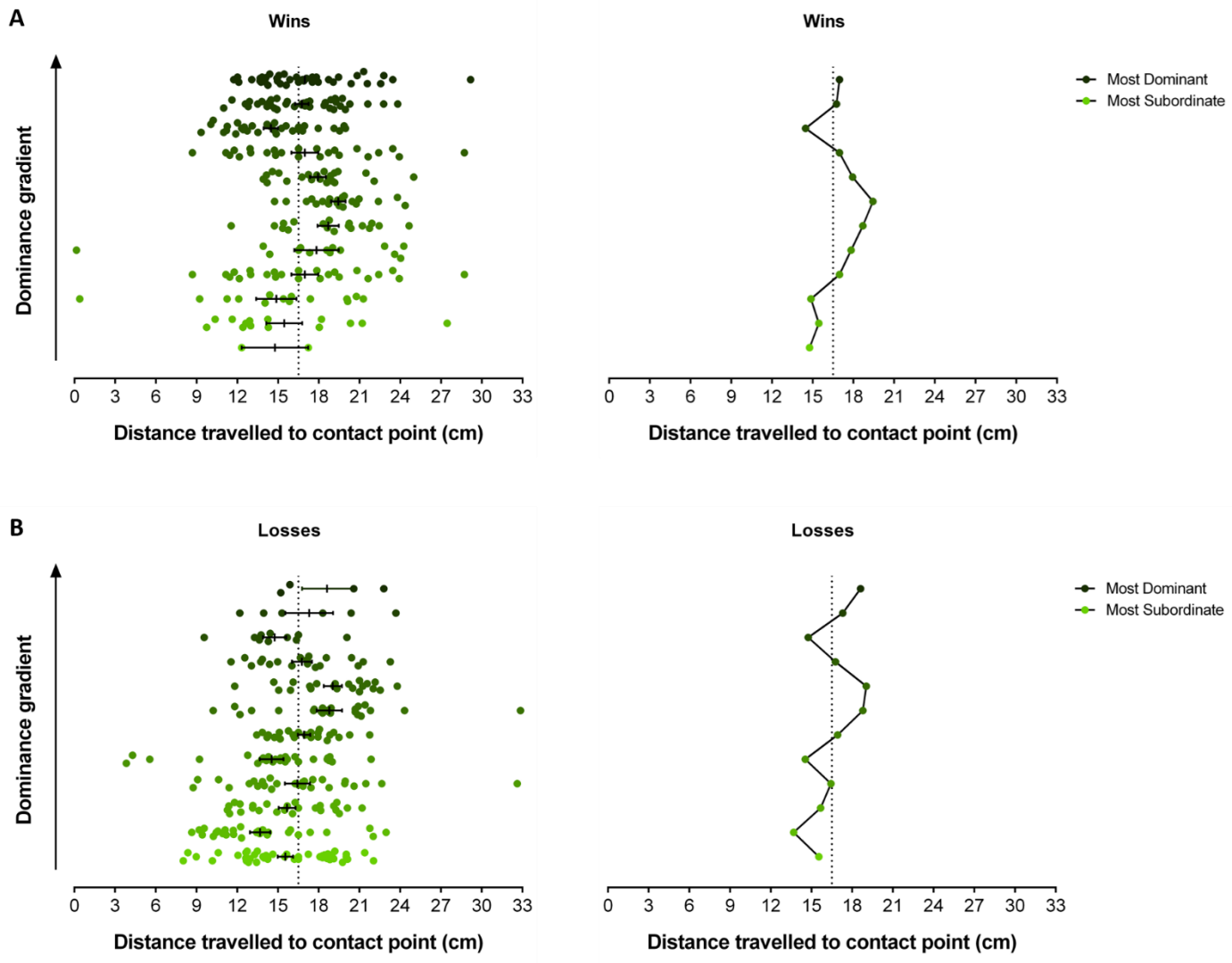


Figure 14 – First contact-point location within the tube does not appear to influence the outcome of a trial. (A) First contact-points from all trials over 4 tournaments, associated with winning and **(B)** losing. First contact-point pattern is considerably similar between wins and losses. Data regarding all subjects was normalized to the left side of the tube. *X* axis represents tube length and dashed the middle of the tube. Dominance attribution considers the cumulative/averaged ranking after 4 Round-Robin Tournaments. Dot-plot graphs are depicted as the mean ± SEM and mean connected graphs present only the mean.

Behavioral traits: effect on social dominance and *vice-versa*

All behavioral manipulations followed the experimental timeline illustrated in the **Figure 9A**. Approach-avoidance anxiety, sociability and depressive-like behaviors were analyzed in this order, before and after social hierarchy development by Round-Robin Tournaments with the tube-test. To characterize the animals regarding these traits we used respectively the elevated plus maze, the three-chamber social test and the forced swimming test. Two cages were not subjected to tube test trials and served as a control and to account for animal age before and after tube test trials **(Figure 9A)**.

Approach-avoidance anxiety

Firstly, we investigated whether anxiety-like behavioral profiles could be predisposing some individuals to certain social ranks, and if frequent exposures to the tube test and eventual dominance acquisition could alter this behavioral characteristic.

When considering the full population of subjects, no significant differences are observable between both timepoints **(Figure 15A and 15B)**, however latency to enter an open arm in the EPM, slightly tends to increase after Round-Robins **(Figure 15A)**.

Moreover, when considering animals that were subjected to tournaments and those who were not, we have found that exposure to the tube test does not induce significant differences, neither in the latency to enter an open arm, nor in the total time spent in the open arms **(Figure 15C and 15D)**.

Furthermore, of those animals subjected to the tube test, we verified that none of the parameters used is predictive of social rank acquisition **(Figure 15E and 15F)**. Prediction would be possible if any difference before social stratification by Round-Robin Tournaments was depicted between animals that would become dominant and/or subordinate. Similarly, dominance acquisition is not accompanied of changes in anxiety-like behavioral parameters, and also, dominants do not differ from subordinates after social stratification in both parameters measured **(Figure 15E and 15F)**.

Sociability

We next asked whether social skills among subjects in our population could be influenced by repeated tube tests and consequent dominance-subordinate stratification, or itself predict dominance. At the first we analyzed all animal (subjected to tube test and not), and found that there is normal social behavior, measured as an increased preference for social interaction and preference for social novelty. However, when the entire population of animals is tested at a later time point (after tube test trials) social preference is no longer observed **(Figure 16A)**.

We then went to dissect if this effect was due to the animals being subject to tube testing or because the animals are being probed at an older age. We find that before Round-Robin Tournaments, animals manifest strong social novelty preference **(Figure 16B).** The animals not scheduled to run still show a strong trend for high sociability, although not statistically significant most likely due to the lower number of mice used (n=12 vs n=8). Nevertheless, those subjected to repeated tube testing appear to lose their preference for social interaction at the later timepoint, whereas, animals that did not undergo this manipulation, display a similar social behavior as in the first timepoint **(Figure 16B and 16C)**.

Moreover, we also observe that social rank can not be predicted by social and social novelty preferences, and does not lead to changes in social-like behavior. Also, in both timepoints, dominants and subordinates do not differ between them **(Figure 16D and 16E)**.

Depressive-like behavior

Finally, we aimed to assess if depressive-like behavior is a trait that determines and conditions dominance, and also if it is shaped upon social stratification and consequent rank acquisition.

For all subjects, total time immobile does not change between both timepoints **(Figure 17B)**, but latency to stop swimming decreases significantly **(Figure 17A)**. This decrease is mainly due to individuals that were not subjected to the tube test **(Figure 17C)**. Importantly, mean latency to stop is not significantly different between subjected and non-subjected individuals before and after tournaments **(Figure 17C)**. After unfolding this data, those animals subjected to the tube test manipulation in accordance to their dominance acquired after Round-Robins, we found out that latency to stop swimming was not predictive and that it does not change with this process **(Figure 17E)**.

On the other hand, total time immobile slightly tends to be increased in the animals destined to be controls of tube test manipulation, however, both conditions do not present any significant evolution in a second time point. Thus, total time immobile between both populations is similar at both time points **(Figure 17D)**. Likewise, upon dominance ranking none of the analyses show significant difference across this parameter **(Figure 17F)**.

This array of experiments revealed that approach-avoidance anxiety-like behavior does not show pronounced alterations. Animals that undergone social stratification after tube test have lower social and social novelty preferences, but part of this effect may be explained by testing being performed in older animals, since mice not tested in the tube, also showed modest changes **(Figure 16B and 16C)**. Finally, tube test manipulation induced an improvement in a feature associated with depressive-like behavior, where animals subjected to the tube test do not see the latency to stop swimming decrease at a later timepoint **(Figure 17C)**.

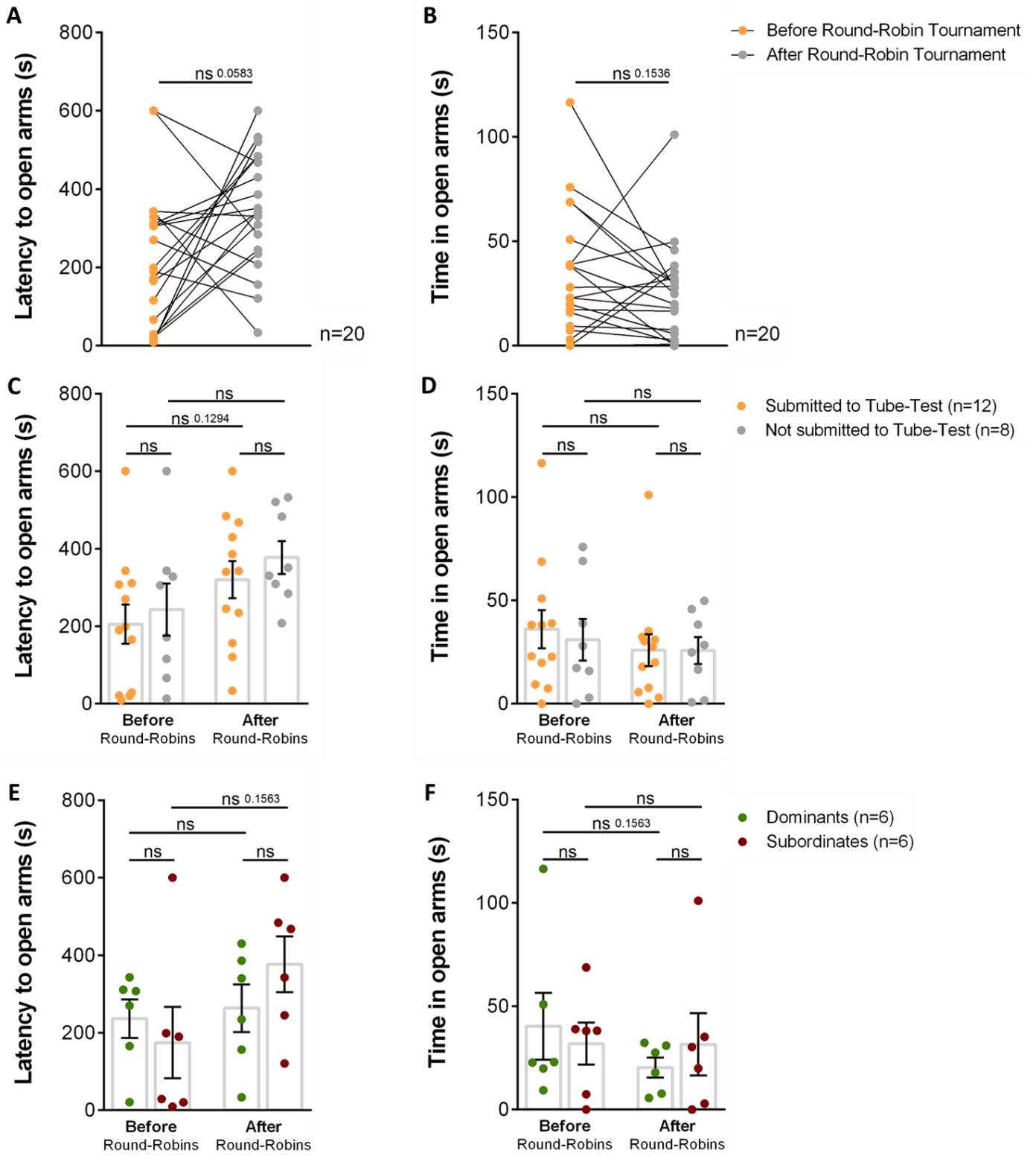


Figure 15 – Approach avoidance anxiety-like behavior does not predict, nor is influenced by dominance. (A) Latency to enter an open arm and **(B)** total time spent in open arms of the EPM, before and after 4 Round-Robin Tournaments, for all subjects. **(A)** Latency to enter an open arm does not change significantly, as well as **(B)** time in open arms. **(C)** Latency to enter an open arm and **(D)** total time spent in open arms, before and after tournaments, for individuals subjected to tube test trials and those not subjected. Tube testing does not induce significant differences neither in the **(C)** latency to enter an open arm, nor in the **(D)** total time spent in open arms. **(E)** Latency to enter an open arm and **(F)** total time spent in open arms are not predictive of dominance neither are influenced by dominance. Dominance attribution considers the cumulative/averaged ranking after 4 Round-Robin Tournaments. All date is depicted as the mean ± SEM. Wilcoxon matched-pairs signed rank test was used in all "before and after" analyses and Mann Whitney t-test for analyses within each timepoint, p value ns > 0.05.

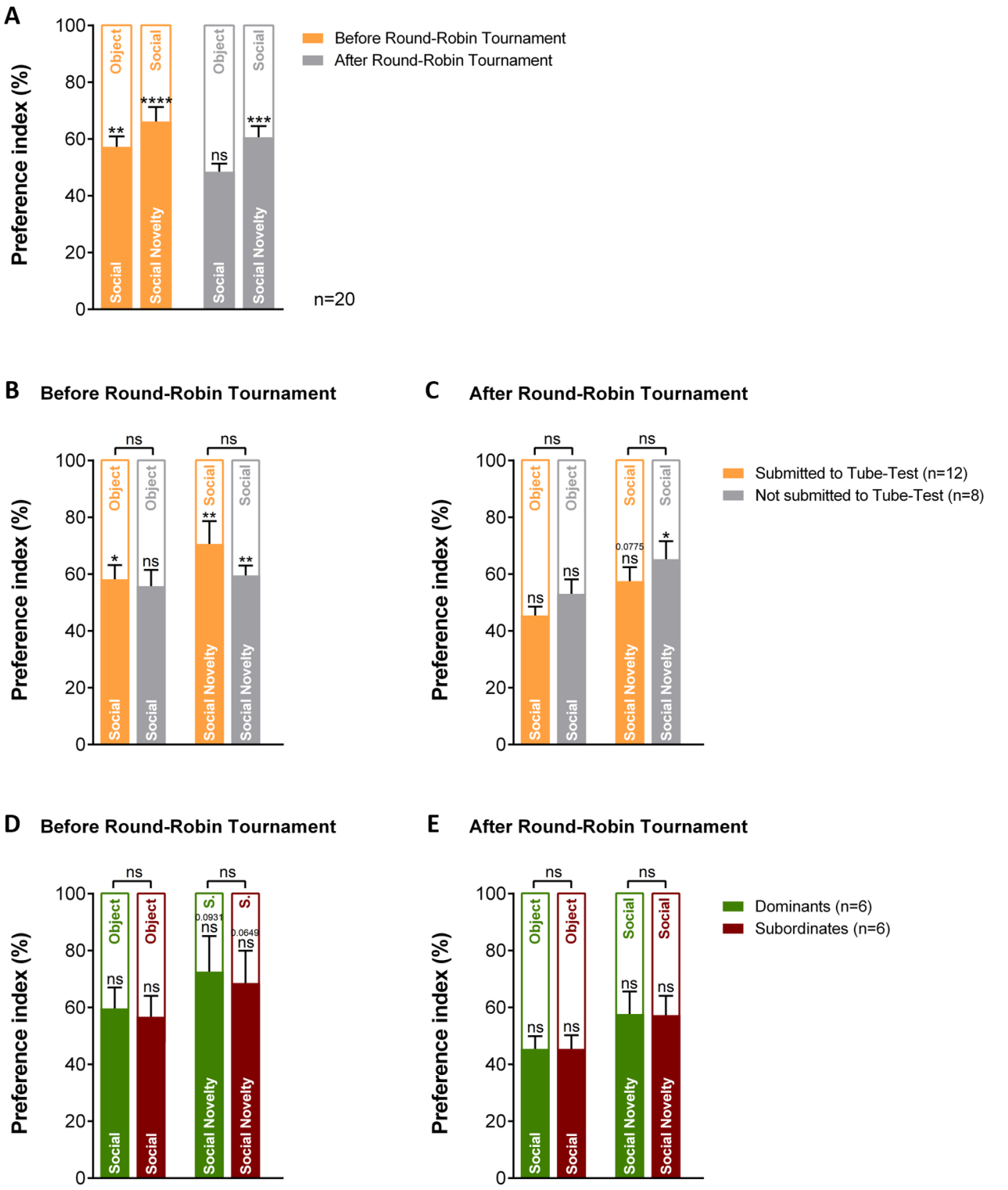


Figure 16 – Sociability does not predict, nor is influenced by dominance. (A) Preference for social and social novelty environments, before and after 4 Round-Robin Tournaments, considering all individuals. **(A)** All individuals display an increased preference for the social novelty environment, in both time points, whereas social preference is only significant before tournaments. **(B and C)** Preference for social and social novelty environments by subjected and non-subjected individuals to the tube test, **(B)** before and **(C)** after tournaments. **(B)** Before tournaments, only the cages destined to be subjected to the tube test display a significant social preference, but both showed significant social novelty preference. **(C)** After Round-Robin Tournaments, subjected animals lost their social and social novelty preference, whereas non-subjected maintained the same profile. **(D and E)** Preference for social and social novelty environments by dominant and subordinate individuals, before and after Round-Robin Tournaments. **(D)** Neither social preference, nor social novelty preference are predictive of social dominance and **(E)** also are not altered by dominance acquisition. Dominance attribution considers the cumulative/averaged ranking after 4 Round-Robin Tournament. All data is depicted as the mean ± SEM. Mann Whitney t-test was used for all analyses within each timepoint, p value ns > 0.05, * < 0.05, ** < 0.01, *** < 0.001.

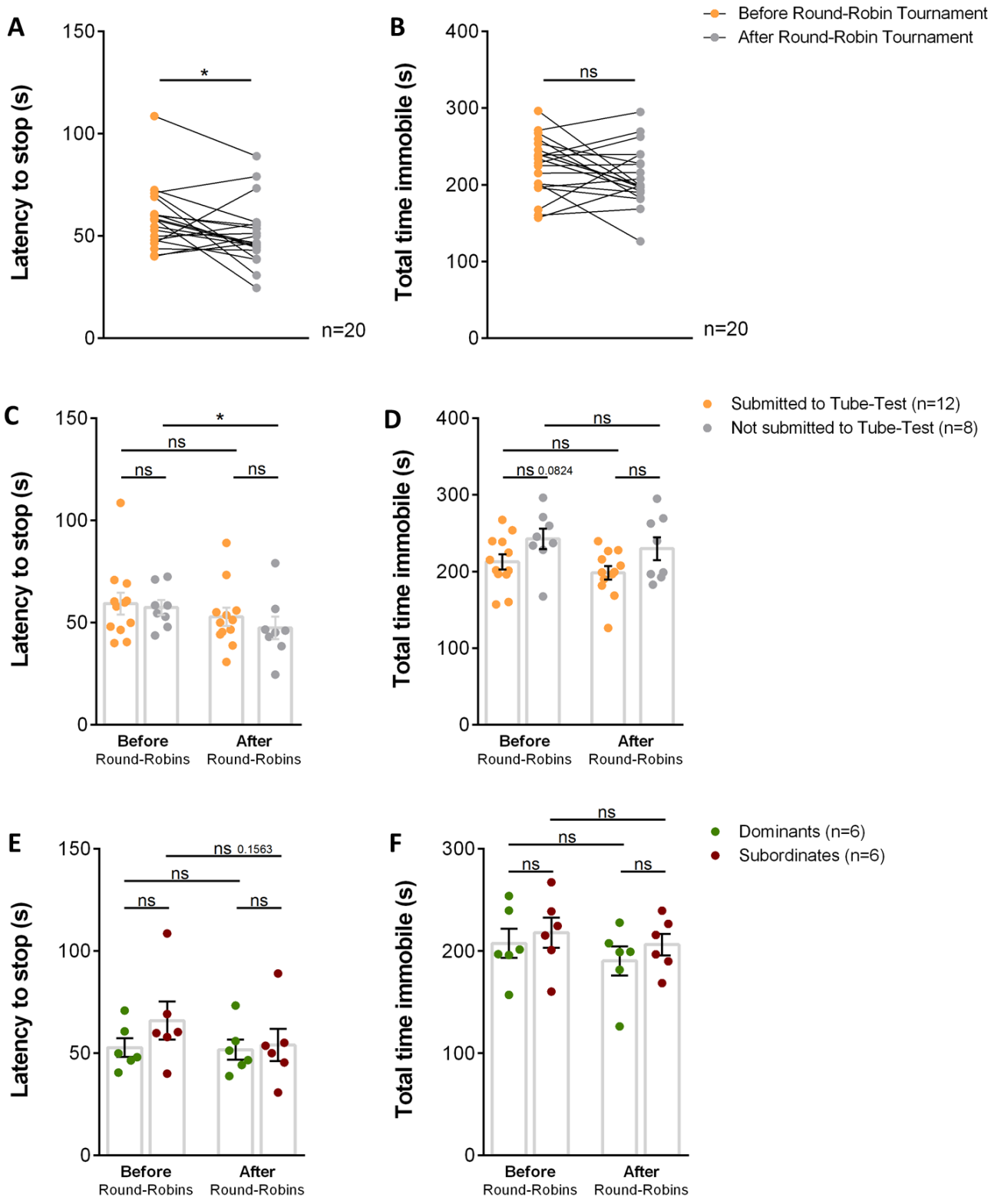


Figure 17 – Depressive-like behavior does not predict, nor is influenced by dominance. (A) Latency to stop swimming and **(B)** total time immobile in all subjects, before and after tournaments. In all subjects **(A)** latency to stop swimming, but not **(B)** total time immobile is significantly altered towards a decrease, after tournaments. **(C)** Latency to stop swimming and **(D)** total time immobile before and after tournaments, for individuals subjected to tube test trials and those not subjected. **(C)** Animals not subjected to the tournaments presented a significantly lower latency to stop swimming after 4 Round-Robin Tournaments, whereas subjected animals remained unchanged. **(D)** Total time immobile is not significantly influenced by exposure to the tube test. **(E)** Latency to stop swimming and **(F)** total time immobile do not predict, nor are influence by dominance. Dominance attribution considers the cumulative/averaged ranking after 4 Round-Robin Tournaments. All date is depicted as the mean ± SEM. Wilcoxon matched-pairs signed rank test was used in all "before and after" analyses and Mann Whitney t-test for analyses within each timepoint, p value ns > 0.05, * < 0.05.

Molecular correlates of social dominance

To verify if the observations made by Fei Wang and colleagues (2011) could also be replicated in our hands and in the context of a Round-Robin trial, we tested whether dyadic encounters in the tube test and consequent establishment of dominance relationships, induce changes in expression patterns of cFos in the medial prefrontal cortex.

Interacting within the tube with an opponent and eventual *win* or *loss* of a trial tends to induce more cFos expression in the medial prefrontal cortex, with different contributions from different sub-regions, when comparing to animals that just cross the tube (Neg TT). Notwithstanding, this expression shows a moderate tendency that is more pronounced in subordinates than dominant mice **(Figure 18A and 18B)**.

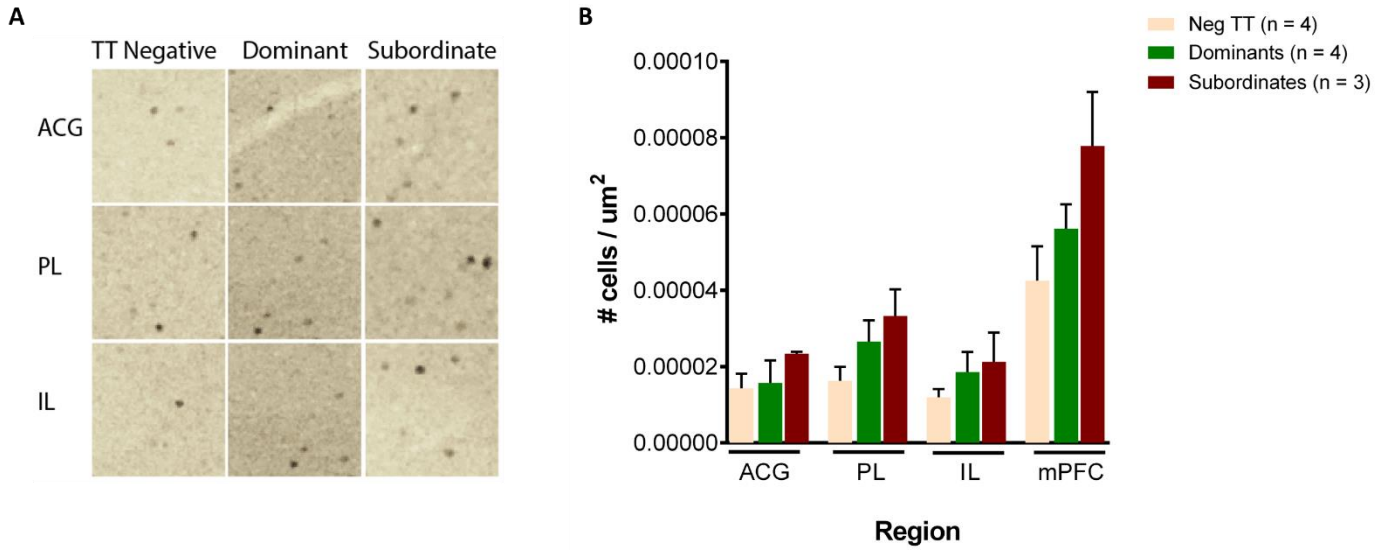


Figure 18 – Establishment of dominance relationships through the tube test tend to increase cFos expression in the medial prefrontal cortex of subordinate mice. (A) Representative image of cFos positive cells in different regions of the medial prefrontal cortex in dominants, subordinates and controls. **(B)** Quantification of cFos positive cell density in the different regions. Although both dominants and subordinates shown a non-significant trend to express more cFos than subjects that only cross the tube test without facing an opponent (Neg TT), subordinates tend to present a higher density of cFos positive cells than dominants in all regions depicted. Dominance was attributed according to the outcome of the last 24 trials performed by these subjects. All data is depicted as the mean ± SEM. ACG, anterior cingulate; PL, prelimbic region; IL, infralimbic region; mPFC, medial prefrontal cortex. Image kindly provided by Lara Franco.

Due to the altered expression of several genes in the RNA sequencing results we performed in our stress model, we specifically focused on genes with overlapping roles in receptor activity and behavior **(Figure 7)**. From these eight targets we aimed to test if they would be altered due to stress protocol or due to the lower hierarchical rank phenotype the stress animals develop. Upon quantification by quantitative real-time PCR, we found that the type 1a dopamine receptor D1 (Drd1a) and the type 1 neuropeptide Y receptor (Npy1r) had a significantly higher mRNA expression *ratio* in subordinates comparing to more dominant mice **(Figure 19A)** and that mean percentage of *wins* from the last 5 tournaments correlates negatively with the Npy1r expression *ratio* **(Figure 19B)**.

Next, we intended to understand if this altered gene expression could also reflect alterations in the protein levels of the same candidates. In an attempt to explore this finding, we isolated postsynaptic densities

of medial prefrontal cortex to identify if Npy1r is present in this subcellular compartment. Samples were collected from the same regions as those for RNA sequencing. We determined if the isolation protocol was performing properly **(Figure 20A)** and tested the signal related to the Npy1r **(Figure 20B)**. Although, we could not perform a formal quantification, we can observe a moderate enrichment of the PSD-95 signal, and a trend towards a decrease in the synaptophysin signal, suggesting a successful purification (PSD fraction) **(Figure 20A)**. Importantly, we successfully detect the Npy1r signal in SPM and PSD fractions **(Figure 20B)**. Additionally, isolation of postsynaptic densities and detection of Npy1r protein seems to be slightly similar between the two amounts of tissue tested (17 and 6 tissue hole punches) **(Figure 20A and 20B)**.

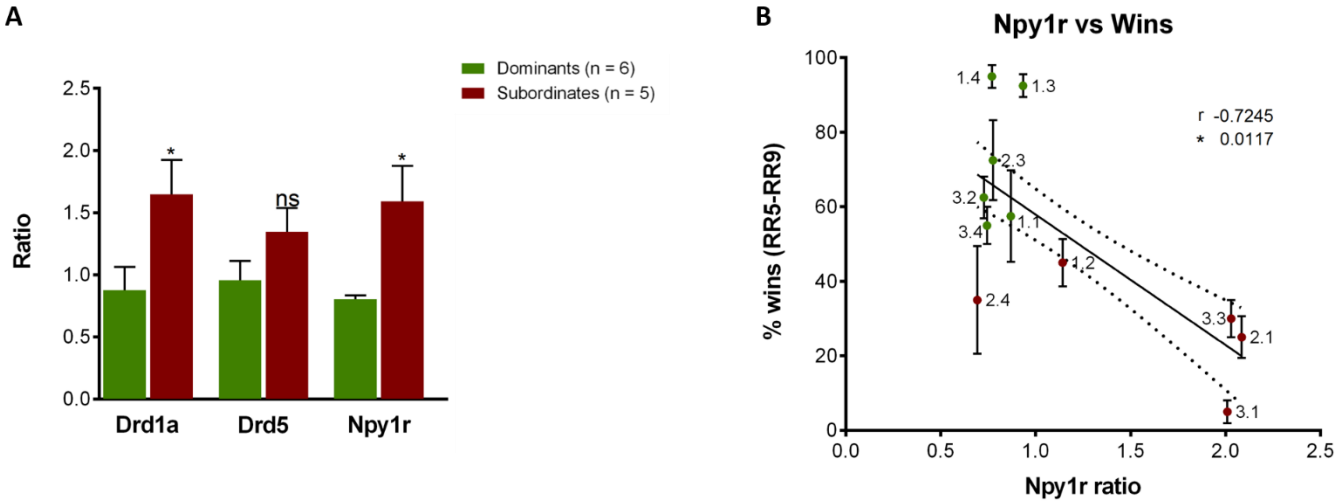


Figure 19 – Neuropeptide Y receptor type 1 expression in the medial prefrontal cortex is correlated with animal dominance in the tube test. **(A)** Ratios between candidate gene expression and *house-keeping* control gene expression in dominants and subordinates. Type 1a dopamine receptor D1 (Drd1a) and type 1 neuropeptide Y receptor (Npy1r), but not dopamine receptor D5 (Drd5), are significantly more expressed in subordinates than dominants. Dominance attribution considers the cumulative/averaged ranking of 5 Round-Robin Tournaments. **(B)** Correlation between percentage of wins in the last 5 Round-Robins Tournament and Npy1r ratio. Npy1r expression is negatively correlated with dominant behavior. All data is depicted as the mean ± SEM. Unpaired t-test with equal standard deviation was utilized to analyze candidate genes ratios and Pearson correlation to correlate Npy1r with dominance, p value * < 0.05. Images kindly provided by Lara Franco.

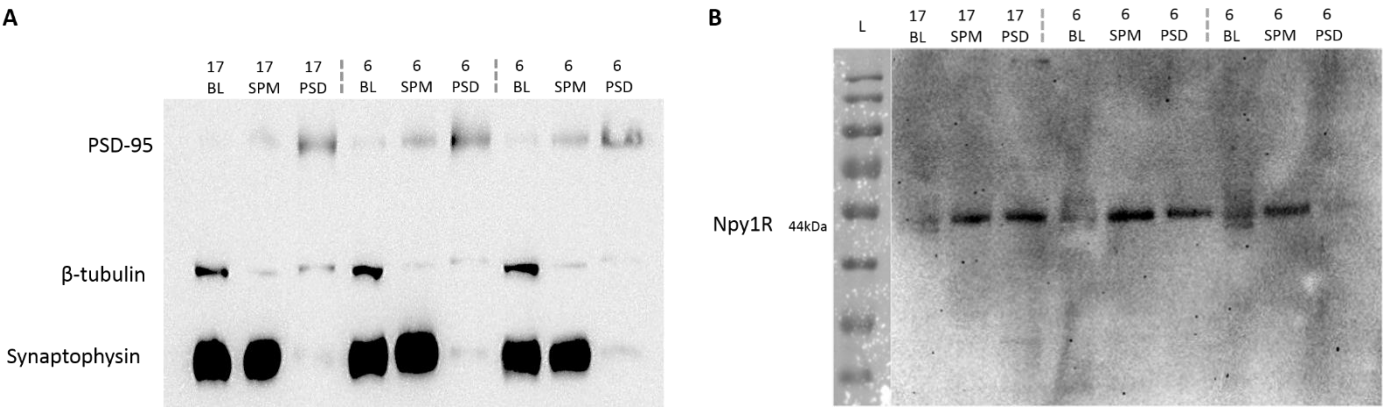


Figure 20 – Isolation of postsynaptic densities control and Npy1r protein labelling from medial prefrontal cortex tissue samples. **(A)** Western-blot showing specific markers for presynaptic terminals (Synaptophysin) and postsynaptic terminals (PSD-95), together with the loading control (β-tubulin). Increasing levels of postsynaptic density protein 95 (PSD-95) along purified fractions of pre and postsynaptic terminals, together with a decrease in synaptophysin signal, demonstrate that isolation was sucessful. **(B)** Western-blot for Npy1r. Npy1r signal appears to be more defined along purified fractions of postsynaptic densities. L, ladder; BL, brain lysate; SPM, synaptosomal membrane-enriched fraction; PSD, postsynaptic density fraction; Numbers - 17 and 6 - denote amount of 2mm diameter tissue punches used; kDa, kilodaltons.

Chapter IV | Discussion

Animals in a social group interact and establish dominance relationships that culminate in social stratification and the formation of a hierarchy. This process is initially characterized by frequent agonistic encounters and circular loops where dominance across individuals is not clearly defined. Eventually stability is reached, dominance relations become well defined and aggressive encounters decrease in frequency. At this point, hierarchy becomes linear and cooperation is more prevalent, shaping access to food, sexual partners and nesting places. Therefore, social stratification is crucial for group dynamics and fitness. However, hierarchy is dynamic in all its phases[1,25]. Individuals of a group try to avoid any event that could bring disorder to the group, such as in the case of territorial conflict. This makes hierarchy in animal societies to become unidimensional. However, and not so often, situations of "intergroup dominance" occur in nature. This are generally associated in situations where territories are shaped in a spatiotemporal fashion, in other words, when groups occupy one territory for specific times, then change to other place but eventually return. In cases where the same territory is shared in different time points there is some risk of more than one group be at the same place at the same time. These situations generate what Edward O. Wilson describe as "intergroup dominance". We took advantage of this observation to create a Round-Robin Tournament scheme that would allow individuals of different cages (different groups) to interact and develop hierarchies beyond their cage dimension **(Figure 9B)**.

We used the tube test to assess dyadic encounters and determine animal dominance. We observed most individuals become well stratified and clear dominance relationships are produced **(Figure 10A)**. Consecutive *wins* and *loses* displayed by the most segregated ranks might represent what is described as the *Winner* and *Loser* effect. This, has been characterized to be dependent on prior experience and in some molecular mechanisms, such as those related to androgen activity/action and cyclic adenosine monophosphate (cAMP)-dependent signaling[10,81–85]. In a very similar paradigm as the one we used, van den Berg *et al* (2014) verified that this effect occurs naturally in the tube test, but when the outcome of a trial is forced, the effect shows a strong dependence on androgen-mediated mechanisms[86]. Additionally, Oliveira *et al* (2009) further supports these results as it observed that the *winner*, but not the *loser* effect, is androgen-mediated[84]. Likewise, a study performed in crayfish showed that the *winner* and *loser* effects are also dependent on serotoninergic and adrenergic-like mechanisms, respectively. Antagonists of both types of activity, selectively prevented the development of *winner* and *loser* effects. Due to the G protein-coupling of each receptor (serotonin receptor 5HT1 is negatively coupled, whereas adrenergic-like receptor are positively coupled), the authors manipulated the cAMP levels in crayfish to assess if the same consequence in the *winner* and *loser* effects was achievable without direct action on the receptor. They found that enhancing cAMP levels in dominant animals prevent the *winner* effect, whereas depleting cAMP levels in subordinates abolished the *loser* effect[81]. Similarly, serotonergic function in the brain has also been shown to influence hierarchical development in male vervet monkeys. Subjects treated with pharmacological compounds able to enhance (tryptophan and fluoxetine) or decrease (fenfluramine and cyproheptadine) serotonergic function, moved upward and downward in the hierarchical ladder, respectively[87]. Given the role of serotoninergic activity in motivational behavior[88], the association between *winner* effect and serotoninergic activity may be

interpreted as an increased motivation to engage in dyadic encounters[8] and establish dominance relations that is reinforced with every *win*.

Thus, *winner* and *loser* effects may have a particular importance for hierarchy formation[82,83,85]. These rapidly allow to select hierarchical extremes and thus, can reduce the number of individuals with similar dominance rankings. Thus, circular and triangular loops, characteristic of unstable phases **(Figure 1)**, decrease in number and incidence. Nonetheless, our data also shows the presence of more undefined relationships, which seem more characteristic of intermediate ranks. This has been described in Edward O. Wilson book *Sociobiology: The New Synthesis*, where Wilson reviews and summarizes a hypothesis made by Ivan Chase (1973, 1974), which considers that in a Round-Robin Tournament *"… most will be just moderately successful."*, which in turn complicates the formation of linear hierarchies[1]. This shows up in the course that hierarchical strength (Landau's index) is closely related to number of individuals in a group. In fact, Thorleif (1922) also has previously described that number of individuals within a group of fowls closely influences hierarchical dynamics and stability[6,20]. Therefore, our results might be an intrinsic consequence of the paradigm adopted, since we are increasing the amount of possible relationships between subjects, which only contact each other during the test and then return to their home cages. In fact, cage hierarchies composed of 4 subjects (inferred considering the intracage trials present in the Round-Robin Scheme), appear to be much more linear than the meta-hierarchy **(Figure 10C to 10E)**. This may be due to the fact these animals are housed together and therefore can address their dominance-relationships more frequently. Supporting this idea, other line of evidence shows that groups of 12 mice living together in a large vivarium, establish well defined linear hierarchies approximately in 48-96 hours[89]. Therefore, moderate instability towards intermediate rankings in our data may be due to lack of further opportunities of some subjects to enforce dominance relationships. Nonetheless, given the *all* vs *all* design of our Round-Robin Tournament scheme, we are able to extrapolate cage hierarchies from the meta-hierarchy **(Figure 10B)**. This allow us to have some degree of confidence regarding the possibility of our observations being also manifested within home cages. However, an important observation is the display of some bias in animals of the same cage towards certain ranks in the meta-hierarchy **(Figure 10A)**. This might be a consequence of the manifestation of different degrees of "natural" dominance among cages. Cages where animals are "naturally" more submissive, "strategies" used by individuals to acquire dominance may be different than those used for animals in more competitive environments. Also, the fact that social hierarchy can be learned[59], could explain why cage mates would rank at a similar level in the meta-hierarchy. A research group created a paradigm where human volunteers could visualize social confrontations, to which the authors attributed a relative dominance discrepancy. They found that besides learning of social hierarchy, watching social confrontations also biases threat learning, which, in mice, could be particularly influent in how an animal behaves inside the tube[59].

Taking all these observations together, although social hierarchy formation is highly influenced by extrinsic factors (e.g. group size, stochastic events, group environment/context), characteristics inherent to the individual might play the most important role and dictate if it will reach specific social ranks - which may

explain why none of the cages are entirely submissive or dominant **(Figure 10A)**. In this view, the Round-Robin Tournament in the tube test appears as a strategy that clearly enables the selection of the most prominent dominance phenotypes to further test and explore the neural correlates of dominance.

The strategies one adopts, as just discussed, might deeply dictate who will acquire dominance. Therefore, we aimed at revealing clues of how a tube test trial is conducted, and we find that dominance and prior experience shape, differently, trial duration and time to first contact within the tube **(Figure 11 to 13)**. Trials where a subordinate lose, independently of the opponent, are always shorter than those where dominants lose **(Figure 11).** Dominance relationships are unidirectional, and therefore, any situation that opposes it will tendentiously be more challenging. Thus, it is expectable that dominants take longer to lose than subordinates. Importantly, it is also observable that along tournaments, both tend to become shorter in duration **(Figure 11B)**, might resemble learning of social ranking of each individual, which is also described to be more related to hierarchy stability[1,6,20]. First group members need to interact, establish relations of dominance and crystalize their position in the group. So, it is expected that at the first tournament, trial duration is larger, as a result of necessity to investigate each other and establish dominance relationships. Also, it is the first time the animals are exposed to this behavioral paradigm, which may carry some confounding factors that influence trial duration. Afterwards, once settled, the main process in tube test dyads would be the perception on the social ranking of the opponent. Communication of social hierarchy in rats, was described to be a process that occurs through sniffing pattern during face-to-face interactions[90]. The tube test, due to its design, only allows individuals to communicate through face-to-face interactions, therefore it assures to the animals communication of their status by this process. This goes in agreement to what we found regarding social rank discrepancy and trial duration **(Figure 13A)**. A similar analysis was performed by Fei Wang *et al* (2011), but restricted only to a context of cage hierarchies[34]. When social rank between opponents is largely dissimilar, perception of social rank is facilitated[59], and for that reason it would be reasonable to expect that trials where social ranking is highly disparate, are shorter than those where ranking is similar between opponents. As was already discussed, subjects are unevenly distributed along the meta-hierarchy and this predisposes the discrepancy in rank to be more frequent towards a specific range of "ΔRanks". Specifically, we have observed that large rank discrepancies are less frequent. Therefore, we can deduce that the majority of trials with lower rank discrepancy belong to those animals whose dominance relations are more unstable and not well defined, so, trial duration becomes longer.

We also asked whether previous social knowledge (including social status) regarding opponents, would have an impact on trial duration, when compared to situations where individuals did not know each other previously. Intriguingly, we found that trials between individuals of different cages are not different in duration when compared to those between cage members **(Figure 11C to 11F)**. Social recognition in 4 to 8 month old C57/BL6 adult male mice has been described to last up to 7 days[91], which largely covers the period spent since they are collected from their cage until they interact in the tube. Therefore, two further questions emerge: (1) do the animals still recognize their opponent, yet this recognition brings no influence to trial duration?; (2) Does the tube test prevent the ability of individuals to perceive cues important for social

recognition? Mice perform recognition through a variety of mechanisms, comprising odors, hormones, vocalizations and touch, in different types of investigations, such as face-to-face, flank and ano-genital investigations[90,92]. Of these, the tube test, due to its structure, prevents ano-genital and probably at some degree, flank investigations. However, ano-genital interactions are more characterized to initial/first encounters[92], hereupon, the tube test does not seem to prevent the capability of animals from the same cage to social recognize each other. Therefore, these results may be simply due to absence of influence of social recognition in trial duration. If so, trial duration would not evolve along tournaments as we clearly observe in our data **(Figure 11B)**. Thus, this implies that social rank perception can be a parallel process regarding social recognition or that is dependent on context, and so, hierarchy perception inside the cage becomes a different process than status perception in the tube test. Also, it is not excluded that animals attribute some degree of reward and/or aversion to the tube itself, and therefore influence their performance. Social dominance hierarchies are widely studied in the context of striatal function[51–53], and therefore is becomes reasonable to ask whether animals in the context of the tube test could develop some degree of *winning*-associated reward[8]. Moreover, corticostriatal communication in these context promising, since both structures present evidences to be deeply involved in dominance contexts and also, due to its anatomical and biological relationship.

Duration to first contact, although there is no overall difference between intracage trials and intercage trials **(Figure 12A)**, after trial segregation according to rank of the individuals engaging in the trial, we observe that in intercage trials, homologous dyads among subordinates are significantly longer than the remaining conditions. The same does not happen in intracage dyads, with no significant differences observed between conditions **(Figure 12C and 12D)**. Duration to first contact inside the tube may reflect neophobia, and therefore, animals may dedicate some time to better analyze their opponent before engaging. In this case, prior social cognition may have an influence. Hence, individuals with prior knowledge of their opponent, as in the case of intracage battles, would approach each other more rapidly when compared to intercage trials. However, the reduced sample of subordinate vs subordinate in intracage trials may be confounding the results. Furthermore, the fact this result is more directed towards subordinates may reflect the decreased predictability and lack of control felt by subordinates as Sapolski described in primates[25]. If this holds true, the immunohistochemistry results for cFos expression **(Figure 18)** may be explained by the fact that activity in the mPFC may possibly be recruited also as a consequence of reduced predictability[25,27,93] and therefore explain why cFos tends to be more expressed in subordinates. However, it would still be required to confirm if this activity, measured by cFos expression, derives from principal cells and/or interneurons. This would also be relevant to evaluate coherence between our results and those from Fei Wang *et al* (2011), since they showed that dominant behavior was more associated with increased cFos expression.

In addition, the fact this observation does not happen in the presence of a dominant (Dominant vs Subordinate), might be explainable if in these interactions, the dominant is the one approaching. Self-reported dominants (humans) were described to perform worse in social hierarchy learning[59] and also, those who display a higher degree of *aggressive-dominance*, appear to rely less on social learning[21,59]. Simultaneously,

dominant primates only displayed higher attention to those above them or at the same level in their hierarchy[21,94,95]. Therefore, in these trials, dominants could be advancing farther and faster. In homologous trial between dominants, according to these explanations, the most dominant would be the one advancing more in the tube.

Location of the first contact in the tube test was hypothesized as an aspect that could confer initial advantage or disadvantage for the course of one trial. It would be reasonable to state that an animal that contacts in the tube test at a longer distance from the entrance side than its opponent, would have some sort of advantage, since the distance to expel it would be shorter. In this view, we observed that this parameter is independent of the outcome of a trial **(Figure 14)**. Animals show similar location of the initial contact in trials they have won **(Figure 14A)** and in those they have lost **(Figure 14B)**, and therefore, instead of being a parameter that may influence dominance acquisition, may be an observation that is subject-dependent. Considering this, the shorter duration to first contact in heterologous trials (between subordinates and dominants) **(Figure 12C)** may be a consequence of dominants being those engaging in interaction, which could reflect a contact point shifted to the opposite side of entrance. In our results, although the most dominant animals present a tendency to a shift more deviated towards the opposite side than most subordinates, both are relatively close to the midline. On the other hand, intermediate ranks present the most shifted contact points towards the opposite side, but this population is comprised of both dominant and subordinate individuals **(Figure 14)**. Therefore, our results regarding contact point in the tube test do not contain the detail required to prove this hypothesis. To do so, similar analyses should be performed considering the rank of the opponent. However, our results give some insights that, serendipity might not be the most influent factor in establishing dominance relationships. Every individual shows a considerably wide range of contact points over tournaments, some even win or lose a trial in what would be the most unfavorable and favorable situation, respectively. Therefore, how animals react and behave upon these situations is possibly more relevant for trial outcome than stochastic events experienced in the tube. This stresses that a detailed and scrutinized examination of the behavior of the subjects within the tube test may be an interesting task to be performed and that would allow a better knowledge of how dominance is acquired in the context of the tube test. Precise description of animal behavior inside the tube could allow further analyses to test which epochs during the trials are more relevant, which sequence of events might me associated with the process of *winning* or *losing* and how strong would be these chains of events in conferring dominance when manipulated.

Notwithstanding, our results regarding social hierarchy formation and more detailed behavioral analysis in the context of the tube test confer a first line of evidence that so far is not described, and that with further processing of this information, could be of major relevance in unveiling how hierarchy formation using the tube test occurs.

We also assessed whether behavioral traits, specifically approach-avoidance anxiety, sociability and depressive-like behaviors, could be predisposing some individuals to become dominants and/or subordinates, and whether acquisition of dominance could have an influence in those same traits.

Interestingly, we found that none of the measured parameters predisposes for or is altered upon dominance acquisition **(Figure 15 to 17)**. The group of Carmen Sandi has characterized the relationship between social status and anxiety in male wistar rats. In their work they found that highly anxious rats, more likely become submissive individuals with low social status, in comparison to those with a low anxiety profile. Also, they observed that neither high-anxious, nor low-anxious rats were different in their social behavior[19]. Moreover, mutant mice for the *Shank3* gene were characterized to be highly dominant, but in contrast displayed anxious-like behavior together with social deficits[17]. Additionally, mutations in the μ-opioid receptor gene (*OPMR1 A112G)*, led to a dominant mouse model with also increased sociability[18]. These discrepant relations of dominance with anxiety-like and social behavior may suggest that these behavioral traits are linked to dominance in a very subtle manner and may depend in a range of different factors. In terms of experimental methodology, these studies are also very dissimilar. On one hand, Sandi group used social isolated rats and assessed dominance based on aggressive interactions between individuals. We have approached dominance with the tube test and used mice as animal model, which were socially housed. Therefore, dominance may be occurring differently and also variability between species may be a confounding factor. Also variability within the population may be influencing this aspect, since we addressed this question with a total population of 12 animals, whereas Sandi group used 24 rats per group of high- and low-anxious animals. The higher the population size the higher are the chances to capture subtle differences. However, Fiona Hollis *et al* (2015), as we also found, does not observe a relation between dominance with sociability. Suggesting that different behavioral traits may be linked to dominance differently.

On the other hand, other groups have looked at specific mutant mice, and their performance in the tube test to evaluate dominant behavior. This, further supports that dominance may have several genetic mechanism underlying it, and that these may in parallel be accompanied by differences in other behavioral traits. In other words, this might mean that different functional pathways (e.g. *Shank3*-related or *OPMR*-related) may in fact influence dominance, but simultaneously influence also other behavioral traits (e.g. anxiety and sociability). On the contrary, could be that these genetic modifications could be causing different anxious and/or social profiles, and because of those, animals would behave in a way that confer them low and/or high social dominance. Moreover, if differences according to dominance in these behavioral traits are more dependent on genetic features, because our animals share a great similarity at the genetic level, they would unlikely be expressed in these animals. Regarding depressive-like behavior, some evidence points towards a cause for submissive behavior[12–14], whereas others point as a consequence of subordinate behavior[15,16]. Although associations exist between depressive-like behavior and dominance, to our knowledge, it is still not clear that there is any direct and precise mechanism that could explain the relation between both. However, several piece of evidence suggest that serotoninergic function correlates with the display of dominant behavior[8,81,87]. Also, the fact that this type of activity has been widely studied as dysfunctional in psychiatric mood disorders[88], leads to the current hypothesis that depressive-like behavior generates a submissive-like behavior due to serotoninergic function deficits[8].

In our data, we found no changes in depressive-like behavior when considering social dominance **(Figure 17)**. This may also be due to similar reasons as those discussed previously for the remaining traits. Individuals that are very similar among themselves will unlikely display a behavioral differences *a priori* that could lead them to become dominants or subordinates. However, dominance hierarchies are characterized by imposing social norms and enforcing status over group members, which leads to an uneven stress incidence[5,23,25]. In fact, this might explain why some animal models are selected as depressive-like models after social defeat paradigms[15,16]. Therefore, we asked whether these traits could also be altered between dominants and subordinates after social stratification. The fact we did not observe any differences in dominants and subordinates for every trait we have tested, may derive from the fact that dominance classification was made considering the meta-hierarchy. Although this type of stratification enables us to predict rank of one individual inside its home cage **(Figure 10B)**, these relations between intercage animals are not maintained after trials finish, and although, we can conclude who is the most dominant and subordinate, we are probably preventing the chance of enforcing their dominance upon others.

To further support our findings regarding social stratification process we also analyzed the same traits before and after tournaments, but without further segregation in dominant and subordinate groups **(Figure 15 to 17)**. Anxiety-like behavior in our animals is more likely being only influenced by age, since both conditions (subjected and not subjected to the tube test) evolve similarly. In fact, EPM parameters are altered between young and adult rats[96,97]. Differently, sociability appears to be lost in the population of animals subjected to the tube test **(Figure 16B and 16C)**. The only explanation that may address this observation would be some degree of social aversion displayed by the animals as a consequence of repeated exposures to the tube test, nevertheless, this still requires further investigation. Moreover, regarding the results from the forced swimming test, we have determined that “older” animals (not subjected to tube test) acquire a lower latency to stop struggling, which could be interpreted as a depressive-like behavior **(Figure 17C)**. In other words, being tested in the tube test might have prevented the occurrence of this observation. Tube test tournaments are a demanding and dynamic procedure that causes animals to lose considerable amounts of weight (data not shown) after each tournament that is eventually recovered after a few days. This aspect may be looked at as physically demanding, and therefore, may indirectly affect performance in the forced swimming test. Besides weight, age of subjects was also reviewed as an influent factor that may influence the results in the forced swimming test - older animals become more depressive-like[98].

Finally, some results from our RNA sequencing were further confirmed to be more related with social hierarchy, of which Npy1r appears the most interesting, since it correlates negatively with dominance **(Figure 19B)**. Npy1r has already been described to be particularly enriched in the frontal cortex of rats[68]. As briefly mentioned in the introduction (Chapter I – “Role of neuropeptides in social hierarchy”), it is unlikely that NPYergic activity in the mPFC derives from hypothalamic control, however, this type of activity can also reflect GABAergic activity (gamma-aminobutyric acid – GABA), since co-release of both molecules has been confirmed[99–101]. In fact, NPY classifies one type of GABAergic interneurons and also labels approximately 50% of somatostatin-positive (SST) interneurons[102]. So, the role of this neuropeptide might also be related

to local interneuron activity. Manipulation of Npy1r-related activity in the IL was shown to have a profound impact in fear extinction recall. This observation was achievable by administration of NPY during both initial and late phases of fear extinction separately. The effect was mediated by Npy1r, since administration of an Npy1r antagonist together with NPY totally prevented this observation. A dampening of the IL layer V excitatory pyramidal neurons excitability, as measured by patch-clamp recordings, appeared to underlie these observations. The authors observed a significant enhancement of the inhibitory postsynaptic currents (IPSCs) amplitude, with no significant effects on the excitatory postsynaptic currents (EPSCs) in these neurons. These results led the authors to suggest that NPY, towards its action on Npy1r, is optimal for controlling prefrontal excitatory outputs[103]. Npy1r is a $G_{i/o}$-coupled protein receptor, with an apparent dual function when considering $GABA_A$-mediated activity and Glutamatergic activity. Activation of this receptor has been proposed to, through different signaling pathways, culminate in decreased activity of the protein kinase A (PKA) and exchange protein activated by cAMP (EPAC), which respectively cause a potentiation of $GABA_A$-mediated currents and a decrease in NMDA-mediated currents[104]. Hence, NPY, when co-released with GABA, might act towards a potentiation of GABAergic currents.

These results are particularly interesting for the following reasons: (1) prefrontal excitatory output was the parameter that Fei Wang *et al* (2011) suggested as underlying differences and transitions in social ranks, by acting according to the model discussed before **(Figure 3A)**; (2) Npy1r-mediated activity in the mPFC (IL), significantly enhanced IPSCs amplitude, and this was sufficient to modify behavior. Our data, verified that expression of the Npy1r followed a dominance gradient **(Figure 19B)**, and thus, based on the results demonstrated by Lauren L. Vollmer *et al* (2016), it might be possible that modulation of the NPYergic function in the mPFC could influence and trigger behavioral changes in a context of social hierarchy.

Other line of evidence points towards the role of NPYergic activity on anxiety-like behavior. Inactivation of Npy1r gene specifically in excitatory neurons of the forebrain, triggered the development of anxiety-like behavior in mice[105,106], which, as previously discussed, relates both with dominant and submissive behavior. Thus, NPYergic activity might also be linked to dominance behavior through its action and role on anxiogenesis.

To conclude, the manifestation of submissive behavior might be a consequence of a decreased excitatory output from layer V mPFC neurons, as a result of suppressed excitability, due to overexpression of Npy1r in those neurons. Therefore, GABA, when co-released from NPY-positive or SST- and NPY-double positive interneurons in the mPFC, would trigger an abnormal inhibitory action. If Wang's model holds true, this inhibitory action would trigger a specific top-down control over several subcortical regions, each one responsible for a specific dominance-related behavior. As discussed previously, these types of behaviors may range among social perception, hierarchy learning, reward-related, motivation to confront, salience and aggression.

Chapter V | Conclusion and Future Perspectives

In this work we reveal pieces of evidence that intergroup hierarchies in wildtype mice is a defined process, supported by precise temporal dynamics during dyadic encounters inside the tube test. Also, we have shown that these variables may evolve in parallel with hierarchy stabilization, and reflect hierarchy inside of each individual group. Moreover, we have verified that behavioral traits in our animal model are not predictive, neither change with intergroup social stratification, but that repeated exposure to the tube test alters the normal development of depressive- and social-related behaviors. Finally, we have also found that dominance relationships carry an array of molecular correlates, such as trend towards increased cFos expression in the medial prefrontal cortex of submissive individuals and a strong negative correlation of the Npy1r with dominance status.

Nonetheless, much of the present work may be complemented by future experiments. Additionally a detailed collection of behavioral correlates inside the tube test, aiming to scrutinize which epochs and actions may be the most relevant and prominent in dictating a trial outcome, will give further insights in how dominance relationships are established in a precise manner. Furthermore, it would also be of particular interest to assess if action and manipulation of NPYergic function in the mPFC would be sufficient to trigger rank transitions in the hierarchy and how these animals would behave during those transitions. Additionally, and to have further detailed information about neuronal encoding of social hierarchy, *in vivo* electrophysiological characterization of these processes (hierarchy formation and manipulation) could be performed. Also, and due to the wide range of possible dominance-related behaviors, addressing how communication between the mPFC and other subcortical regions occurs, is a next critical step. Finally, specific tracing experiments are essential to identify the source of NPYergic activity in the mPFC.

Chapter VI | References

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