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THE ROLE OF SIRT1 PROTEIN IN NUCLEUS ACCUMBENS IN RELATION TO ANXIOUS BEHAVIOUR AND SOCIAL DOMINANCE

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ABSTRACT

Introduction. This study investigates the role of Sirt1 protein in anxiety-like behaviour and in social dominance. The purpose of the experiment was to verify whether the ablation of the protein in a specific brain region, the nucleus accumbens, could affect the behaviour of the mice in terms of social dominance and anxiety levels.

Methods. 24 weaned mice were hosted in 6 separated cages, each one with four mice. Mice from three cages received a viral microinjection by stereotaxic surgery which prevented Sirt1 gene expression specifically in the nucleus accumbens, while the other ones received the same stereotaxic surgery treatment with a different virus that had no effect on the gene expression.

Results. Data obtained from light/dark box test showed a difference between the knock-down group and the control one in the latency of first entry in the light compartment. In fact, the knock-down group displayed a significantly greater latency compared with the control group. This result is related to a greater anxiety-like behaviour but was not confirmed by the other variables analysed.

No interaction effect was found between Sirt1 knock-down, social dominance and anxiety but, interestingly, a significant difference emerged in the number of novel object approaches between dominant mice and subordinate ones. In fact, subordinate mice displayed a greater exploratory behaviour (more approaches to novel object) than the dominant one.

Conclusions. We can assume from the results obtained that less concentration of Sirt1 protein in nucleus accumbens seems to be potentially related with an anxious behavioural phenotype.

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Chapter 1: Introduction

1.1 Social dominance

Human social systems have evolved based on social hierarchy, which have emerged to increase the probability of survival in hazardous situations. (N. Watanabe & M. Yamamoto, 2015)

Social hierarchy is a phenomenon among social animals and a fundamental self-organizing scheme that has a deep impact on individuals' survival, reproductive success, health, (Z. Fan, H. Zhu, T. Zhu et al. 2019) and behaviour.

The establishment of social hierarchies is evolutionary conserved across species from insects and fishes to rodents, in which both intrinsic (physical and mental factors that are inherent and located within, for example body size/strength, courage/fear, grit/persistency, stress level) and extrinsic factors (factors that are not inherent, acting from the outside, for example, environment, state of ally and opponents, experience such a history of winning/losing) contribute to social status determination. (T. Zhou, C. Sandi et al. 2018)

Moreover, social hierarchy helps to reduce conflicts within the group because it sets rules about, for example, accessibility to resources (like food, water, potential mates etc.) where, generally, dominant individuals have a preferential access. (K. B. Leclair, et al. 2021)

Dominant individuals are important for social hierarchy as well as subordinate ones.

In this study we focus on social dominance, characterized by consistently winning at points of social conflict because, for example, the motivational priorities of two (or more) individuals are incompatible (F. Wang, et al. 2014).

In rodents, social dominant behaviour is characterized by winning in conflict situations with peers, displaying agonistic behaviour[1], getting the first access to food, marking of territory, a prominent order in grooming, proactive courtship, and a low participation in labour. (F. Wang, H. W. Kessels & H. Hu, 2014).

Social dominance relationships are common for most laboratory animals and are often determined by observing predictable patterns regarding which animal chases or retreats during social interactions. These predictable patterns are organized in a hierarchical fashion where each animal has a social rank within the group. For example, groups of laboratory mice commonly form transitive or despotic hierarchies.

Transitive hierarchies are composed with individuals with unique dominance ranks (e.g., alpha, beta, gamma), while despotic hierarchies are comprised of one individual with a defined alpha rank and undefined subordinates ranks cage-mates. (J. A. Varholik, et al. 2018)

In this study we considered the linear transitive model to determine the social rank of the mice.

Furthermore, a famous paradigm to evaluate social dominance in laboratory rodents is the tube test. It is a nonviolent conflict situation created ad hoc to force only one of the two rodents (mice or rats) to pass through the tube. (F. Wang, H. W. Kessels & H. Hu, 2014)

Interestingly, the stability of a social hierarchy seems to decrease with an increased number of mice per cage. For example, aggressive behaviour in group-housed male

[1] Agonistic behaviour: social behaviour related to fighting that includes threats, displays, retreats, placating aggressors, and conciliation.

mice decrease by housing the animals in small groups of three to five animals (Pascalle L.P. et al.; 2001).

Aggressivity has often been associated with social dominance. For example, dominant male's agonistic behaviour may serve to solidify social hierarchies and avoid fighting, but only when the subordinates do not respond with appropriate submissive behaviour, violence seems to be used by the dominant. (Wang F. et al. 2014)

Indeed, several studies suggest that aggressive behaviour seems to depend more on social context as a result of a cost-benefit analysis, than on social dominance itself. (Hillman, K. L. 2013 - Wang F. et al. 2014) For example, group-housed familiar mice do not exhibit extensive aggressive behaviour towards cage-mates, presumably owing to the suppression of aggression by a stable social hierarchy. In fact, even when mice are tested in the resident-intruder paradigm[2] against unfamiliar standard opponent mice and aggressive behaviours can be observed, the resident-dominant mouse of the cage did not appear to be more aggressive. (Benton, D. et al. 1980 - Wang F. et al. 2014)

To conclude, although aggression may initially be necessary to determine a hierarchy, once established, it can suppress further aggression and unwanted fights among the group members. (F. Wang, et al. 2014)

However, social hierarchy in mice is not fully stable and, although the presence of dominance hierarchies is found in cages of male and female mice, social dominance can change over time.

Furthermore, social dominance, in humans as well as in animals is related to a specific brain activity (N. Watanabe, M. Yamamoto, 2015) and to a different behavioural

[2] Resident-intruder paradigm: an intruder animal is put in the cage of a resident animal who will display a specific aggressive behaviour towards the intruder.

phenotype. For example, in rodents, dominant individuals usually barber the hair and plucks the whiskers of their cage-mates (the Dalila effect[3]), deposits small drops of urine to mark their territory and emit significantly more vocalizations when receiving a female stimulus. (Wang F. et al 2011)

Furthermore, J. A. Varholik, et al. 2018 found that social dominance (i.e., dominance rank and degree of dominance behaviour) accounted for phenotypic variation in mice. For example, they found a relationship between social dominance and exploratory behaviour in which subordinate mice expressed more exploratory behaviour than the dominant ones. Interestingly, the study also reported several dynamic hierarchies among cages, where mice formed unclear ranks. This finding contradicts the general assumption that mice often form stable and transitive dominance behaviour hierarchies.

To conclude, social hierarchy is a universal phenomenon among animals and can help to reduce conflicts by setting rules. For example, dominant individuals usually have preferential access to resource.

In rodents, social dominance relationships are very common and can be expressed in several different ways (i.e., despotic or transitive) where aggressivity is not always necessary and it does not depend necessary on social dominance. In fact, in stable social hierarchies aggressivity can be greatly reduced.

Lastly, recent studies also report the existence of dynamic hierarchy in mice, where the relationship between social dominance and phenotypic profile seems to be more complex and poorly known.

[3] Dalila effect: excessive type of grooming observed among laboratory mice in which the dominant mouse barbers the hair and plucks the whiskers of its cage-mates.

1.2 Neural substrates of social dominance

In the last decade, neuroscience discovered interesting findings to clarify neural substrates underlying social dominance and determine which brain regions are involved in the establishment of social hierarchy.

The amygdala, hippocampus, intraparietal sulcus (IPS), ventromedial prefrontal cortex, lateral prefrontal cortex, and the striatum seem to be involved in several aspects of social dominance, including perception, learning, and formation of social dominance. (N. Watanabe & M. Yamamoto, 2015)

Each region contributes to different aspects of social dominance, and reciprocally connecting with one another. (N. Watanabe & M. Yamamoto, 2015)

The amygdala is generally considered as the centre of emotional responsiveness. (Ledoux, 2007; mentioned in N. Watanabe, M. Yamamoto, 2015) Additionally, this brain region has high sensitivity to social information such as trustworthiness and social rewards. (Adolphs, 2010; mentioned in N. Watanabe, M. Yamamoto, 2015)

The first study to investigate the function of amygdala in terms of the social behaviour in non-human primates found that high-ranking monkeys with surgical lesions of this brain region lost their status in the social dominance hierarchy and became extremely submissive. (Rosvolt et al. 1954 mentioned in N. Watanabe & M. Yamamoto, 2015)

Subsequent experiments reported that monkeys with selective bilateral lesions of the amygdala showed insensitivity to threatening social signals. (Machado & Bachevalier, 2006 mentioned in N. Watanabe & M. Yamamoto, 2015)

These findings suggest a crucial role of the amygdala in social dominance perception and maintenance. (N. Watanabe & M. Yamamoto, 2015)

The second brain region involved in the establishment of social hierarchy is the hippocampus (HPC) as well.

Evidence from a wide range of species support the hypothesis that the HPC is essential for both episodic and semantic memory. (Eichenbaum H. 2017)

In rodents, the ventral HPC (vHPC; anterior HPC in primates: aHPC) is involved in modulation of motivated behaviours via connections to the hypothalamus and amygdala, and in valence associative learning (Fanselow M.S. & Dong H-W, 2010).

The vHPC is very important for social memory because of its role in integrating valence information from hypothalamus and amygdala connections. For example, inhibition of the vHPC in mice can impair both memory encoding and recall of individuals. (Okuyama T, et al., 2016)

Furthermore, one study suggests a role of vHPC in learning social ranks in a context of social hierarchies. In fact, BDNF[4] which enhances neural plasticity required for learning, is upregulated in the HPC of dominant mice after winning agonistic interactions (Taylor S.L., et al. 2011), potentially reinforcing their dominant social status.

So, there is strong evidence that the role of the HPC in encoding individual social memories is conserved across social species, but further studies are required to verify its specific role in the establishment of social hierarchy.

Much information related to social hierarchies seems to be processed in the prefrontal cortex (PFC), the centre for executive behavioural control.

[4] BDNF: brain derived neurotrophic factor.

Monkey studies suggest a role if PFC in registering elements of social state. In fact, neuronal activity in the PFC increased in dominant monkey and decreased in subordinated ones during social engagement. (Fujii N. et al. 2009)

A study reported how lateral PFC (LPFC) neurons of monkey represented winning and losing in competitive video games. (T. Hosokawa & M. Watanabe, 2012) In the experiment, monkeys played a video shooting game, either competing with another monkey or the computer, or playing alone without a rival.

Results reported that they were more motivated in the competitive than in the non-competitive games, in fact, LPFC neurons showed differential activity between the competitive and non-competitive games showing winning- and losing-related activity. Thus, activities of prefrontal neurons differed depending on whether the competition was between monkeys or between the monkey and the computer. (T. Hosokawa & M. Watanabe 2012)

Instead, morphology of the rostral PFC correlated with social dominance. For example, dominant monkeys that live in large social groups have a larger volume of grey matter in the rostral PFC. (Sallet J. et al. 2011)

In mice, the prelimbic cortex[5] is activated upon social interaction with an unfamiliar conspecific, and lesions of this region changed their behaviour during social interactions. (Avale M.E. et al. 2011)

To resume, the PFC seems to be involved in the recognition, representation, and regulation of social status.

The striatum is another brain area that is emerging as critically involved in social competitiveness and, consequently, in the early stages of social hierarchy formation.

[5] Prelimbic cortex: dorsal anterior cingulate cortex (dACC) in human.

The striatum codes value, saliency, and reward-prediction-error signals. (Matsumoto and Hikosaka, 2009; Samejima et al., 2005)

The nucleus Accumbens (NAc) is one of the main components of the striatum and has been recently shown to play a crucial role in social hierarchy establishment, particularly in mediating the influence of anxiety on social competition. (Hollis F, et al. 2015; Goette L., et al. 2015)

In fact, rats with high anxiety trait showed low social competitiveness and impaired mitochondrial function in the NAc mediated this effect. Moreover, these features led to a subordinate rank of the rat. (Hollis F, et al. 2015; Goette L., et al. 2015) Instead, boosting NAc mitochondrial function (with intraaccumbal infusion of nicotinamide) improved social competitiveness and prevented the development of a subordinate status in high-anxious rats. (Van der Kooij M.A., et al. 2017)

Furthermore, several neurotransmitters are involved in social dominance and hierarchy formation. For example, 5-HT, dopamine, oxytocin, and NPB/W (neuropeptide B and neuropeptide W) are modulated by the formation of the social hierarchy of a group.

5-HT system seems to contribute to the formation of social hierarchy. In fact, using measurement of 5-HT obtained from circulating blood collected from adult male vervet monkeys housed in groups lead to the conclusion that 5-HT levels depended on the social rank of a monkey, such that dominant monkeys had approximately twice the 5-HT concentrations of subordinate monkeys. (Raleigh et al. 1984)

In the same study, 5-HT levels of dominant monkeys were sensitive to the presence of subordinates, in fact, when a dominant monkey was temporarily isolated, its 5-HT levels diminished to approximately the same level as those of the subordinate monkeys.

On the other hand, the transition from a subordinated to a dominant position in the social hierarchy was accompanied by an increase in 5-HT levels. (Raleigh et al. 1984) The dopamine apports his contribute as well, in fact in a positron emission tomography (PET) study of social hierarchy (Grant et al. 1998), dominant monkeys had greater binding of a D2R[6] ligand (fluoroclebopride), which has high affinity for D2Rs, in the basal ganglia than did subordinate monkeys.

Moreover, Morgan et al. 2002 reported that, in monkeys, the formation of a social hierarchy produced a D2R gradient, in which the binding of D2R ligand increased in all monkeys, especially in the dominant ones.

Oxytocyn instead, plays an important role in the regulation of complex social cognition and social behaviours such as attachment, social recognition, social exploration, aggression, and anxiety. Michopoulos et al. 2011 has demonstrated the influence of oxytocin on the formation and maintenance of a social hierarchy. In fact, dominant female macaque monkeys had higher serum oxytocin levels than those of subordinate monkeys.

Neuropeptide B (NPB) and Neuropeptide W (NPW) system show limited localization (N. Watanabe & M. Yamamoto, 2015), such as the hypothalamus, hippocampus, ventral tegmental area, and central nucleus of the amygdala in rodents. (Tanaka et al. 2003) Neuropeptide B/W contributes to the perception of dominance, and we can find it in neurons that have a limited projection primarily to the amygdala.

To conclude, the amygdala, hippocampus, intraparietal sulcus (IPS), ventromedial prefrontal cortex, lateral prefrontal cortex, and the striatum are involved in several aspects of social dominance, like perception, learning, and formation of social

[6] D2R: Dopamine receptor D2

dominance (N. Watanabe & M. Yamamoto, 2015) but also several neurotransmitters, such as 5-HT, dopamine, oxytocin, and NPB/W.

1.3 The nucleus accumbens

The nucleus accumbens (NAc) is one of the major components of the ventral striatum and a key region mediating a variety of behaviour, including reward and satisfaction. (S. Salgado & M. G. Kaplitt, 2015)

This brain region is often referred as the pleasure centre of the brain for its primary role in positive affect and feelings of reward, but it also plays an important role on motivation, behaviour, and a limbic-motor interface (Floresco, 2015; Salgado and Kaplitt, 2015).

Moreover, the NAc is a subcortical structure, located anterior to the posterior border of the anterior commissure and specialized part of the striatal complex that is closely related to the caudate-putamen and separate in function and composition from the septum.

The nucleus accumbens can be divided into a central core surrounded by a shell.

Core and shell are defined by various histochemical, electrophysiological, connectional, and cellular criteria. For example, on a molecular level, studies have noted core-shell differences in the distribution of several neuroactive substances and receptors, including substance P (Prensa L., et al. 2003), dopamine (DA), serotonin (Deutch A.Y. & Cameron D.S., 1992) and serotonin receptors (Patel S., et al.1995), with a tendency for these substances to be located in the shell.

The shell of the NAc not only has characteristics similar to those of striatal tissue, but also contains features analogous to the amygdala. For examples, immunohistochemical

similarities (such as the presence of areas rich in neurotensin, cholecystokinin and opioid peptides) as well as connectional similarities, including efferents to the lateral hypothalamus and afferents from the basolateral complex of the amygdala. (Heimer L, et al. 1997) This has led to the notion that the shell area of the accumbens could be conceived as a transitional zone between the striatum and the extended amygdala (Heimer L. & Alheid G.F., 1991).

Furthermore, the NAc is the main input nucleus of the basal ganglia (Nicola S.M. 2007), as it receives both indirect input via the mesolimbic dopaminergic projections from the ventral tegmental area (VTA) and substancia nigra as well as direct input via glutamatergic projections from the subiculum and amygdala, hippocampus, thalamus, prelimbic and prefrontal cortex. (S. Salgado & M. G. Kaplitt, 2015)

The main neuronal cell type of the NAc is the medium spiny neuron[7] which projects to various areas, for example, hypothalamus, thalamus, globus pallidus, but also amygdala and septum. (S. Salgado & M. G. Kaplitt, 2015)

Due to its input from the limbic system as well as output and cytochemical similarity to the motor nuclei of the basal ganglia, the NAc seems to be the functional interface between the limbic and motor systems, suggesting its role in controlling the biological drives necessary for survival and reproduction.

Indeed, studies have demonstrated that the NAc plays crucial roles in locomotion (Roberts M.D., et al. 2012), learning (Everitt B.J., et al. 1991), avoidance (McCullough L.D., Sokolowski J.D. & Salamone J.D. et al 1993), impulsivity (Basar K., et al. 2010), risk-taking behaviour (Kuhnen C.M. & Knutson B. 2005), feeding behaviour (Kelley

[7] Medium spiny neurons (MSNs): are a special type of GABAergic inhibitory cell which represents 95% of neurons within the striatum.

A.E., et al. 2005), sexual motivation, as well as incentive and reward (Rebec G.V., et al. 1997). For example, the shell seems to mediate the reinforcing properties of novelty, feeding behaviour, rewarding substances and drug relapse, while the core plays a crucial role in spatial learning conditioned responses, motivated responses, impulsive choices, and seems to be involved in mood disorders. (Salgado and Kaplitt, 2015)

One of the methods used to study the functional properties of the NAc in animals is through ablation surgery. Several ablative procedures based on various physical principles, have been used to date in the field of neurosurgery. For example, stereotaxic surgery is a powerful method to manipulate the brain of living animals and allows to target deep structures of the rodent brain consistently and accurately. This technique can be also combined with virus-mediated gene transfer, where the expression of a gene in a specific area can be altered. In our experiment we used stereotaxic ablation with the injection of a specific that interfere with the SIRT1 gene expression. (See chapter 2.2)

However, the NAc has also been implicated in numerous other psychological disorders, such as addiction, obsessive-compulsive disorder, anxiety, depression, Alzheimer's disease, Huntington's disease, Parkinson's disease, and obesity (Salgado and Kaplitt, 2015).

For example, bilateral ablation of the NAc has been attempted for the treatment of opiate and alcohol addiction. (Zhao H, et al. 2012; Wu H-M., et al. 2010 cited in Salgado and Kaplitt, 2015)

Interestingly, recent rodent studies (Cherix A. et al. 2020; Hollis F. et al. 2015; Larrieu T. et al. 2017) report the implications of social dominance in stress susceptibility with a special focus on nucleus accumbens. For example, in Larrieu T. et al. 2017 study emerged how high rank mice were more susceptible to display social avoidance

following exposure to chronic social defeat stress (CSDC)[8], while low rank mice were not affected.

In Cherix A. et al. 2020, they report that high (ranks 1 and 2) and low (ranks 3 and 4) rank mice displayed different phenotypes for anxiety-like behaviour and metabolic profile in the nucleus accumbens. For example, high rank mice showed higher time spent in open arms of elevated plus maze test (EPM), as well an increased latency to enter to the centre of open field (OF). At the opposite, subordinate mice displayed a less anxiety-like behaviour. Moreover, high rank mice showed also reduced levels of several energy-related metabolites in the NAc that support a higher susceptibility of high social rank to develop depressive-like behaviours.

Next, they found that using a mitochondria-boosting supplement (Acetyl-L-carnitine, LAC) in nucleus accumbens in chronically stressed mice had antidepressant-like effects, especially in high rank mice which showed reduced levels of several energy-related metabolites in the NAc.

To resume, nucleus accumbens is involved in many aspects of behaviour, for example in motivation and reward.

It is a subcortical brain region divided into a core and a shell, which present difference at histochemical, electrophysiological, connections and cellular levels.

Medium spiny neurons are the main neuronal cell type and project to several brain regions, like hypothalamus, thalamus, globus pallidus, but also amygdala and septum. Experiments on mice highlight the involvement of this structure in social dominance and his implications in develop stress susceptibility.

[8] Chronic social defeat (CSDC) consisted of a daily bout of social defeat by an aggressive male mouse over 10 consecutive days followed by a social interaction test 24 hr later.

Following this line of research, in our study we focused on NAc with the purpose of investigating the role of Sirt1 protein in relation to social dominance and anxious behaviour. In this way it is possible to identify how this brain structure affect the behaviour and the social status of the animals.

1.4 Anxiety behaviour

Anxiety is defined as a temporally diffused emotional state caused by a potentially harmful situation (D. Daviu et al. 2019) and is characterized by subjective experiences and physiological changes including sweating, dizziness and increase in blood pressure and heart rate. (G. G. Calhoon & K. M. Tye, 2015)

Historically, it has been differentiated in two types: state anxiety and trait anxiety.

Both, state, and trait anxiety responses represent an evolutionary advantage to anticipate and avoid danger and are not mutually exclusive (Goes et al. 2018, Takagi et al. 2018 cited in N. Daviu et al. 2019).

The main difference between these two types of anxiety is their duration. For example, state anxiety is an acute response to a potential threat, while trait anxiety is chronic, expressed constantly during the life of the individual and is therefore considered as a trait of an individual's personality.

In this study we focus on state anxiety, which is particularly suitable on being investigated in animal model.

State anxiety entails hypervigilance in anticipation of a threat that can be triggered by acute stress and has the primary function of avoiding dangerous situations and to facilitate memory consolidation. (D. Daviu & M. R. Bruchas et al. 2019)

Although the underlying neural substrates have not yet been fully elucidated, in the last decades research is getting many interesting results.

For this purpose, animal models are widely used in neuroscience and especially the mouse has emerged as a model of choice in genetic research, for several reasons:

- The limbic circuits, involved in anxious behaviour, are well-conserved across species, especially in mammals.
- Rodents' lifespan allows for experimentation (1-3 years).
- Rodents, in particular mice are small in size and generally cost less to maintain.

The most popular tests used for assessment of anxiety-like behaviour in rodents include the open field, elevated plus maze and light-dark box. (N. Kulesskaya & V. Voikar, 2014)

These tests are ethologically based and refers on the innate, conflicting drives of rodents to explore novel spaces and to avoid open, exposed and brightly illuminated areas where they might be more vulnerable to environmental threats.

Furthermore, recent technological developments have been instrumental in advancing the understanding of the neural substrates of anxiety measured in these behavioural assays. (G. G. Calhoon & K. M Tye, 2015) For example, the use of genetic manipulations allows deeply investigate the anxiety-like behavioural manipulating genes and proteins in the brain, allowing the establishment of causal relationships between the activity of neural circuits and anxiety.

A key structure encoding emotional valence and therefore guiding animal behaviour is the basolateral nucleus of the amygdala (BLA), which receives sensory inputs of multiple modalities, and projects to output structures controlling behavioural responses. (D. Daviu & M. R. Bruchas et al. 2019)

This central connectivity has made the BLA a focus for identifying the neural substrate of valence processing. Moreover, several studies showed that BLA integrity is critical for processing positive and negative valence. (Tye et al. 2008, LeDoux et al. 1990, McDonald, 1998; cited in D. Daviu & M. R. Bruchas et al. 2019)

A recent study revealed that photostimulation of BLA neurons synapsing in the core/shell section of Nucleus Accumbens support reward seeking (Namburi et al. 2015 cited in D. Daviu & M. R. Bruchas et al. 2019).

Lastly, BLA neurons projecting also to the medial section of the central amygdala (BLA-CeA) and mediate place avoidance. (Namburi et al. 2015)

Another region projected by amygdala is the ventral hippocampus (vHPC), which it is involved in inducing anxiogenic effects. At the contrary, an inhibition of the projections BLA-vHPC causes an anxiolytic effect (Felix-Ortix et al. 2013).

These findings support the important role of BLA in processing of learned emotional valence (BLA-NAc and BLA-CeA) and innate emotional state (BLA-vHPC).

Furthermore, another brain region involved in the evaluation for threat interpretations is the mPFC, which regulates subcortical responses to threatening stimuli.

As a neocortical structure, the mPFC is organized into six layers in humans (I–VI; layer IV is absent in rodents) containing excitatory pyramidal neurons and a diverse array of inhibitory interneurons. (G. G. Calhoon & K. M Tye, 2015)

Reciprocal connections between the mPFC and the amygdala have been extensively studied in the learned fear response, as well as in anxiety disorders. (G. G. Calhoon & K. M Tye, 2015)

Finally, the nucleus accumbens plays a role as well. For example, one study reports a role of NAc in the relationship between anxiety and social dominance.

In fact, Hollis F. et al 2015 found that trait anxiety on rats directly influenced social dominance identifying an important mediating role of mitochondrial function in nucleus accumbens. For example, high-anxious animals were prone to become subordinate during a social encounter with a low-anxious conspecific exhibiting a reduced mitochondria function and respiratory capacity (for a decreasing of ATP[9] as well) in NAc.

Interestingly, evidence supports a relationship between Sirt1 protein and anxiety behaviour.

Sirt1 protein is a nicotinamide adenosine dinucleotide-dependent deacetylase involved in several functions in the organism. (See the next paragraph)

In Libert S. et al. 2011 study the global impact of Sirt1 protein on brain have been evaluated in mice. In the experiment Sirt1 knock-out mice were used (which have a domain of Sirtuin gene deleted in the nervous system) and wild-type littermates.

Sirt1 knock-out mice showed a reduced anxiety-like behaviour than the wild-type group and similar anxiolytic effects were achieved pharmacologically (Phenelzine and Fluoxetine). Moreover, Sirt1 knock-out mice showed lower levels of MAO-A in their brains than the wild-type group.

These results indicate that Sirt1 mediates levels of anxiety and exploratory drive by activating transcription of the gene encoding the monoamine oxidase A (MAO-A)[10] to reduce serotonin levels in the brain.

[9] ATP: adenosine triphosphate (ATP) is a nucleotide found in the mitochondria of all plant and animal cells. It is the major source of energy for cellular reactions, this energy being released during its conversion to ADP.

[10] MAO-A: is an enzyme that in humans is encoded by the MAOA gene. MAOA gene is one of two neighbouring gene family members that encode mitochondrial enzymes which catalyse the oxidative deamination of amines, such as dopamine, norepinephrine, and serotonin. It has also been associated with a variety of other psychiatric disorders, including antisocial behaviour.

To conclude, state anxiety is an acute response to a potential threat and in neuroscience rodent model seems to be the best solution to identify the underlying neuronal substrates. The amydagala (basolateral nucleus of the amygdala, BLA), ventral hippocampus (vHPC), prefrontal cortex (mPFC) and striatum (especially NAc) are the main brain regions involved.

An interesting role has emerged from the Sirt1 protein as well, that seems to mediate anxiety and exploratory behaviour in the brain. In our experiment we measured both (anxiety and exploratory behaviour) to identify if variation in the concentration Sirt1 in NAc could affect the behaviour of the mice.

1.5 Sirt1 protein in the brain

Sirt1 is a member of the sirtuin family and a nicotinamide adenosine dinucleotide-dependent deacetylase which orchestrates several functions and remove acetyl groups from histones and nonhistone proteins.

It is a protein that is encoded by the SIRT1 gene and has been implicated in a wide range of biological processes, including gene expression, metabolism, development, aging, obesity, cancer metabolism, cardiac function, apoptosis, and circadian rhythms. (Kim H. et al. 2016 – Rahman S. & Islam R., 2011) For example, manipulation of Sirt1 levels in the liver affects the expression of several genes involved in glucose and lipid metabolism. (Rodgers J.T. & Puigserver P., 2007)

Other studies demonstrated that modest overexpression of Sirt1 in the liver has a protective effect against high fat induced hepatic steatosis ang glucose intolerance. (Banks A.S. et al., 2008; Pfluger P.T., et al., 2008)

Sirt1 presents high levels mRNA in heart, brain, spinal cord and in embryos, suggesting an important role in development as well. In fact, Sirt1 knockout mice show important developmental defects. (Rahman S. & Islam R., 2011) For example, only 20% of these mice survive to adulthood, they are also visibly smaller than their control mice, develop more slowly and show perturbations in eyes morphogenesis. Both males and females that survive are sterile, which can be due to a hormonal inefficiency.

Furthermore, recent data also support a novel role for Sirt1 in higher-order brain functions such as memory and synaptic plasticity (Gao et al., 2010), anxiety and exploratory drive (Libert S. et al., 2011), and depression-like behaviour (H. Kim, et al., 2016). For example, recent experiments showed that Sirt1 loss-of-function impairs memory and synaptic plasticity, in fact, mutant mice lacking Sirt1 in the brain showed a decreased memory performance in a novel object recognition task, which relies on the hippocampus and cortex. (Gao et al. 2010; Michàn et al. 2010). Moreover, another study highlights the role of protein in Alzheimer disease, where Sirt1 seems to reduce the production of Aβ amyloid peptide[11] and improve symptoms of the neurodegenerative pathology in a murine model (Donmez et al. 2010).

In the same study, the use of an antibody against synaptophysin (SVP)[12] revealed significant decreases in SVP immunoreactivity in the hippocampal striatum radiatum of mutant mice lacking Sirt1, as well as reduced SVP protein content in the hippocampus, compared to control group. Moreover, Golgi impregnation demonstrated that the

[11] The amyloid β peptide (Aβ) is a critical initiator that triggers the progression of Alzheimer's Disease (AD) via accumulation and aggregation, of which the process may be caused by Aβ overproduction or perturbation clearance. Aβ is generated from amyloid precursor protein through sequential cleavage of β- and γ-secretases while Aβ removal is dependent on the proteolysis and lysosome degradation system.

[12] Synaptophysin (SYP) is an integral membrane glycoprotein localized to neurons, neuromuscular junctions, paraganglia cells, hypophysis, pancreatic islet cells, and adrenal cells.

dendritic spine density of CA1 pyramidal neurons was significantly decreased in the hippocampus of the experimental group. These results confirm a role of Sirt1 in synapse formation, synaptic plasticity, and memory formation. (Gao et al. 2010)

Sirt1 plays an important role in mood and behaviour as well. (Libert S. et al. 2011; Kim H. et al. 2016) For example, Kim H. et al. 2016 study showed the involvement of Sirt1 protein in anxiety-and-depression like behaviours in nucleus accumbens. In the experiment mice were subjected to chronic social defeat stress and this led to an increased Sirt1 levels in nucleus accumbens.

Moreover, using pharmacological activators of Sirt1 protein (rasveratrol), depression- and anxiety like behaviours increased as well. At the opposite, an intra-Nac infusions of a Sirt1 antagonist reduced these behaviours.

A viral-mediated gene transfer was used to increase sirt1 levels directly in NAc and, again, an increase in depressive-and anxiety-like behaviours was measured in open field, elevated-plus maze, and forced swim tests. (H. Kim, et al., 2016)

To conclude, Sirt1 protein is implicated in a wide range of biological processes, like gene expression and metabolism.

Moreover, studies in the brain reveal an important role of this protein in memory and synaptic plasticity, anxiety and exploratory drive, and depression like-behaviour.

In this thesis we focused on the role of this protein in the brain, particularly in the nucleus accumbens where last evidence highlighted its implication in mood and behaviour.

CHAPTER 2: METHODS

2.1 Animals

28 males C57BL/6J mice born in the laboratory of Lausanne were used in these studies. Upon weaning (postnatal days 21) they were housed in ventilated cages, in group of four, on a 12 h light/dark cycle (0700-1900 h) with ab libitum access to food and water and abundant pine bedding.

Mice were hosted in a controlled facility with controlled temperature and radio turned on. Every week they were weighed, and their food intake was measured.

Each mouse received the same treatment conditions and during the experiment, the animals were subjected to several behavioural tests for the evaluation of the anxiety-like behaviour and the social hierarchy in each cage.

After each behavioural test or any kind of manipulation, mice always had a rest time.

Cage 4 was removed during the experiment (after 3 months) as it represented an extra control cage with a great variability compared to other control cages.

2.2 Materials

a. Elevated plus maze test

Before surgery, animals were tested for a general anxiety assessment with elevated plus maze test (EPM) and a baseline was obtained to verify the absence of significant differences between the mice.

It consisted of two opposite open arms (30 x 5 cm) ant two enclosed arms (30 x 5 cm) surrounded by a 15 cm-high black wall elevated 75 cm from the ground.

Animals were gently placed at the centre of the maze for each individual trial lasting 5 min. The number of entries (frequency), the time spent in open arms, and the latency of the first entry in open arms were collected from the computer software (Ethovision from Noldus) and analysed.

Higher anxiety behaviour is indicated by a lower frequency of entries into open arms, less time spent in open arms and a long latency of first entering in open arms.

At the opposite, lower anxiety behaviour is indicated by a higher frequency of entries into open arms, with more time spent there and a shorter latency to first entry.

Before the test:

EPM was placed in a specific room straight under a camera, with all the zones placed to be visible on the video and was stucked on the floor with a tape.

The camera, directly above the arena, recorded the scene and the software Ethovision was used to track the movements of each animal and collect the data for the analyses.

The computer was located behind a curtain, to be invisible to the mice.

The light has been set up by using the variable light on the wall, the luxmeter.

The day of the test:

After turning on the radio, the recording for each trial was 5 minutes per animal.

Every mouse was gently taken from the cage, and it was put in the small transport box.

Then, for each trial, every mouse was introduced into the centre of the EPM (always the same position for each mouse) with the head in the closed arm and the experimenter hidden behind the curtain.

The mice were tracked by the computer and then were taken out with the transport box in their home cage.

After the test:

Every time each mouse finished the test, the platform was cleaned with Et-oh 5%.

Ethovision software tracked the amount of time the mice spent in the closed arms versus the open arms throughout a 5-min session. It also tracked the number of the entries in open arms and the latency to first entry.

b. EchoMRI

Whole body composition was determined by nuclear magnetic resonance (NRM)-based technology (echoMRI, Echo Medical systems).

EchoMRI was performed three times during the experiment, the first time on postanal days 84, the second one on postanal days 116 (before the beginning of the tube test) and the last one on postanal days 179.

Each mouse was gently placed briefly (approximately one minute, no anaesthesia required) in the echoMRI machine, inside an appropriate plastic tube.

Whole body fat and lean content were measured and expressed as a percentage of total body weight.

Together with the information about the body composition, body weight of each animal was measured with a specific scale.

EchoMRI data were used to check for any variation of body composition between the knock-down group and the control one during the experiment.

c. Knock-down of SIRT1 gene in NAc by stereotaxic surgery

After the EPM test and the first echoMRI all the mice were subjected to stereotaxic surgery.

Targeted deletion of the SIRT1 gene was achieved using the Cre/loxP site-specific recombination system through bilateral stereotaxic delivery of an adeno-associated virus expressing Cre-recombinase (AAV-Cre) into the nucleus accumbens.

Cre-loxP system is a widely used technology for mammalian gene editing and is based on the action of Cre recombinase, which is one of the tyrosine site-specific recombinases and is produced from *cre* (cyclization recombinase) gene of bacteriophafe P1.

Cre recognizes the specific DNA fragment sequences called loxP (locus of x-over, P19) site and mediates site-specific deletion of DNA sequences between two loxP sites.

The mechanism of Cre-loxP system is based on the recognition of two directly repeated loxP site by a single Cre recombinase and the excising of the loxp flanked (floxed) DNA. (H. Kim, et al., 2016)

Half of the mice (3 cages) received an injection containing AAV2-SYN-eGNP-T2A-GFP-T2A virus which led to a knock-down of SIRT1 gene in NAc and the other ones (3

cages) received AAV2-SYN-eGFP-WPRE, a control virus with no effect on SIRT1 gene expression.

Adeno-associated virus (AAV)-mediated gene transfer was performed in mice aged 10-11 weeks. Previously, groups were counterbalanced for body weight, body composition and anxiety (EPM).

Before surgery, all mice received an injection of analgesic (buprenorphine, temgesic at 0.1 mg/kg) and were anesthetized by isoflurane inhalation (induction 4% isoflurane and maintenance 2.5% isoflurane in O2 at a flow of 4 L/min) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with a mouse adaptor.

1 uL of AAV solution (1:2 dilution of stock AAV solution in saline) was infused at a rate of 0.1 μL/min using a Hamilton syringe (Hamilton Bonanduz AG) fitted with a 33-gauge needle aimed at the NAc (coordinates taken from bregma in mm: A.P. +1.6, M.L. ±1, D.V. -4.8).

The needle was left in place for an additional 10 min before being slowly withdrawn. Moreover, an ophthalmic ointment was applied in both eyes to prevent corneal desiccation.

Viral mediated gene transfer for selective ablation of SIRT1 gene were microinjected specifically in neurons of nucleus accumbens.

Only three cages received the virus expressing Cre-GFP, while the control group received a different virus with no effect on Sirt1 protein.

After the surgery, mice were treated with paracetamol (200-300 mg/kg, Dafalgan) for seven days and allowed to recover for at least 3 weeks.

Unfortunately, due to a carelessness of the facility operators, for one week after the surgery all cages did not receive its bedding.

However, for one week the animals were checked every day and for each one the body weight, the appearance, the behaviour, and the surgical wound were collected and analysed to avoid any possible post-operation consequences, such as excessive weight loss, an unhealed scar or anhedonia etc. In few days, all mice totally recovered from the surgery.

d. Open field test with novel object

Open field test is commonly used for the evaluation of the exploratory behaviour and the general activity of the animals. More spent time in the edges of the box and less time spent in the centre of the box is interpreted as anxiety-like behaviour.
The open field test with novel object was performed in this experiment to determine the possible effects of Sirt1 knock-down in exploratory and anxious behaviour.

Before the test:

The test room was dark and sound isolated. A video camera positioned directly above the arena was used to record the movements of each animal and then a specific software (the Observer XT1) tracking it.

The test:

The mice were gently placed in the centre of a white plastic open-field arena (50 cm x 50 cm) divided in 4 separated compartments within the centre of each ones a novel plastic bottle. The animals were allowed to explore freely for 5 min and for each trial 4 mice were gently placed, one for each compartment.
After each trial the arena was carefully cleaned with Et-oh 5%.

After the test:

The time spent in centre zone, the latency of entering in centre zone and the number of sniffing were collected to measure the anxiety-like behaviour.

Higher anxiety behaviour is indicated by a less time in centre zone, less entries in centre zone, while, at the opposite, lower anxiety behaviour is indicated by a greater time in centre zone, more frequency of entries into centre zone.

The frequency of sniffing the novelty object indicate the exploratory behaviour.

Data were scored using Observer software.

e. Light/dark box

The test aimed to evaluate the anxiety-like behaviour like the EPM test and OFNO test. The purpose of the test was to detect any possible variations in the anxiety-like behaviour after the surgery in the two groups (CTL and KD).

Light/dark box was conducted on 126 postnatal days, before the tube test and two days after the open field test with novelty object.

The structure consists in a cage (21x42x25) divided into a small dark compartment (one-third) and a large, illuminated compartment (two-thirds) by a partition with a door. The mouse was allowed to move freely between the two chambers with the door open for 5 min.

Based on the innate aversion of rodents to brightly illuminated arenas and spontaneous exploratory behaviour of the mice, the time spent in the dark chamber could serve as an index of anxiety-like behaviour.

Before the test:

The arena of LDB was placed in the room straight under the camera to make sure all zones are visible on the video during recording.

The LDB was taped on the table to avoid movements during the test.

The computer was hidden by a curtain.

The light was set up by using the normal light on the ceiling by the luxmeter, which was kept in the drawer of the main room.

Light parameters: on the light area is above than 200/190 Lux in the open arms and completely black in dark area.

The test:

After turned on the radio on the computer by internet the video was set up. 10 min was the total time, and the test was 6 min per animal.

The stopwatch started after each animal was in the maze.

After started recording and checked everything was working, the animal was taken from the cage in the dark area, with a small transport box.

Each animal was gently placed in the dark area and closed it with a lid. The door of the dark area was open after 5 seconds.

The stopwatch started after the opening of the door of the dark zone.

The mice were observed by the computer for 5 minutes and then they were taken out with the transport box and put back in their home cage.

After the test:

Between each trial the box was cleaned with Et-oh 5% and at the end of the experiment the entire room was cleaned.

The videos of the test were saved on an external hard drive and a software (Ethovision from Noldus) was used to do to measure quantified variables: time spent in light box, latency to first enter in light box, number of entries in light box.

f. Tube test

The tube test is used to evaluate the social rank in rodents.

In this test, two mice enter a narrow tube from opposite ends and meet in the middle.

The mouse that forces the opponent out of its way is designed as the “winner”.

The one that retreats out of the tube first is designate as the “loser”.

Mice from the same cage were tested against each other with a round robin design, resulting in 6 contests per day per cage.

Then, the mice were sorted by individual cumulative wins. The ones who won the most were labelled dominant (rank 1 and 2) and the ones who lost the most subordinate (rank 3 and 4).

Before the test:

One transparent Plexiglas tube with 30cm length and 3cm inside diameter has been used.

This diameter of the tube was a size just sufficient to permit the adult mice to pass through without reversing the direction.

T maze was used to place the tube test inside to create an arena containing the two mice after the trial, when they exit of the tube.

A camera was used to watch the movements of the mice during each trial.

Every tube used was cleaned between each trial with 5% ethanol and dry with paper.

A dim light was set above the arena with the radio on. The test included two main parts: training phase and test phase.

Training phase:

The training stage aims to familiarize the mice with the test procedure and environment. It is important for the mouse to know the consequence of walking through the tube and from each side. Nothing was recorded in this phase.

Each animal was given 3 trials per side of the tube per day, over 2 days, for a total of 10-15 min per mouse.

A trial consisted of releasing a mouse at the end of the tube and leaving it to run through the tube, sometime with the help of a plastic stick pushing very gently at its back.

If the animals did not cross the tube in 30 sec, the mouse was pushed inside gently.

After the training of each mouse, the tube was carefully cleaned.

Test phase:

In the testing stage, mice are tested in a pair-wise fashion in the tube for 12 days.

This phase was recorded.

Two mice for each trial were released simultaneously into the opposite ends and care was taken to ensure that they met in the middle of the tube.

The mouse that first retreated from the tube within 2 minutes was designated the loser of the trial.

Each mouse met all the mice of the cage. So, each one had 3 encounters per day.

When a mouse lost, he was scored zero. When a mouse won, he was scored 1.

After each trial the tube was carefully cleaned.

The mice are put back into the home cage and left to rest with their cage mates before starting the next trial, in order to reduce the potential immediate impact of recent winning or losing.

The social rank was considered stable if all four cage mate mice maintain the same ranking for at least four consecutive days based on the following observation.

Close the arena, in the camera area of recording there were paper with the number of the cage and the number of the pair mice of each trial. It allowed to understand which mouse is put there and from which cage.

A video device was used to record the whole test procedure and precisely capture specific behavioral epochs in the tube test.

By the video recording it was possible to classify the behaviors into push, initiation, pushback, resistance, retreat, or stillness.

2.3 Procedure

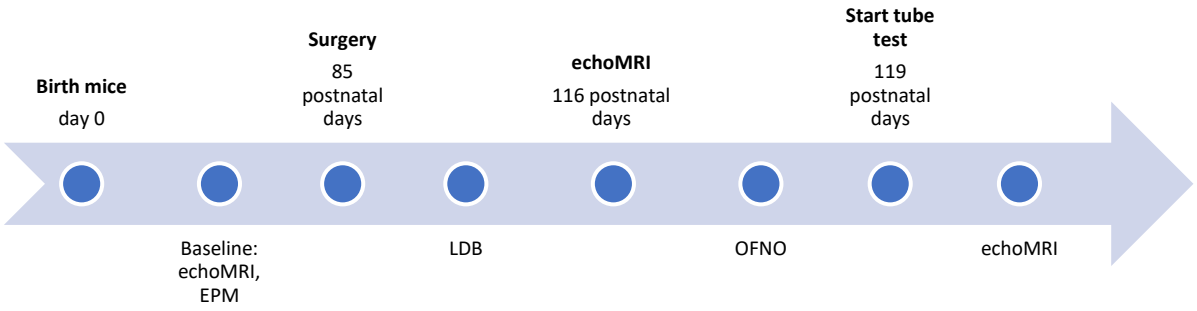

Figure 1. Timeline of the experiment.

Seven male mice cages, 4 mice per cage, were subjected to several behavioural tests, stereotaxic surgery and three echoMRI.

The experiments took place in the Behavioural Genetics Lausanne Laboratory (Vaud, Switzerland), at École Polytechnique Fédérale de Lausanne (EPFL).

Study was approved by Commission Cantonale d'éthique de la recherche sur l'être humain (CER-VD).

Data were collected from October 2021 to January 2022, for a total sample of 28 mice.

The experiment is still in progress, only part of the results is discussed here.

As independent variables we have: the group (control and knock-out), the rank (subordinate and dominant) and the time.

The dependent variables measured are:

- EPM (Elevated plus maze test), time spent in open arms, number of entries in open arms (frequency) and latency to first entry in open arms.
- OFNO (open field with novel object), time spent in centre zone, number of entries in centre zone /frequency), latency to first centre zone and number of sniffing the novelty object.
- LDB (light/dark box test), time spent in light box, number of entries in light box (frequency) and latency to first entry in light box.
- Tube test, time spent to win/lose, winner/loser.
- echoMRI, fat mass level (%), lean mass level (%).
- Body weight (g) and food intake (g).

In total there were four control (CTL group) and three experimental (KD group).

Subsequently, cage 4 was removed because it was not considered sufficiently representative of the control group.

Each subject in the experiment received the same treatments (except the different type of virus injected) and was subjected to the same experimental measures.

Each experimental test was administered by a single experimenter during the light period.

To induce ablation of Sirt1 transcript in NAc, mice of 85 postnatal days were subjected to stereotaxic surgery using a viral delivery of Cre recombinase.

After the surgery they were checked to ensure a correct recovering.

During the experiment the animals were subjected to several behavioural tests in different days for the evaluation of the anxiety-like behaviour and the social hierarchy in each cage.

After each behavioural test or any kind of manipulation, mice always had a rest time.

EchoMRI was performed three times to measure the body composition of each mouse and check any possible variation over time between the two groups.

All behavioural manipulations were performed by experimenter's blind to treatments groups.

All animals' procedure were approved by the Cantonal Veterinary Office (Vaud, Switzerland).

2.4 Statistical analyses

All statistical analyses were performed with Rstudio (R version 4.03) and Prism 7.0 (GraphPad).

Assumptions of normality of error distribution, and homogeneity of variance were examined graphically and with specific statistical tests: Shapiro-Wilk test (normality

test), Breusch-Pagan test (Homogeneity test), Durbin-Watson test (autocorrelation errors test).

Based on these inspections, no transformations of data were performed.

Unpaired Student's t.test with two tailed p-value was performed to verify differences between groups. One-way ANOVAs was performed to determine significance for conditions in which both genotype and social rank were taken into account.

All values included in the figure legends represents means ± SEM.

All data needed to evaluate the conclusions are present in this paper.

CHAPTER 3: RESULTS

3.1 Sirt1 ablation in NAc does not affect body weight and food intake

Mice increased their body weight over time (p=0,0001).

However, Sirt1 KD did not affect body weight (no main effect of genotype nor an interaction between time and genotype). (Figure 1)

Food intake was not affected by time nor genotype or the interaction between these. (Figure 2)

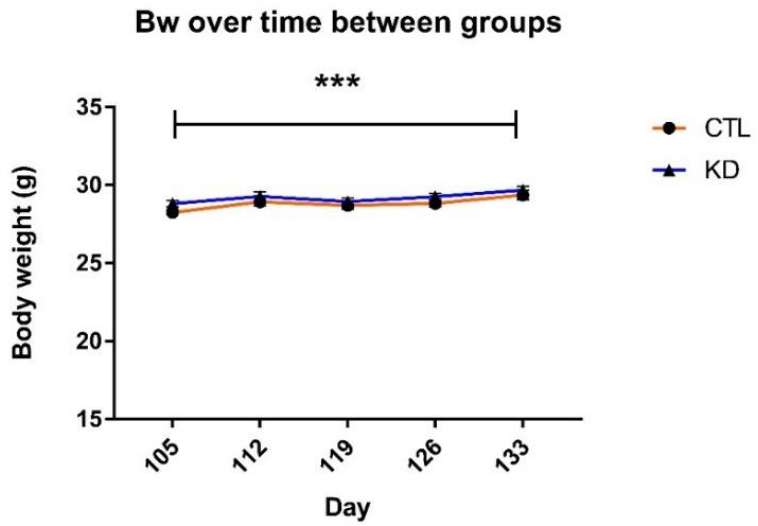

*Figure 1. Variation of weight over time in all mice. Period: postnatal days 105 to postnatal days 133. Data are mean ± SEM (*P < 0.05, **P<0.01, ***P<0.001, two-way ANOVA, n=24 Time: F (4, 88) = 11,27 P<0,0001*** Model linear assumptions respected.*

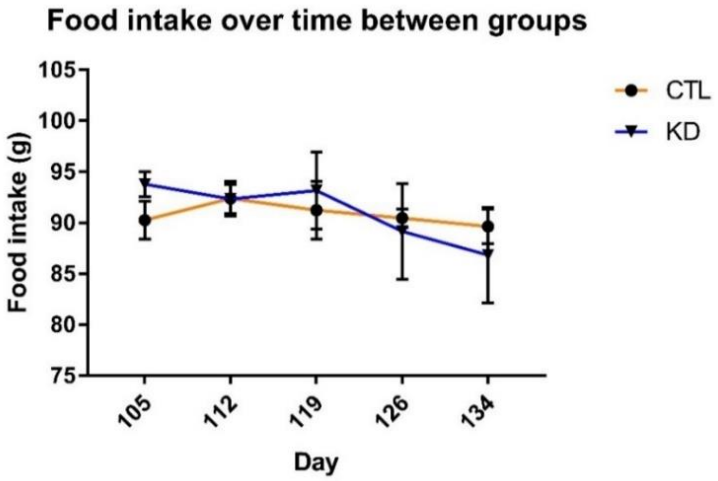

Figure 2. Variation of food intake over time between groups. Period: postnatal days 105 to postnatal days 133. Data are mean ± SEM. Two-way ANOVA, n=24. Interaction: F (4, 16) = 1,16 P=0,36 Time: F (4, 16) = 2,44 P=0,08 Group: F (1, 4) = 0,005 P=0,94. Model linear assumptions respected.

3.2 Anxiety baseline data and body composition

Elevated plus maze test was used to verify the anxiety-like behaviour at the beginning of the experiment before the stereotaxic surgery in NAc.

Sirt1 ablation was induced and performed to balance the two groups (CTL vs Sirt1 KD) on anxiety levels.

Based on these results, at the beginning of the experiment, no statistical variation was found between CTL and Sirt1 KD groups. (Figures 3)

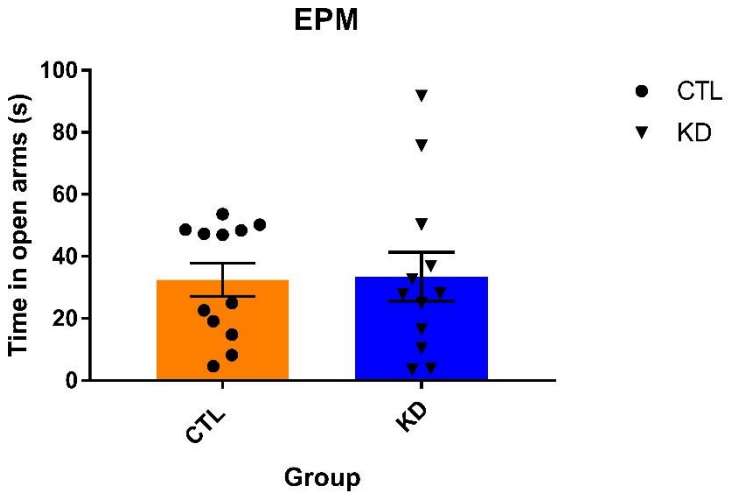

Figure 3. Elevated plus maze – time in open arms. Data are mean ± SEM Unpaired t test, n=24; P value= 0,91; Two-tailed; t=0,11 df=22; F test to compare variances (F, DFn, Dfd= 2, 206, 11, 11, P value= 0,20); Model linear assumptions respected. No influential cases.

EchoMRI was performed to understand whether Sirt1 KD affected fat and lean mass levels (Figure 4-7).

It was performed in three different times during the experiment; the first time before the surgery (postnatal days 84 – figure 4), the second time one month after the surgery and before the first tube test (postnatal days 116 – figure 6) and one last time (postnatal days 179).

All the echoMRI detections reported no significant differences between the two groups, except the variation of fat mass levels over the time (p=.0006).

From this data we can assume that the absence of Sirt1 expression in NAc did not affect the body composition of KD group.

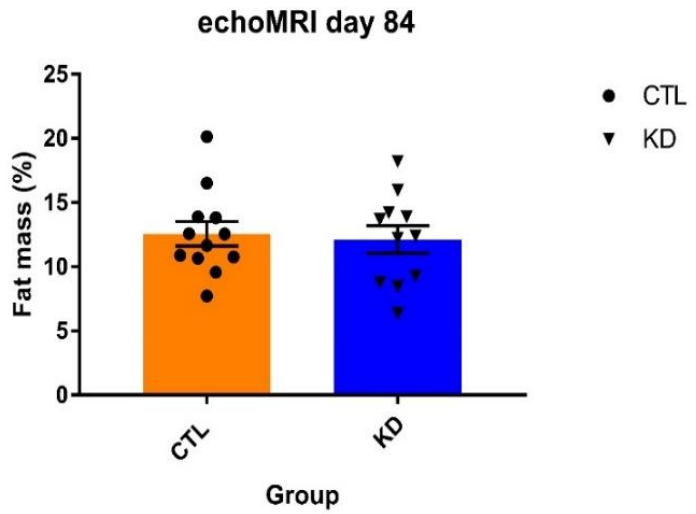

Figure 4. echoMRI – at mass level (%) postnatal days 84. Data are mean ± SEM. Unpaired t test, n=24, P value= 0,52; Two-tailed. t=0,66 df=22. Model linear assumptions respected, no influential cases.

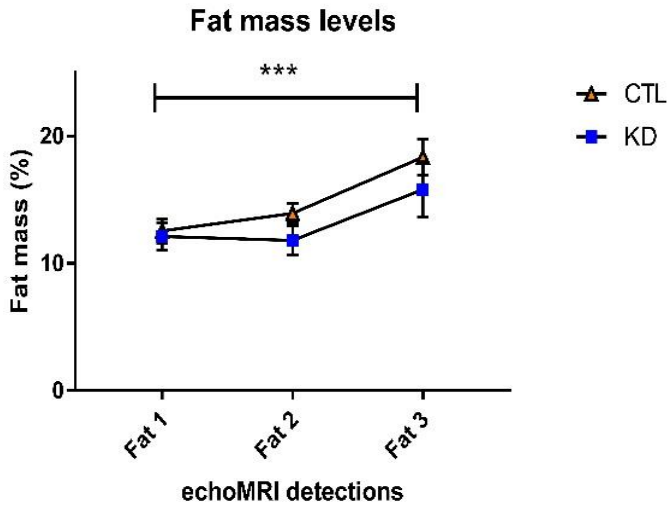

*Figure 5. echoMRI detections – fat mass level (%) postnatal days 84, 116 and 179. Data are mean ± SEM. (*P <.05, **P <.01, ***P <.001), Unpaired t test, n=23 Two-way ANOVA, n=23. Interaction: F (2, 42) = 0,41 p=0,66 Time: F (2, 42) = 8,96 p=0,0006***. Group: F (1, 21) = 1,98 p=0,17. One influential case was removed (mouse 12, group=KD) from analyses (systematic error during echoMRI detection).*

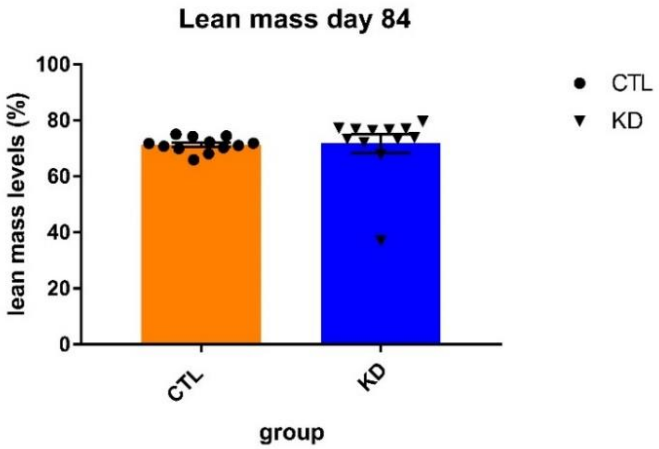

Figure 6. echoMRI – lean mass level (%) postnatal days 84. Data are mean ± SEM. Unpaired t test, n=24, p= 0,90; Two-tailed. t=0,1242 df=22. Model linear assumptions respected, no influential cases.

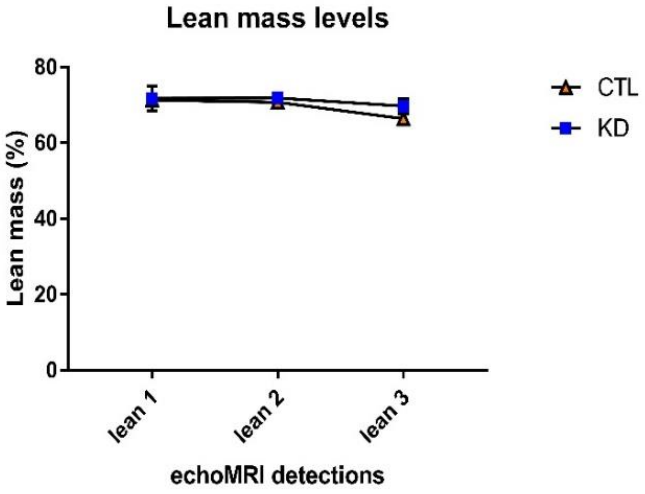

Figure 7. echoMRI detections – lean mass level (%) postnatal days 84, 116 and 179. Data are mean ± SEM. Two-way ANOVA, n=24. Interaction: F (2, 44) = 0,47 p=0,63; Time: F (2, 44) = 3,19 p=0,051; Group: F (1, 22) = 0,80 p=0,38. Model linear assumptions respected, no influential cases.

3.3 Knock-down mice expressed a heightened anxiety-like behaviour

As the purpose of the experiment was to investigate the role of Sirt1 in social dominance and anxiety-like behaviour, OFNO (open field with novelty object) and LBD (light/dark box) were used 30 days after the stereotaxic surgery to detect any possible variation in anxiety-like behaviours between KD and CTL groups.

Results showed a significant difference in the time of latency of entries in light compartment (figure 8). In fact, the knock-down group expressed a higher latency than the control group. (p=0,01)

The same trend was found for the latency of OFNO test (figure 9), but without a significance. (p=0,23)

Data suggests that the knock-down group seems to display a greater anxiety-like behaviour than the control group. (See Appendix for other analyses)

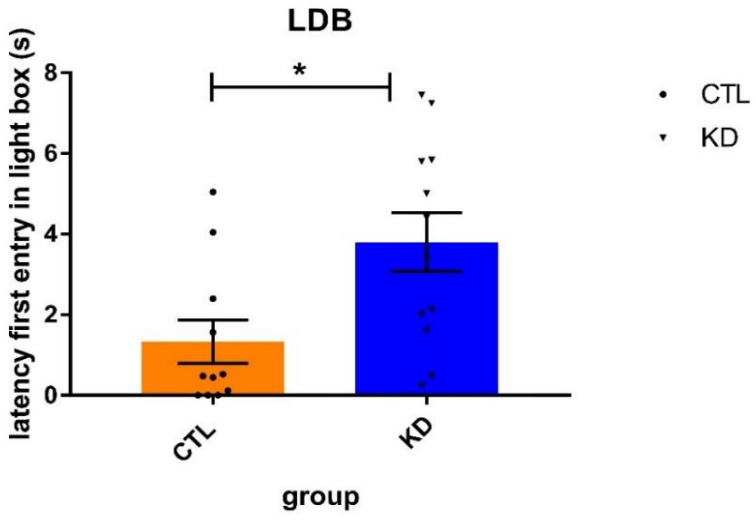

*Figure 8. Latency to first light field (LDB). Data are mean ± SEM (*P <.05, **P <.01, ***P <.001, Unpaired t test, n=23); p= 0,01* (d=0.07); Two-tailed; t=2,70 df=21; Model linear assumptions respected. One influential case was removed from the analyses (mouse number 18 group: CTL)*

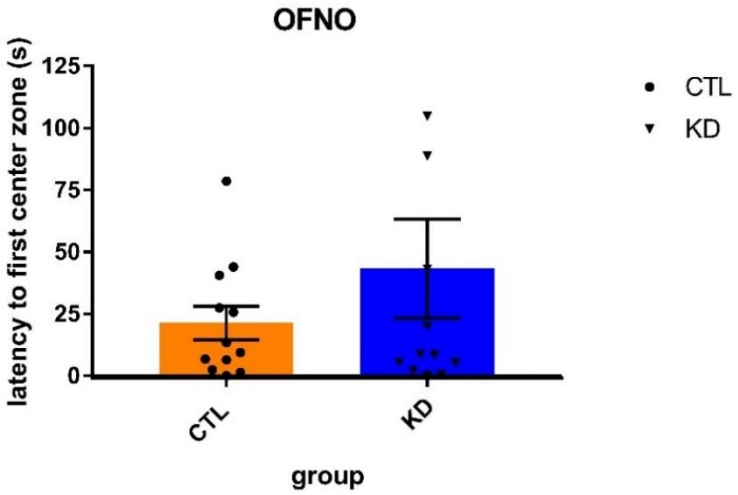

Figure 9. Latency to first centre zone (OFNO). Data are mean ± SEM. (Unpaired t test, n=24); p= 0,31; Two-tailed; t= t=1,04 df=22; d = -0.42; Model linear assumptions respected. No influential cases.

3.4 Tube test: dominant mice expressed reduced sniffing behaviour

The tube test did not show a stable hierarchy over time in every cage. (See the appendix)

To determine the social rank of each mouse in the cage, z-scores of the cumulative wins over the days were calculated. Then, z-score of the average time of winning was calculated. For the loser of each duel, 120 seconds were considered in the analyses, that is the total duration of a trial.

Rank 1 and 2 were considered dominant mice, rank 3 and 4 were considered subordinate mice.

Next, we analysed whether dominant and subordinate mice differed in their anxiety-like and exploratory behaviour, searching for an interaction effect between genotype and social rank. However, no significant effect was found.

We found a significative difference between dominant and subordinate mice in the number of object approaches in the novel object task (OFNO). In fact, dominant mice approached the object significantly less often than subordinate ones (p= 0,019).

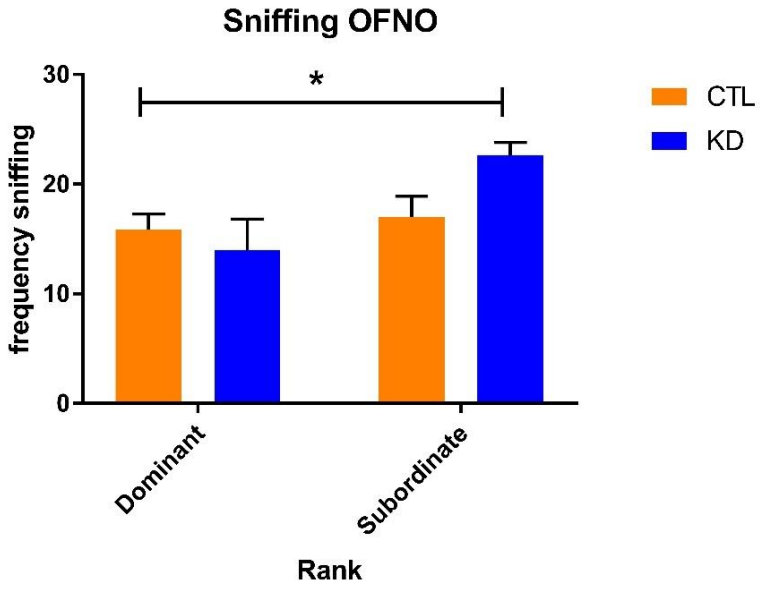

*Figure 10. Time of sniffing the novelty object (OFNO). Data are mean ± SEM. Two-way ANOVA.(*P <.05, **P <.01, ***P <.001) Interaction: F (1, 20) = 3,75 p=0,07 Rank: F (1, 20) = 6,44 p=0,02* (d= -0.97) Group: F (1, 20) = 0,98 p=0,33. Model linear assumption respected. No influential cases.*

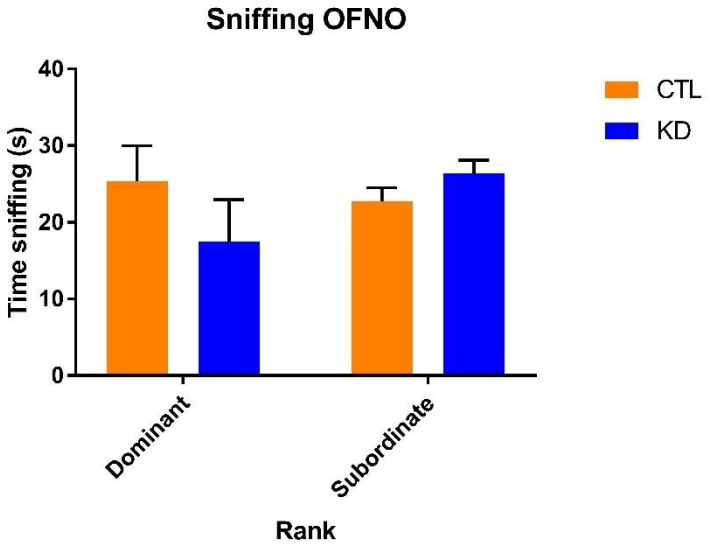

Figure 11. Frequency of sniffing in open field (OFNO). Data are mean ± SEM. Two-way ANOVA. Interaction: F (1, 20) = 2,30 p=0,14 Rank: F (1, 20) = 0,69 p=0,42 Group: F (1, 20) = 0,31 p=0,58; Model linear assumption respected. No influential cases.

No significant difference was found in the time of novel object approaches between group (CTL and KD) and between social ranks (dominant and subordinate mice). From these data we conclude that only the social rank had a significant effect on exploratory behaviour, in fact subordinate mice expressed a more exploratory behaviour by the number of object approaches.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

Based on the results presented here, Sirt1 KD group did not show any statistical difference in fat mass and body weight. Instead, a mild anxiety-like phenotype was observed in the light/dark box. In fact, Sirt1 KD group showed a greater latency to first entry in light compartment compared to the control group.

Moreover, in open field test with novel object (OFNO) a statistical variation in the number of approaches emerged between dominant and subordinate individuals.

These results suggest that Sirt1 protein knock-down in male adult mice has limited effects on behaviour and no influence on body composition and weight.

In fact, Sirt1 protein seem to potentially mediate anxious behaviour, in which less Sirt1 in NAc is related to a more anxious behaviour.

In Kim H. et al. 2016 study, the authors report a different direction in the effect of the protein in the brain. Indeed, the effects of Sirt1 ablation in the NAc lead to a decrease of depression and anxiety-like behaviour.

In the same study, a pharmacological inhibition of Sirt1 in NAc led to the same results, that is a decreased of anxiety-like and depression-like behaviour. While, at the opposite, its pharmacological activation induces an increased susceptibility of anxiety and depression-like behaviours.

Generally, latest studies on this protein confirm its involvement in mood-related behaviour (such as depression or anxiety) and the greater the expression of the SIRT1 gene, the greater the susceptibility. (Libert S. et al., 2011; Kim H. et al. 2016)

In our experiment we however show an opposite effect the hypothesis of Sirt1 protein on anxiety-like behaviour. However, the surgical ablation of SIRT1 gene was performed

only in neurons (and not glial cells) of the NAc and this specific target may also have influenced the animals' behavioural effects.

Lastly, there are some limitations that are important to mention.

First, the low stability of the social ranks obtained from the tube test. In fact, the test was conducted to evaluate the social hierarchies of the mice, in each cage.

Despite it was conducted for 12 days, several cages showed an instable social hierarchy. This aspect may have interfered with the obtained results, in fact instability in hierarchies may have hidden a possible interaction effect between genotype and rank on the anxiety scores.

In literature the tube test is considered one of the most suitable tests as well as being one of the most used to determine the social hierarchy in mice. (Fan Z. et al. 2019)

An explanation for the absence of stability of social hierarchies could be due to the surgery. In fact, the stereotaxic surgery procedure focused on an extremely important brain region in the establishment of the social hierarchy (NAc) and both groups reveal unstable social hierarchies (Sirt1 KD and control group). However, further studies need be carried out to deepen this point and clarify the possible effect of this kind of surgery in mice.

Second, mice were adults when the surgery was performed. This may have interfered with the ablation effect since the mice had already completed their development.

It could be interesting to verify whether the Sirt1 ablation in NAc, in new-born mice, leads to the same results or, at the opposite, shows some variation in the growth of the animals (such as weight) or metabolism.

In fact, from our results Sirt1 protein seems to not affect the general metabolism either, with no statistical variations in the food intake between control cages and Sirt1 KD.

However, the food intake was assessed per cage, not individually.

It would be interesting to check if a different food intake is associated also with a different social rank. This could also clarify if Sirt1 ablation influences the metabolism in mice and if this effect is related to the different social ranks as well.

To conclude, although we cannot assume a role of Sirt1 protein in body weight and body composition, our data suggest its potential involvement in anxiety-like behaviour. In fact, less concentration of this protein seems to be related to a more anxiety susceptibility and further studies are necessary to clarify its role in NAc and determine a possible causal relationship between anxiety and Sirt1.

Because of the multiple functions of Sirt1 protein in the organism and in the brain, understanding its neurobiological mechanisms can clarify its role in mood and behaviour by offering new insights into research.

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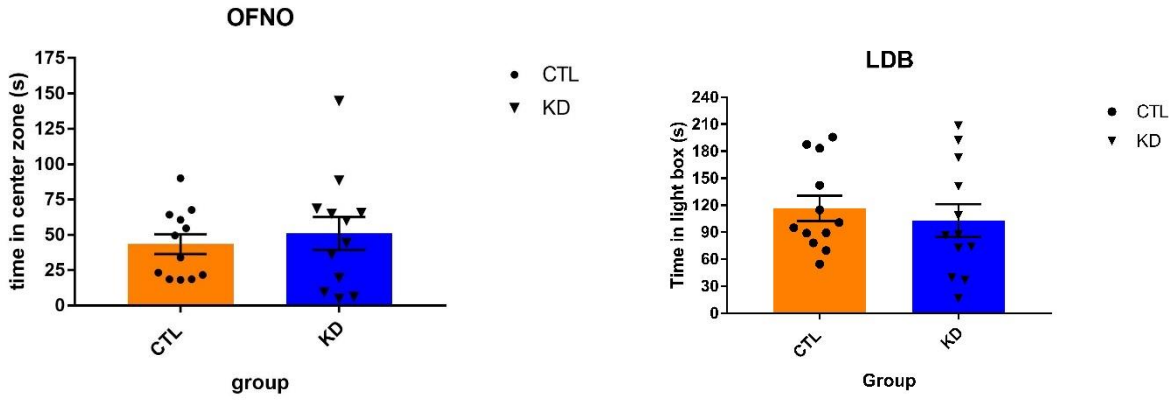
APPENDIX



Figures A. To the right: time in center zone (OFNO). Data are mean ± SEM. Unpaired t test, n=24, p= 0,58; Two-tailed. t=0,55 df=22. Model linear assumption respected. No influential cases.

To the left: time in light box (LDB). Data are mean ± SEM. Unpaired t test, n=24, p= 0,56; Two-tailed. t=0,59 df=22. Model linear assumption respected. No influential cases.

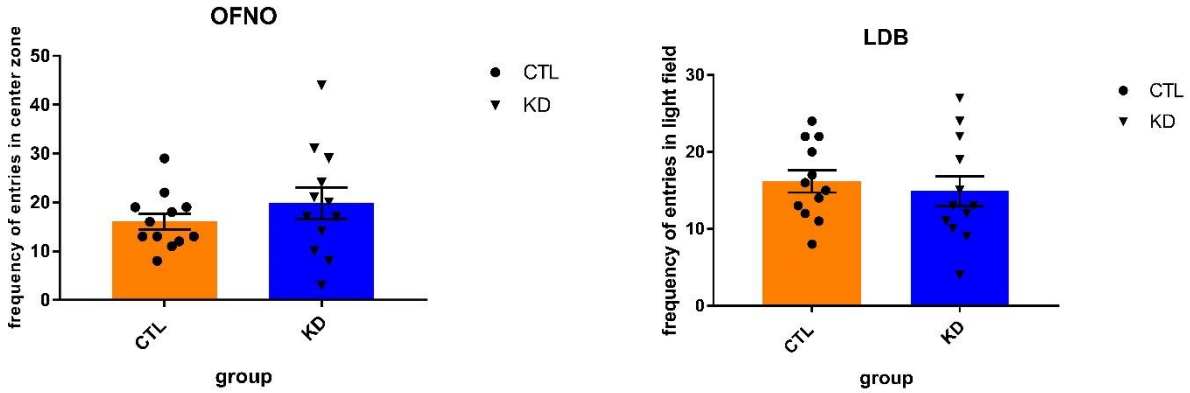
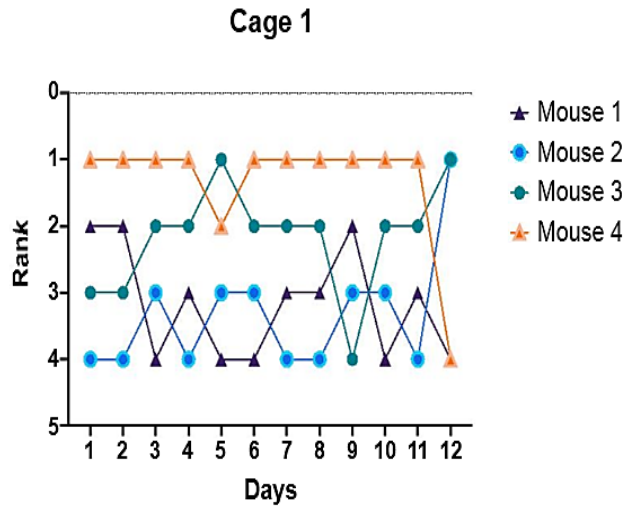
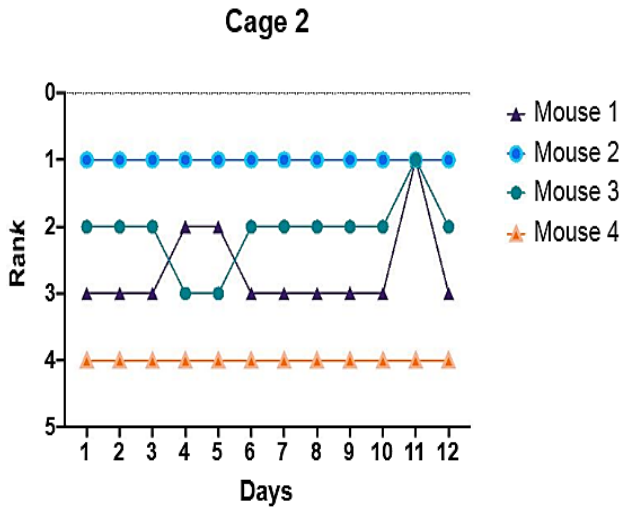
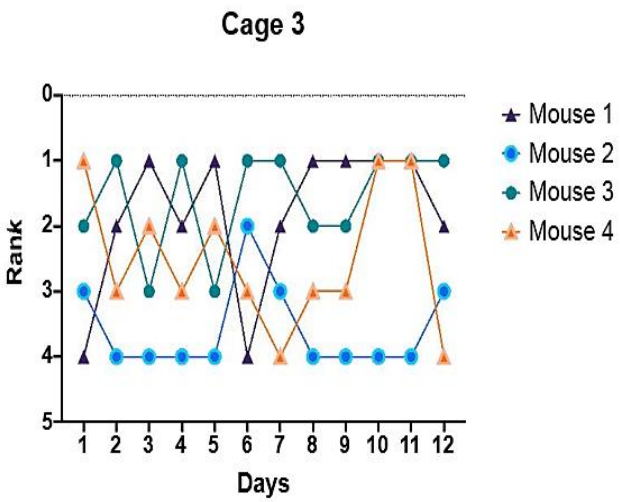


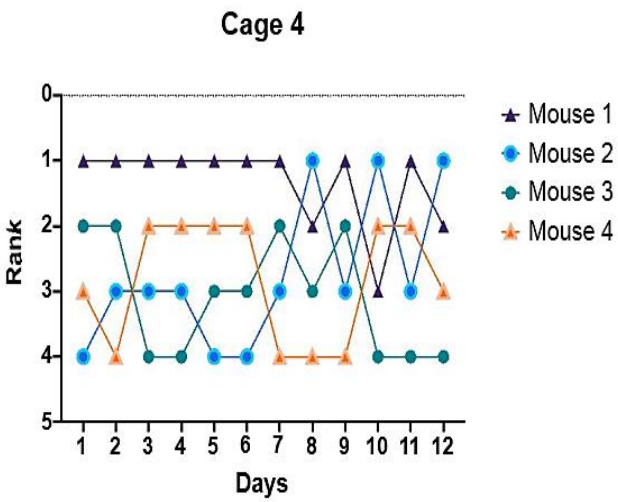
Figure B. To the right: frequency of entries in center zone (OFNO). Data are mean ± SEM. Unpaired t test, n=24, p value= 0,31; Two-tailed. t=1,03 df=22. Model linear assumption respected. No influential cases

To the left: frequency of entries in light box (LDB). Data are mean ± SEM. Unpaired t test, n=24, p= 0,61; Two-tailed. t=0,51 df=22. Model linear assumption respected. No influential cases.


Cage 1
Rank
Days
Mouse 1
Mouse 2
Mouse 3
Mouse 4


Cage 2
Rank
Days
Mouse 1
Mouse 2
Mouse 3
Mouse 4


Cage 3
Rank
Days
Mouse 1
Mouse 2
Mouse 3
Mouse 4


Cage 4
Rank
Days
Mouse 1
Mouse 2
Mouse 3
Mouse 4

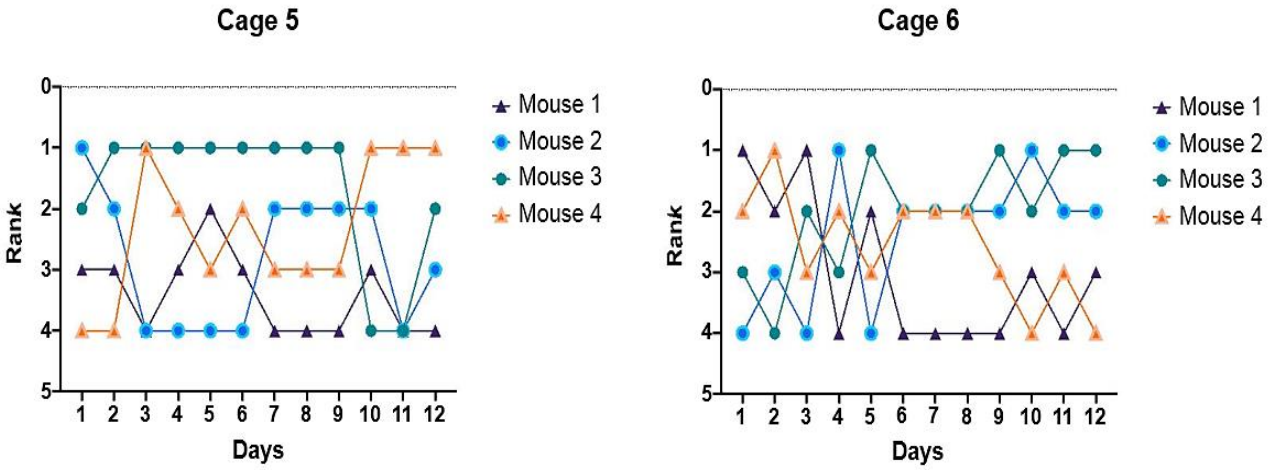


Figure C. cage z-scores of the cumulative wins over the days calculated by Dr. Silvie Ruigrok (the supervisor of the experiment).