Impact of individual differences in glucocorticoid adaptation to stress on behavior, neurophysiology and metabolism

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Preface

In the following dissertation, I present a selection of the work conducted in the course of my doctoral thesis. There is a general introduction on the current literature on stress and its implications on cardiovascular, metabolic and aging health. Thereafter, there are three research chapters focused on addressing how selection for constitutive differences in stress responsiveness during pre-puberty influences the biobehavioral phenotype of rats with a specific focus on the autonomic nervous system (chapter 1), the metabolism (chapter 2) and aging (chapter 3). None of the chapters of this thesis is yet published. Following the general discussion, in Annex, there are two documents. The first annex is description of an experiment performed in order to validate the model used to select the rats in the three chapters of the thesis. The second annex is a published review article on the interactions between physiology during development and programming of psychopathological aggression.

Summary

The stress system is a key modulator of homeostasis and allows organisms to adapt to environmental changes. Proper survival is dependent on the appropriate stress response, for example initiating food (energy) intake or provoking physical reaction. However, long-term activity of the stress system is related to cardiovascular diseases, metabolic syndromes as well as accelerated aging and cognitive impairments. In order to assess the impacts of stress regulation on cardiac, metabolic and aging health processes, we used lines of rats selected for their differential glucocorticoid responsiveness to stress during juvenile period, in the different experiments presented here. The three lines of rats showed low, intermediate or high response to stress and elicited differences in biobehavioral phenotypes.

Cardiovascular diseases are highly exacerbated by stress exposure and autonomic imbalance. Reduced vagal modulation has been related to a lower stress flexibility and deleterious effects on cardiac health. In a first study, we investigated the autonomic nervous system modulation of heart rate in the three lines of rats (differing in their responsiveness to stress). Electrocardiographic recordings were performed at rest and following autonomic pharmacological manipulations. Rats with intermediate reactivity to stress had a higher resting parasympathetic (vagal) modulation and a reduced heart rate compared to rats with low or high stress responses. Furthermore, pharmacological treatments showed that the sympathetic regulation of the heart was not impaired in rats with low and high responsiveness to stress.

Stress can affect social interactions and, in return, social interactions can be the cause of critical stress. Furthermore, since the stress system is related to key metabolic mediators, we investigated in a second study, the general metabolism of rats from the three lines. Moreover, we paired rats from the different lines together, in mixed-line dyads, and we evaluated the differences in social interactions and the long-term effects of mixed-line pairing on metabolism. We used indirect calorimetry and mitochondrial respirometry to measure energy expenditure and mitochondrial function. We observed that the selection for differences in glucocorticoid responsiveness induced constitutive differences in energy expenditure and fuel use. Moreover, we showed that the biobehavioral phenotypes affected the social interactions between the different lines. Finally, long-term mixed-line pairing affected global and central metabolism of the rats, with rats from the low and intermediate responsive lines being more susceptible to changes.

In a final experiment, we studied the interaction of two risk factors for cognitive decline, the secretion of corticosterone and aging. Indeed, dysfunctions of the stress system contribute and facilitate aging and increased glucocorticoids induce cognitive alterations. We assessed anxiety, stress responsiveness, coping-style and cognitive functions in a Morris water-maze at early-aging. Results indicated that the phenotype of the lines were stable throughout life and that learning, swimming strategies and reversal ability were different between the lines.

Overall, we showed that this model is suitable to study the systems related to stress regulation. Future research may use this animal model in order to investigate further the relationship between opposite stress regulation and health.

Keywords

Stress, juvenile period, HPA axis, corticosterone, stress habituation, biobehavioral phenotype, coping style, autonomic nervous system, heart rate variability , social behaviors, metabolism, energy expenditure, aging, cognition

Résumé

Le système de stress est un modulateur de l'homéostasie permettant à un organisme de s'adapter aux changements environnementaux. La survie nécessite une activation appropriée des réponses au stress, par exemple en initiant l'apport d'énergie ou en provoquant une réaction physique. Cependant, une activité prolongée du système de stress est liée aux maladies cardiovasculaires, au syndrome métabolique, au vieillissement et aux déficiences cognitives. Pour déterminer l'impact du stress sur la régulation cardiaque, métabolique et le vieillissement, nous avons utilisé des lignées de rats, sélectionnés pour leurs différentes productions de glucocorticoïdes face au stress pendant la jeunesse. Les lignées de rats ont une faible, moyenne ou forte réponse au stress et ont des phénotypes bio-comportementaux différents.

Les maladies cardiovasculaires sont liées au stress et à un déséquilibre du système autonome, puisqu'un contrôle parasympathique réduit est lié à une plus faible flexibilité et à des effets néfastes sur la santé cardiaque. Dans la première étude, nous avons étudié le contrôle du cœur par le système nerveux autonome dans les trois lignées de rats. Nous avons enregistré l'électrocardiogramme au repos et suite à des manipulations pharmacologiques du système autonome. Les rats ayant une réaction intermédiaire au stress montrèrent une plus grande modulation parasympathique et un rythme cardiaque au repos plus faible. De surcroit, les traitements pharmacologiques démontrèrent que la modulation sympathique du cœur n'était pas modifiée chez les rats ayant une faible ou haute réponse au stress.

Le stress peut changer les interactions sociales et, en retour, les interactions sociales peuvent induire un stress important. Le stress interagit avec les régulateurs majeurs du métabolisme et donc, dans la deuxième étude, nous avons étudié le métabolisme des rats provenant des trois lignées. De plus, nous avons apparié des rats des trois lignées dans des paires mixtes et nous avons mesuré leurs interactions sociales ainsi que les effets à long-terme sur le métabolisme. Nous avons observé que les lignées avaient des différences sur l'utilisation d'énergie, que les phénotypes bio-comportementaux affectaient les interactions sociales et que cela affectait, à long terme, le métabolisme global et central des rats.

Dans l'expérience finale, nous avons étudié les interactions de deux facteurs de risques du déclin cognitif, la sécrétion de corticostérone et le vieillissement. En effet, des dysfonctionnements du système de stress facilitent le vieillissement et l'augmentation de glucocorticoïdes peut induire des altérations cognitives. Entre le milieu de la vie et le début de la vieillesse, nous avons étudié l'évolution des phénotypes bio-comportementaux des trois lignées. Nous avons évalué l'anxiété, la réponse au stress et le style d'adaptation à différent moments du vieillissement, ainsi que les fonctions cognitives dans une « piscine de Morris ». Les phénotypes bio-comportementaux des lignées étaient stables durant la vie et l'apprentissage et la mémoire à long terme étaient différents entre les trois lignées.

Nous avons aussi montré que ce modèle est approprié pour l'étude des systèmes liés au stress et que de futures recherches l'utiliser pour étudier plus en détail les liens entre la régulation du stress et des processus biologique liés à la santé.

Mots-clés

Stress, jeunesse, axe HHS, corticostérone, habituation au stress, phénotype bio-comportemental, style d'adaptation, système nerveux autonome, variabilité du rythme cardiaque, comportements sociaux, métabolisme, utilisation d'énergie, vieillissement, cognition

Abbreviations

ACh: Acetylcholine
ACTH: Adrenocortropic Hormone
AgRP: Agouti related Peptide
ANOVA: Analysis of Variance
ANS: Autonomic Nervous System
ARC: Arcuate Nucleus of the Hypothalamus
ATP: Adenosine Triphosphate
AUC: Area under the Curve
AVP: Arginine Vasopressin
BAT: Brown Adipose Tissue
BDNF: Brain Derived Neurotrophic Factor
BNST: Bed Nucleus of the Stria Terminalis
Bpm: beat-per-minute
BW: Body Weight
cAMP: cyclic Adenosine Monophosphate
CAST: Corticosterone Adaptation Stress Test
CeA: Central Nucleus of the Amygdala
CORT: Corticosterone
CRH: Corticotropin-Releasing Hormone
CVD: Cardiovascular Disorder
CVLM: Caudal Ventrolateral Medulla
DMH: Dorsomedial Nucleus of the Hypothalamus
DMNV: Dorsal Motor Nucleus of the Vagus
EC: Endocannabinoid
FST: Forced-Swim Test
GABA: Gamma Amino Butyric Acid
GLP-1: Glucagon-Like Peptide-1
GPCR: G Protein-Coupled Receptors
GR: Glucocorticoid Receptor
HPA: Hypothalamic-Pituitary-Adrenal
HR: Heart Rate
FKBP51: FK506-binding protein 51
LC: Locus Coeruleus
LSD: Least Significant Difference
LTP: Long Term Potentiation
MAP: Mean Arterial Pressure
MCxR: MelanoCortin x Receptors
mPFC: Medial Prefrontal Cortex
MR: Mineralocorticoid Receptor
mRNA: Messenger RNA
NA: Noradrenaline
NAmb: Nucleus Ambiguous
NMDA: N-Methyl-D-Aspartate
NPY: Neuropeptide Y
NTS: Nucleus Tractus Solitarius
PFC: Prefrontal Cortex
PKA: Protein Kinase A
PL: Pre-Limbic
PNS: Parasympathetic Nervous System
POMC: Pro-Opio Melanocortin
PTSD: Post Traumatic Stress Disorder
PVN: Paraventricular Nucleus of the Hypothalamus
RNA: Ribonucleic Acid
RSA: respiratory sinus arrhythmia
RVLM: Rostral Ventrolateral Medulla
SA: Sinoatrial node
SAM: Sympatho-Adrenomedullary
SEM: Standard Error of the Mean
SNS: Sympathetic Nervous System
TH: Tyrosine Hydroxylase
VMH: Ventromedial Nucleus of the Hypothalamus

General Introduction

The stress concept: homeostasis and allostasis

Stress can be defined as a state in which homeostasis of an organism is threatened and behavioral and physiological adaptive responses are induced to re-establish homeostasis (Chrousos, 2009). Homeostasis is a process wherein physiological parameters are maintained in a dynamic equilibrium (McEwen and Gianaros, 2011). Within the brain, a wide range of systems is activated to adapt behavioral and physiological responses in order to meet the demands imposed by the stressor. Those short-term and adaptive processes constitute the "allostasis" and long maladaptive responses are entitled the *allostatic load* (McEwen and Gianaros, 2011) and *allostatic overload*, which the "wear and tear" on the body and brain that results from being "stressed out" (McEwen, 2005). The *Darwinian concept of stress* is balancing the benefits of allostasis with the costs of allostatic load in order to analyze repercussions on health and diseases (Korte et al., 2005). Indeed, in order to maintain homeostasis, the stress-activated systems of an organism will act globally throughout the body, in order to offer survival and adaptation (McEwen and Wingfield, 2010). Stress is constituted of environmental and biological components, in a way that the environmental stress is shaping adaptation and evolution of the biology of the organism experiencing it (Bijlsma and Loeschcke, 2005). During disruption of homeostasis by a stressor, different brain systems are activated to prepare the organism to respond accordingly. The different pathways and neuromodulators involved in the stress response are the autonomic-nervous system (ANS), with fast activation of the sympatho-adrenomedullary (SAM) axis, and the hypothalamic-pituitary-adrenal (HPA) axis with production of glucocorticoids as final effector (Ulrich-Lai and Herman, 2009).

The SAM and HPA axes

i. The ANS and SAM axis

The autonomic nervous system is the central regulator of homeostasis and contains two different axes. The sympathetic nervous system (SNS), which is the fast-acting part, leads to stress reactivity and to "fight-or-flight" responses. The parasympathetic nervous system (PNS) is the low reactive division bringing the organism back to its resting homeostatic state and controls the "rest-and-digest" response (Feher, 2012).

The sympatho-adrenomedullary arm increases quickly heart rate and blood pressure by excitation of the cardiovascular system by the SNS, while the PNS rapidly reduces ANS activation, resulting in short-term responses (Ulrich-Lai and Herman, 2009). When the SAM is activated (Figure 1), preganglionic sympathetic neurons project to vertebral ganglia which project to the end organs. The SAM also projects to chromaffin cells of the adrenal medulla, leading to the release of catecholamines (adrenaline and noradrenaline (NA)) into the circulation (Guyenet, 2006). Into the bloodstream, circulating levels of adrenaline (from the adrenal medulla) and noradrenaline (from sympathetic nerves), increase heart rate, blood pressure, vasoconstriction, and energy mobilization in response to stress (Ulrich-Lai and Herman, 2009). Finally, target glands, organs and muscles are activated according to the response initiated. Once the perceived danger is gone, the PNS takes over to counterbalance the effects of sympathetically induced responses (Ulrich-Lai and Herman, 2009; Gordan et al., 2015; Turner et al., 2012).

ii. The HPA axis

a. General information

The second actor recruited during a stress response is the HPA axis. Stress stimuli are integrated by the paraventricular nucleus (PVN) of the hypothalamus and induce the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the median eminence to stimulate adrenocorticotropic hormone (ACTH) release from the anterior pituitary (Figure 1). Melanocortin 2 receptors (MC2R), in the adrenal cortex, are activated by ACTH and initiate glucocorticoid production (CORT = mainly cortisol in humans and corticosterone in rats).

Circulating CORT affects peripheral organs and promotes stress adaptation by mobilizing stored energy and triggering sympathetic actions (Ulrich-Lai and Herman, 2009). The HPA and SAM axes are closely related and for example CORT can increase vasoconstriction and the adrenal cortex is directly innervated by SNS inputs and facilitates CORT release (Ulrich-Lai and Engeland, 2005). Moreover, at the adrenal cortex level, ANS inputs control ACTH sensitivity and responsiveness (Jasper and Engeland, 1997). The HPA axis and SNS have complementary actions throughout the body, including energy mobilization and maintaining blood pressure during stress.

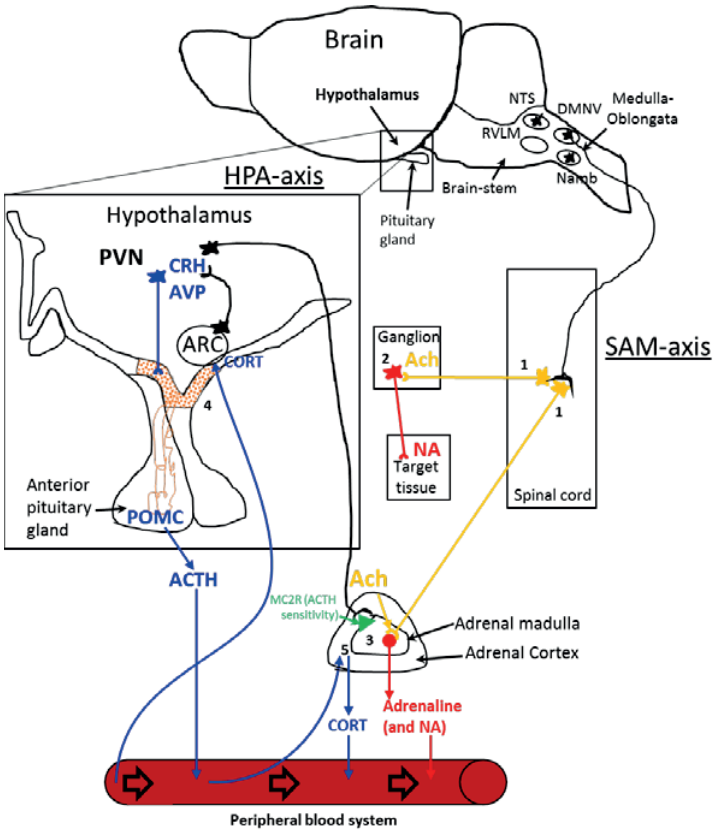

Figure 1. Schematic representation of the SAM and HPA axes (adapted from (Turner et al., 2012). The sympatho-adrenomedullary system ("SAM axis") consists of the SNS and adrenal medulla. Pre-ganglionic neurons (**1**) extending from the spinal cord project to the ganglia and to the adrenal medulla. When activated the pre-ganglionic neurons (**1**) release the acetylcholine (Ach) inducing post-ganglionic neurons (**2**) to release noradrenaline (NA) directly into target tissue. Endocrine chromaffin cells (**3**) in the adrenal medulla release adrenaline (A) and NA into the blood-steam. The hypothalamus-pituitary-adrenal axis (HPA axis) is regulated by corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the paraventricular nucleus (PVN) of the hypothalamus. Those hormones are transported through the median eminence (**4**) to the anterior pituitary gland where they stimulate the synthesis of pro-opiomelanocortin (POMC) resulting in various products including adrenocorticotropic hormone (ACTH). ACTH, secreted into the peripheral blood-stream, acts on the adrenal cortex (**5**) to stimulate synthesis of the glucocorticoids (CORT) which are the final effectors of the HPA axis leading to a wide range of physiological and behavioral adaptations.

Glucocorticoids have both genomic and non-genomic actions throughout the body (Ulrich-Lai and Herman, 2009). The binding of CORT to intracellular and cytosolic glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) controls the genomic actions. These two types of receptors act as ligand-activated transcription factors to affect broad, long-term and long-latency changes in gene transcription (De Kloet et al., 1998). The MR has high affinity for CORT, is largely bound at low circulating levels of glucocorticoids and is important in ambient glucocorticoid signaling processes, by controlling circadian cycle CORT secretion (Herman et al., 2012; Dallman et al., 1989). The GR has lower CORT affinity and reaches extensive binding only at high CORT levels, i.e., during stress responses (Reul and de Kloet, 1985). Action trough GR is the primary mediator of the glucocorticoid inhibition of the HPA axis (de Kloet et al., 2008). By contrast, non-genomic effects arise at target cell membrane, leading to a 'fast' negative feedback-inhibition of the HPA axis. This action occurring within minutes of the rise in circulating glucocorticoids is too fast to be mediated by genomic actions and it has been suggested to be driven by a G-protein-coupled receptor (Groeneweg et al., 2011). The non-genomic action has also been suggested to involve endocannabinoid inhibition (Tasker and Herman, 2011), however there is no consensus on the exact mechanisms.

The GR cytosolic receptor action can be modulated by a set of chaperone proteins (mainly HSP90), and FK506-binding protein 51 (FKBP51), a co-chaperone of HPS90 (Wang et al., 2018) and a potent inhibitor of GR in mammalian cells (Fries et al., 2017). FKB51 is encoded by the gene FKBP5 and, functionally, cytosolic FKBP5 gene expression is promoted by GR activation, which leads to translocation of GR into the nucleus and binding to the promoter region of FKBP5. FKBP51 binds and inhibits GR, leading to GR desensitization (Rein, 2016; Riggs et al., 2003; Wochnik et al., 2005). Thus, FKBP51 is a significant moderator of GR sensitivity and a key element of HPA axis modulation and it has been shown that the higher FKBP5 levels in the PVN, the lower GR binding affinity to CORT, reducing GR translocation to the nucleus (Binder, 2009). High levels of FKBP5 have been related with a more reactive HPA axis and low active coping behaviors (Hartmann et al., 2012).

b. Corticosterone and the negative feedback loop of the HPA axis

The HPA axis activity is auto-regulated by a negative feedback loop, mediated by CORT and principally driven by GR activity (De Kloet et al., 1998). The negative feedback response is not a simple system with a defined feedback 'switch' but instead, requires a network coordinating HPA axis activity with the organism energy needs. The different, but interacting, mechanisms of the negative feedback mainly regulate the CRH neurons from the PVN (Ulrich-Lai and Herman, 2009). The negative feedback involves different sets of neurons and three levels of action of glucocorticoids (Watts, 2005). The negative feedback involves afferent neurons (e.g. NPY/AgRP GABAergic ARC neurons, BNST projections) that have direct synaptic contacts with CRH neurons in the PVN, and neurons (e.g. hypothalamic projections into DMH, amygdala projections to BNST, neurons from ventral subiculum through BNST) affecting indirectly CRH neurons (Watts, 2005). Then, CORT can modulate PVN neurons by acting on neurons from the different level or by indirect physiological feedbacks like changes in energy metabolism. Moreover, CORT itself acts on the negative feedback with three distinct mechanisms. The first mechanism provided by CORT is a fast, non-genomic feedback that is mediated by endocannabinoid (EC) inhibition of PVN glutamate inputs from the dorsomedial (DMH) and ventromedial hypothalamus (VMH). Genomic CORT signaling from the forebrain is involved in the second mechanism, mediated by the pre-limbic (PL) part of the prefrontal cortex (PFC) and by the ventral subiculum (the primary ventral output) of the hippocampus. Since those regions have no direct interactions with the PVN, intermediary synapses translating glutamatergic output into GABAergic inhibition of the PVN through the bed nucleus of the stria terminalis (BNST) are required (Herman et al., 2003). Finally, the negative feedback can take place through a glucocorticoid weakening of ascending excitatory inputs, e.g., the direct projections to CRH neurons from nucleus tractus solitarius (NTS). Indeed CORT can destabilize mRNAs encoding HPA activating neuropeptides such as glucagon-like peptide-1 (GLP-1) and thus diminish/reduce excitatory inputs to the PVN (Ghosal et al., 2016; Ulrich-Lai and Herman, 2009). On the other hand, CORT also plays a role in stress excitation (feed-forward mechanism), with trans-synaptic inputs from the central amygdala (CeA) (Herman et al., 2012).

As previously described, CORT can be controlled by either hormonal cascade with CRH and ACTH actions or by SNS direct projections from PVN to the adrenal gland with the CORT negative feedback mainly being driven by GRs (Tasker and Herman, 2011). However, PVN neurons projecting to the adrenal do not express GRs, so there might be another mechanism involved in transmitting CORT level info to ANS neurons in the PVN (Leon-Mercado et al., 2017). It has been showed that the arcuate nucleus (ARC) of the hypothalamus might be essential in sensing CORT blood levels and controlling glucocorticoid secretion during the feedback response via projections to the PVN (Leon-Mercado et al., 2017). Glucocorticoids penetrate in the ARC through the median eminence and AgRP (Agouti related Peptide) input from the ARC to the PVN transmits glucocorticoids state to control sympathetic splanchnic nerve tone and ACTH sensitivity in the adrenal gland (Leon-Mercado et al., 2017).

c. HPA axis and aging

Dysfunctions of the HPA axis may contribute to aging-related diseases (Gupta and Morley, 2014) by accelerating aging processes (Finkel and Holbrook, 2000; PARE, 1965) and, inversely, aging may decrease adaptive abilities against stress (Sapolsky et al., 1986; Webb and Agnew, 1962). Deleterious effects of aging on learning and memory are well-documented (Bachevalier et al., 1991; Bergado and Almaguer, 2002; Gower and Lamberty, 1993) and some alterations in the neurotransmitter systems (acetylcholine, catecholamines and glutamate) have been described (Bartus, 2000; McEntee and Crook, 1993; Richter-Levin and Segal, 1993). In addition, plasticity processes might also be impaired in aged animals (Barnes and McNaughton, 1985; Bergado et al., 1998) and the synaptic plasticity and neurons survival are reduced with increased CORT levels (Sapolsky, 1999; Kim and Diamond, 2002; Pavlides et al., 2002). The "glucocorticoid cascade hypothesis" from Sapolsky and colleagues (1986) suggests that CORT secreted during stress might desensitize the hippocampus by downregulating GRs. In the long run, in aged individuals, the impairment of the negative feedback loop induced might enhance brain exposure to deleterious effects of glucocorticoids. This may reduce the number of neurons sensitive to CORT in the hippocampus and induce cognitive deficits with aging (Gupta and Morley, 2014). Overall, the literature suggests that aging promotes anatomical and functional deficiencies at the level of hippocampus and amygdala (Bergado et al., 2007, 2011; Frey and Frey, 2008) which leads to learning and behavioral reinforcement deficits (Bergado et al., 2011; Baxter and Gallagher, 1996).

iii. Cardiovascular control

The sinoatrial node (SA) is the main pacemaker of the heart, is situated on the right atrium, and maintains a sinus rhythm (60-100 bpm in humans and around 350 bpm in rats). The heart rate (HR) is constantly controlled by SNS (increases HR) and PNS (decreases HR) with the PNS influence dominating at rest.

a. Sympathetic influences

Cardiac sympathetic pre-ganglionic nerves arise from the upper thoracic segments of the spinal cord (T1-T4) and the excitatory signals to these initiates in pre-motor neurons in rostral ventrolateral medulla (RVLM). The pre-ganglionic neurons of both SNS and PNS secret acetylcholine (ACh) but SNS post-ganglionic neurons release noradrenaline (also called norepinephrine) and sympathetic post-ganglionic fibers are commonly called *adrenergic* fibers. Other central nervous system sites such as the PVN may contribute to the sympathetic modulation depending on physiological and pathological states (Guyenet, 2006). After traveling a short distance, pre-ganglionic fibers leave the spinal nerves and enter sympathetic ganglia and the post-ganglionic fibers extend to the heart (See Figure 2). At the SA level, stimulation by NA from SNS enhances contraction of both the ventricles and atria, which increases the heart rate (Gordan et al., 2015).

b. Parasympathetic influences

Nerves from the PNS originate within the midbrain, pons and medulla oblongata of the brainstem and have long preganglionic neurons, followed by short postganglionic neurons (Gordan et al., 2015). The cell bodies of preganglionic parasympathetic nerves are located in the nucleus ambiguous (NAmb) and the dorsal motor nucleus of the vagus (DMNV) in the medulla oblongata of the brainstem (Freeman and Chapleau, 2013).The PNS neurons release Ach in both the pre- and post-ganglionic neurons and are called *cholinergic* fibers. Acetylcholine binds two types of cholinergic receptors, the nicotinic and the muscarinic receptors. The nicotinic receptors are located at the synapses in-between the pre- and post-ganglionic neurons of the SNS and PNS. Nicotinic receptors produce rapid excitatory responses while muscarinic receptors, located at the end of both post-ganglionic parasympathetic nerves and cholinergic sympathetic fibers, produce relatively slow excitatory response (Gordan et al., 2015). The vagus nerve directly innervates the SA node and decreases HR (negative chronotropic effect). In contrast to sympathetic activity, the PNS does not affect myocardial contractility (vagal afferent may not directly innervate cardiomyocytes). The

arterial baroreceptor reflex has an important excitatory influence on PNS activity (Kunze, 1972). Baroreceptor afferent nerves acts on neurons in nucleus tractus solitarius (NTS) projecting to NAmb and DMNV (McAllen and Spyer, 1978) and provide the major excitatory drive to cardiac vagal neurons under resting conditions, when arterial pressure is normal. The increase in baroreceptor activity, during blood pressure increase, enhance the activity of the PNS, which reduces heart rate (Figure 2). Similarly, a reduction in arterial pressure decreases the baroreceptor activity and leads to an inhibition of the PNS activity and an HR increase (Freeman and Chapleau, 2013).

c. Cellular mechanisms

At the cellular level, sympathetic and parasympathetic receptors are G-protein coupled receptors (GPCRs) which mediates the adrenergic control on heart rate and contractility. The β_1-adrenoceptors, which have higher affinity for adrenaline than NA, is activated by the binding of NA leading to the activation of G-proteins that can activate adenylyl cyclase which convert ATP into cyclic adenosine monophosphate (cAMP). Then, cAMP activates a protein kinase A (PKA) phosphorylates multiple target proteins, binds to ion channels responsible for heart rate increase. The parasympathetic system activates muscarinic (M2) receptors, in the heart, which are inhibitory GPCRs, and reduces PKA activity and leading to opposite effects on ion channels and contractile machinery than sympathetic stimulation (Gordan et al., 2015).

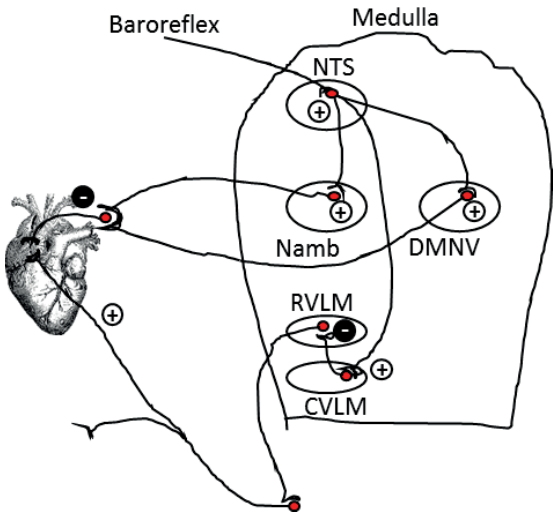

Figure 2. Sympathetic and parasympathetic influences on heart rate are initiated in the medulla oblongata and modulation by baroreflex afferents (adapted from Rahmouni et al., 2016). The parasympathetic regulation of HR in regulated by projections from neurons from the nucleus ambiguous (NAmb) and the dorsal motor nucleus of the vagus (DMNV). The sympathetic control is mainly initiated by pre-motor neurons in the rostral ventrolateral medulla (RVLM). Baroreflex signals are integrated by the nucleus of the solitary tract (NTS) which activates the NAmb, DMNV and the caudal ventrolateral medulla (CVLM). The CVLM sends inhibitory inputs to the RVLM, decreasing the SNS activation. Thus baroreflex signals activate PNS and decreases SNS control of heart rate.

d. Heart rate variability (HRV)

Cardiac vagal activity is dependent on respiratory inputs (Rentero et al., 2002) and breathing modulates vagal activity through direct projections from the central respiratory pattern generator. Moreover, ascending neurons from lung receptors, which are activated during inspiration, trigger GABAergic neurons in the NTS and inhibit vagal neurons in the NAmb. These mechanisms generate the respiratory sinus arrhythmia (RSA), which are rhythmic oscillations of HR around its mean, increasing during inspiration (vagal influence reduced), and decreasing during expiration when vagal influence restarts (Chapleau and Sabharwal, 2010; Carnevali and Sgoifo, 2014). During heart rate variability (HRV) analysis, it is possible to assess the vagal influence by computing the oscillations in the intervals between consecutive heartbeats, which are due to RSA (Task Force, 1996). Thus, HRV analysis is used to estimate the autonomic modulation of the heart in humans and animal models. Although HRV analysis might not be suitable to accurately estimate sympathetic modulation (del Paso et al., 2013), it produces reliable measures of vagal tone (Berntson et al., 1997). A traditional and widely used HRV method is the “time-domain” analysis based on statistical calculation on R-

R intervals (Kleiger et al., 1992), the root mean square of successive R-R interval differences (RMSSD) detects high frequency oscillations of HR, and therefore estimates parasympathetic nervous system activity (Stein, 1994). Interestingly, HRV has been associated with individual differences in emotional responding (Appelhans and Luecken, 2006; von Borell et al., 2007) perceived stress (Dishman et al., 2000) trait anxiety (Fuller, 1992) and depressed mood (Caey and Freedland, 2009).

iv. The Arcuate nucleus of hypothalamus

a. General information

The ARC is located at close proximity to the median eminence and lacks blood-brain barrier, which facilitates access to substances circulating in the bloodstream. It appears that the median eminence acts as a putative route for peripheral molecules to target the ARC (Mullier et al., 2010). The ARC regulates feeding behavior and energy expenditure by sensing circulating leptin, ghrelin and insulin (Schwartz et al., 2000) as illustrated in Figure 3A. Recently, studies have showed the importance of the ARC in the regulation of the negative feedback of the HPA axis (Leon-Mercado et al., 2017) as well as in the autonomic nervous system modulation of heart rate (Rahmouni, 2016). I will briefly review here the implication of the ARC in: i) food intake and energy expenditure, ii) the regulation of the HPA axis and, iii) the modulation of cardiac functions. The goal of this part is to present the ARC as a potential region of interest relating different experimental observations presented in the thesis. I will also introduce ghrelin action as a potential molecular target for further experiments.

b. Regulation of food intake and energy expenditure

The ARC is highly responsive to changes in energy/nutritional state (e.g., fasting) and mediates changes the SNS activity controlling thermogenesis in brown adipose tissue (BAT) (Bartness et al., 2010; Rahmouni and Morgan, 2007). Moreover, ARC neurons that synthesize pro-opiomelanocortin (POMC neurons) or neuropeptide Y and agouti-related peptide (NPY/AgRP neurons) are important for energy homeostasis. NPY/AgRP neurons increase food intake (enhance appetite = orexigenic effect) while POMC neurons decrease appetite (anorexigenic action) and increase BAT thermogenesis. The secretion of ∝-melanocyte-stimulating hormone, a byproduct of POMC, activates melanocortin 4 receptors (MC4R) to suppress appetite and increase energy expenditure (Chen et al., 2000). NPY neurons have a sympatho-inhibitory function in the brain (Münzberg et al., 2016) and GABAergic ARC AgRP/NPY neurons inhibits BAT sympathetic regulation (Shi et al., 2013). Recently, it was shown that tyrosine hydroxylase (TH) neurons in the ARC also make an orexigenic contribution (Zhang and van den Pol, 2016). The ARC TH cells project to the PVN and inhibit POMC neurons by dopamine and GABA co-release (Zhang and van den Pol, 2016). Elevated ghrelin following chronic social stress in mice increases NPY/AgRP expression, caloric intake, and increases the use of carbohydrates as an energy substrate, illustrating its importance in metabolic adaptations (Patterson et al., 2013).

c. Regulation of the HPA axis

As previously mentioned in the part *II.a. Corticosterone and the negative feedback loop of the HPA axis*, the ARC might control CORT production during the feedback response by sensing CORT levels and via AgRP/NPY neuronal projections to the PVN, controlling splanchnic nerve tone and adrenal responsivity (Leon-Mercado et al., 2017). Ghrelin activates CRH neurons in the PVN, which is sufficient to increase acutely plasma glucocorticoid levels (Cabral et al., 2012; Hill, 2012). However, there are no neurons co-expressing CRH and ghrelin receptor in the PVN, which suggests that the HPA axis stimulation by ghrelin is indirect (Cabral et al., 2012). Moreover, recently, a study suggested a mechanism by which ARC GABAergic neurons promote persistent CRH secretion from the PVN neurons into the median eminence (Kakizawa et al., 2016). Furthermore, CRH-expressing neurons located in the PVN express MC4R (Lu et al., 2003; Cansell et al., 2012) and the activity of CRH neurons might mediate some effects of ARC POMC and NPY circuitry.

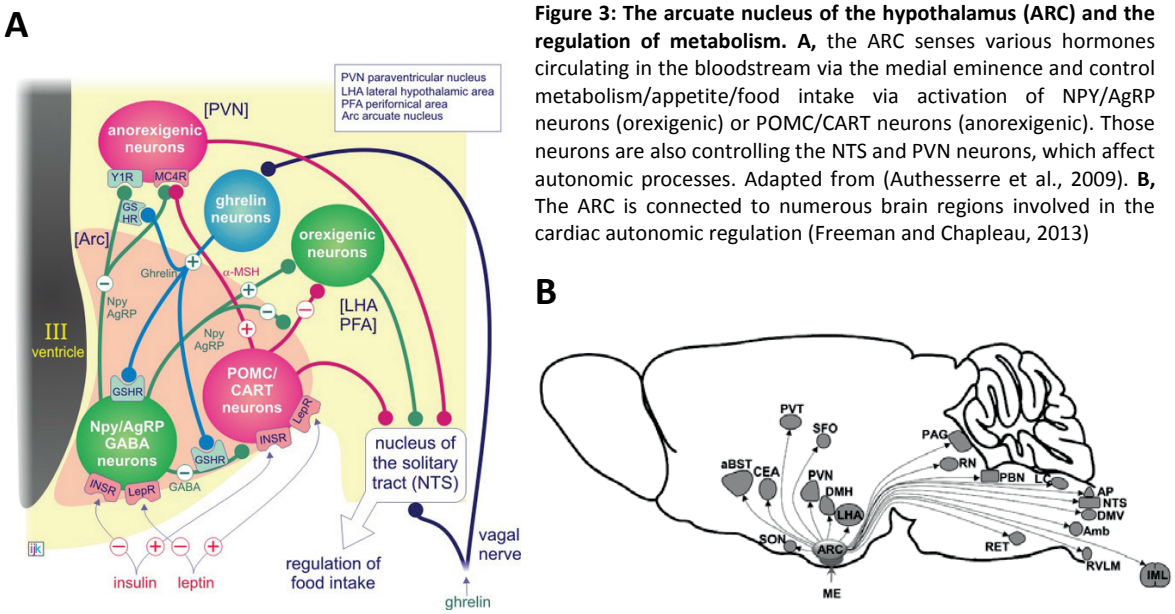

Figure 3: The arcuate nucleus of the hypothalamus (ARC) and the regulation of metabolism. A, the ARC senses various hormones circulating in the bloodstream via the medial eminence and control metabolism/appetite/food intake via activation of NPY/AgRP neurons (orexigenic) or POMC/CART neurons (anorexigenic). Those neurons are also controlling the NTS and PVN neurons, which affect autonomic processes. Adapted from (Authesserre et al., 2009). **B,** The ARC is connected to numerous brain regions involved in the cardiac autonomic regulation (Freeman and Chapleau, 2013)

d. Regulation of the cardiovascular system

The ARC is connected to the various autonomic and cardio-regulatory nuclei as represented in Figure 3B (from Rahmouni, 2016). Recent studies demonstrated a role of ARC in cardiovascular control and both pressor and depressor responses can be elicited by the chemical stimulation of the ARC. Indeed, ARC stimulation elicited tachycardia (Kawabe et al., 2012) that might by mediated by sympathetic inputs to the heart and potential inhibition of vagal inputs to the heart (Nakamura et al., 2009). It was suggested that the outcome of ARC stimulation might depend on basal mean arterial pressure (MAP). Indeed, injections of NMDA (Sapru, 2012) into the ARC, increased MAP and HR when basal MAP was low (Arakawa et al., 2011; Nakamura et al., 2009), but decreased MAP and HR at higher basal MAP (Arakawa et al., 2011; Kawabe et al., 2012). The two different neuronal populations in the ARC, excitatory POMC/CART neurons and inhibitory NPY/AgRP neurons, both projecting to the PVN, might drive the differential responses. Inhibitory effects of baroreceptor afferents, through the NTS and GABAergic neurons from the caudal ventrolateral medulla (CVLM), can explain the inhibitory effects prevailing at normal baseline MAP (See Figure 2). On the other hand, lowering baseline MAP reduces the tonic inhibition on RVLM neurons and unmasks the excitation effects from the PVN to the RVLM eliciting pressor responses (Rahmouni, 2016).

Stress habituation, coping style and stress susceptibility

i. Stress habituation

The process of stress habituation reduces the HPA axis response upon repeated exposure to homotypic stressors and limits deleterious actions of prolonged CORT secretion. Habituation is regulated by limbic stress-regulatory mechanisms and depends, in part, on glucocorticoid feedbacks (Herman, 2013). In humans, the classic adaptation to repeated homotypic stressors is to reduce the HPA axis activation across exposures (Deinzer et al., 1997; Federenko et al., 2004; Gerra et al., 2001; Pruessner et al., 1997), while the organism is able to mount a proper response to heterotypic stressors. Impaired habituation might expose an organism to an allostatic overload (McEWEN, 1998) and studies showed that humans who habituate less to repeated stressors could be considered to engender a depression-like phenotype (Kirschbaum et al., 1995; Kudielka et al., 2006). These studies support a potential link between

differences in stress habituation and allostatic overload, and suggests a potential mechanism linking stress vulnerability and psychopathology development. Stress-related mental disorders (e.g. PTSD and depression) are supplemented by glucocorticoid discrepancies and structural or functional alterations of some limbic circuits, involved in the chronic stress response, suggesting that an inappropriate processing of stressful situations contributes to pathological processes (Herman, 2013).

In order to explain stress habituation, Peters and McEwen presented an allostatic load model involving autonomic, immune, endocrine and metabolic factors (Peters and McEwen, 2015). According to this model, during allostatic regulation, the brain receives extra energy in order to cope with the stressor and stress is defined as a "state of increased cerebral energy need". Thus, in this model, acute stress induces specific mechanisms in order to supply the energy required (Peters and McEwen, 2015). Indeed, in a stress situation, the anterior cingulate cortex and the amygdala process and assess threats (Sarinopoulos et al., 2010). Then, they control the locus coeruleus-noradrenaline (LC-NA) system (Reyes et al., 2011) and leads to cortical NA release which enhances arousal and cortical information transmission (Peters et al., 2017), subsequently leading to increased cerebral energy needs (Hitze et al., 2010). The VMH and PVN activate SNS and HPA axis to produce the extra energy required by the brain (Kubera et al., 2012) and by peripheral muscles involved in the fight-or-flight response (Goldstein, 2010). The SNS accelerates heart rate but increased blood flow may lead to turbulences in the bloodstream and the irregularities induced by turbulences create an uneconomical and under-efficient transport of energy (Peters and McEwen, 2015). Thus, maintaining a laminar (not-turbulent) blood flow, even during the stress response, might be a more effective way to deliver energy to the brain. Rats habituating to stress become low reactive to stressful events, thus saving brain energy and avoiding energy loss in turbulences. On the other hand, chronically reduced allostatic load might have detrimental effects as well (e.g. decreased attention or reactivity in case of emergency).

Stress habituation is mainly directed by the mPFC (Hill and Tasker, 2012), and following a homotypic stressor, the mPFC alters the signaling between amygdala and the LC-NA system and prevents extreme reactive mechanisms (Peters and McEwen, 2015). This process implies the combination of CORT feedback and the endocannabinoid system in a "depolarization-induced suppression of inhibition" (DSI-switch) process (Hill and McEwen, 2010; Hill and Tasker, 2011). In habituators, the mPFC signaling to the PVN is altered and leads to blunted sympathetic and HPA axis responses, which increases protection against cardiovascular and cerebrovascular disorders (Seldenrijk et al., 2012; Hamer and Steptoe, 2012). Moreover, in case of habituation to homotypic stress, the mPFC diminishes the signaling from the amygdala to the hypothalamus, particularly in the VMH and the PVN, contributing to a blunted SNS and HPA axis responses (Patel and Hillard, 2008; Hill et al., 2008). Without increase in SNS and HPA axis activities, the arterial turbulences, due to high bloodstream flow, is less likely (Falsetti et al., 1983; Hanai et al., 1991).

However, some studies have shown that the HPA and SAM axes display different reactivity to repeated stress and that habituation involves different neural processes between HPA and SAM axes. In humans it has been shown that the HPA axis habituated to stress (less CORT production) while the adrenaline response did not change in reaction to repeated psychosocial stress (Schommer et al., 2003). Furthermore, rodents, in response to moderate handling stress, showed important habituation in adrenaline, CORT and ACTH but not in NA release (Dobrakovová et al., 1993). Moreover, it was shown that habituation of autonomic responses is dependent on the type and context of stress, since chronic mild-stress elevated basal HR and decreased HRV in rats (Grippo et al., 2002) but chronic social stress induces partial habituation of HR responses to attack in subordinate but not in dominant mice (Bartolomucci et al., 2003).

Finally, Peters and McEwen (2015) model aimed at explaining influences of stress habituation on energy metabolism. Indeed, a rise in cerebral energy needs triggered by stress is higher in non-habituators (Peters and McEwen, 2015).

This leads to higher use of subcutaneous fat mass in these animals and subsequently induces a lean phenotype with more visceral fat while habituators have more subcutaneous fat. Moreover, differences in phenotype can be explained by different energy sources used and visceral fat grows under chronic stress responses of non-habituators through NPY and glucose uptake pathways (Lumeng et al., 2007). In habituators, the lower reactivity leads to a decrease in the "percentage-of-glucose-allocated-to-the-brain", and therefore the organism has to increase the "total energy intake" to compensate (Peters and McEwen, 2015).

ii. Active and passive coping styles

The repertoire of responses to the stress that an individual has and uses successfully is called *coping style* can be of two opposite types: active/proactive versus passive/reactive coping (Jang et al., 2007). Differences in coping style has been related to differences in disease susceptibility (de Boer et al., 2017). It is widely suggested that susceptibility and coping styles are closely related and that susceptibility to develop depression and cardiovascular dysfunction following stress exposure might be linked to passive coping strategies (de Boer et al., 2017; Wood and Valentino, 2016; Wood, 2014).

Different levels of aggressivity in wild-type rats has been extensively studied as a model of coping styles (Coppens et al., 2010; Koolhaas et al., 2010, 2007; de Boer et al., 2017). A negative correlation between the latency to initiate aggressive behaviors in a resident-intruder test and the amount of burying in a shock-probe burying test was found (Koolhaas et al., 2010). Rats with low attack latency and high burying were classified as active copers. These active wild-type rats showed, in challenging situations, an increase in sympathetic reactivity, higher levels of plasma adrenaline and NA and a larger increase in HR and blood pressure than passive copers (Sgoifo et al., 1996, 1997). These differences were clear in reaction to stress but there were no sympathetic differences during baseline recordings and no differences in HPA axis markers at basal levels (ACTH and CORT levels). In response to stress, there was an inverted U-shape in CORT secretion with low and high aggressive rats having lower CORT response than intermediate animals (De Boer and Koolhaas, 2003). In order to interpret results from the active wild-type rats it is important to note that high aggressive Wistar showed similar aggressivity than intermediate wild-type rats, so there is no Wistar equivalent of the high-aggressive wild-type phenotype.

Other studies assessed HPA and SAM axes activity in active and passive copers and in response to stress, passive copers have an increase in CRH (Herman et al., 2016) and a large HPA axis response (Boersma and Tamashiro, 2015). Furthermore, passive copers have enhanced pro-inflammatory cytokines and oxidative stress systems, leading to depressive-like phenotype (Wood, 2014) as well as cardiac hypertrophy and reduced HRV (Carnevali et al., 2017). Another study showed that active and passive copers had similar increase in CORT and hypothalamic CRH levels in response to stress, but differential adaptation to stress in term of HPA and SAM axes (Pérez-Tejada et al., 2013). Passive copers were more vulnerable to stress in a forced-swim test (FST), had higher secretion of CORT in response to chronic social stress and lower basal CORT than controls and on the first day of stress and a general reduction in NA and adrenaline secretion (Pérez-Tejada et al., 2013; Gómez-Lázaro et al., 2012). On the other hand, animals exhibiting active coping during social stress had a reduction of CRH and NA transmission in the brain, an increased NPY and serotonin activity as well as a resilience to stress (Wood, 2014). Moreover, active animals did not show depressive-like behaviors or decreased HRV (Wood, 2014). Overall, active coping might be linked to increased HRV and cardiac capacity to adapt properly in response to stress, while passive strategy might be related to decreased HRV and global sensitivity to cardiovascular disorders (Carnevali et al., 2017).

Interestingly, a recent study showed that selection of rats based on their active or passive burying did not predict their social status but rather influenced the consequences of being subordinate (Boersma et al., 2017). They showed that active copers were more susceptible to subordination stress and expressed a higher allostatic load (larger

adrenals and increased stress response). This suggests a relationship between coping style and stress susceptibility but importantly, it shows that both concepts are context dependent. It should be noted that coping style is not a binary concept and may encompass different sub-coping strategies depending on environment and context (strain/species, social and demographic context, fitness, previous experiences and internal/emotional states, etc.) and it is a multi-dimensional system (de Boer et al., 2017). The multi-dimensional approach suggests that individuals sharing a coping style tend to share the same coping strategies in different situations and that coping behaviors might share neurobiological and molecular mechanisms. However, importantly, having an active strategy in a specific situation does not necessarily implicate using active strategies in all situations (Veenema and Neumann, 2007), and one should be careful when comparing coping strategies from different experiments and contexts.

iii. Susceptibility to stress

Susceptibility to stress is characterized by an increased stress sensitivity and/or a deficiency in stopping stress response (Ebner and Singewald, 2017) which might be due to an abnormal negative feedback of HPA axis (Carter and Goldstein, 2015; Herman et al., 2016). Susceptible individuals exposed to social defeat display changes in physiological stress responses including cardiovascular parameters, arterial pressure and heart rate (Carter and Goldstein, 2015; Golbidi et al., 2015). They also exhibit different responses of the immune system (Ménard et al., 2017) and increased HPA axis factors, e.g. ACTH and glucocorticoids (Herman et al., 2016). Rodent studies have shown that susceptible animals have lower stress inhibition in hippocampus, mPFC, BNST and significant changes in key elements of the HPA axis, like the GR/MR ratio and FKBP5 levels (Beery and Kaufer, 2015; Ebner and Singewald, 2017). The reward system has also been implicated in differentiating resilient to susceptible animals and stress susceptibility has been correlated with stress induced increase in levels of brain derived neurotrophic factor (BDNF), a key regulator of dopamine release in the nucleus accumbens (Beery and Kaufer, 2015). Moreover, susceptibility to social defeat was further shown to be mediated by enhanced firing of VTA dopamine neurons, with resilience characterized by a lack of activity dependent BDNF release (Krishnan et al., 2007).

An animal model with differential responsiveness and adaptation to stress during juvenile period

The juvenile period is an extremely sensitive period during which stress increases risks of developing neuropsychiatric disorders (Brydges, 2016; Horovitz et al., 2012). Our laboratory has developed a model of stress during this critical period, including pre- and peri-pubertal stressors, and established a key role of stress in the development of psychopathology-like behaviors later in life (Toledo-Rodriguez and Sandi, 2011; Márquez et al., 2013; Tzanoulinou et al., 2014b, 2014a; Cordero et al., 2012). Moreover, there was evidence that enhancing CORT during the peri-pubertal period led to increased aggressive behaviors (Veenit et al., 2013). Interestingly, a truncated version of the protocol, keeping only the juvenile pre-puberty stressors, was insufficient in causing the long-term behavioral alterations previously observed (Toledo-Rodriguez and Sandi, 2007; Tzanoulinou et al., 2014b). However, selecting rats for extremes CORT responses during the truncated protocol induced differences in aggressivity at adulthood (Walker et al., 2017). Specifically, with a 'corticosterone-adaptation-stress-test' (CAST) protocol, breeder rats were selected, for several generations, based on their CORT responses to between postnatal days 28 and 30 (P28-30). Rats having a loe, intermediate or high plasma CORT concentration on the third day of stress were classified as 'Low-', 'Inter-' and 'High-'lines respectively. Rats from the High-line showed a lack of habituation to stress in term of CORT secretion while rats from the Inter- and Low-line habituated, with the Low-line showing significantly lower CORT after 3 days of stress (See Annex 1 for more details). The selection responses were specific as well as equally evident for both sexes, implying that a genetic selection was involved (Walker et al., 2017). Progeny of those animals, not exposed to the

CAST protocol and tested only at adulthood, differed in stress response and anxiety-like behaviors in that Low-line animals produced less CORT in response to restraint stress and were less anxious in the elevated plus maze (Walker et al., 2017; Walker and Sandi, 2018). High-line rats exhibited the opposite biobehavioral phenotype and were more aggressive in a resident-intruder test and floated more in a FST (see table 1, for a summary of the biobehavioral differences between the three lines). Finally, submitting rats from the lines to the peri-pubertal stress protocol increased aggressive behaviors of the Low-line but not of the High-line (Walker and Sandi, 2018).

Gene expression analysis in key elements of the HPA axis was performed on brains from the 6th generation of these lines (Walker et al., 2017). There were no differences in *crh, crhr1* and *crhr2* expressions in the different brain regions. However, the High-line had lower levels of *Nr3c1* in the hippocampus while having higher levels of *Fkbp5* in the PVN and *Avpr1b* in the pituitary and a higher *Pomc* expression as compared to Inter-line (Walker et al., 2017). Those findings were suggested to be contradictory to the efficient negative feedback observed in response to restraint stress in the High-line (Walker et al., 2017). Indeed, *Fkbp5* is a potent inhibitor of GR in mammalian cells (Fries et al., 2017) and decreased *Nr3c1* in the hippocampus has been be associated with impaired CORT negative feedback (de Kloet et al., 2005; Tasker and Herman, 2011). Differences in *Avpr1b* and *Pomc* in the pituitary may illustrate an increased sensitivity to AVP and a higher availability of POMC to generate more ACTH in the High-line. Taken together, these gene expression results suggested a compensatory mechanism involved in the negative feedback of the High-line animals in the PVN and possible hippocampal dysregulations. The gene expression profile from the Low-line animals showed low levels of *Gad1* in CeA, high *Avp* but low *Avpr1a* and low *crhr1* in the PVN as well as high *Mrap* in the adrenals. The CeA is comprised mainly of GABAergic cells but has indirect excitatory (disinhibition) control on the HPA axis activity and thus, a reduction in CeA GABAergic activity could inhibit the HPA axis (Ulrich-Lai and Herman, 2009), in line with the low CORT response of the Low-line. *Avp1a* in the PVN controls vascular tone and might have several roles in the central nervous system (Lolait et al., 2007) and increased *Avp* gene expression in the PVN is linked to elevations of basal HPA activity (Zhou et al., 2011). In normal conditions, there are low levels of crhr1 in the PVN (Henckens et al., 2016) but there is evidence that CRH can regulate its own expression in CRH neurons of the PVN (Aguilera and Liu, 2012). Moreover, activation of CRHR1 in the PVN has been related to anxiogenic effects (Fan et al., 2013) and is generally thought to mediate stress-initiation (van Bodegom et al., 2017) fitting the lower anxiety-like behaviors and lower CORT secretion in Low-line animals. Finally, given the role of MRAP in the adrenal gland in the control of adrenal sensitivity to ACTH (Liu et al., 2013), the higher levels of expression of *Mrap* in the low-line might reflect an adaptation to compensate for the low corticosterone levels observed in these animals. MC2R is regulating the adrenal sensitivity to ACTH and is dependent on Mrap co-expression, thus Mrap is critical for production of CORT (Gorrigan et al., 2011).

Behavioral Test	Measurement	Line	Effect	Reference	
Elevated-plus-maze (EPM)	Anxiety-like behaviors (time in open-arms)	**Low**	↓	Walker et al., 2017 Walker et al., 2018	Behavioral profile
		Inter	↔		
		High	↑		
Resident-Intruder (RI)	Aggressive behaviors	**Low**	↓	Walker et al., 2017 Walker et al., 2018	
		Inter	↔		
		High	↑		
Resident-Intruder (RI) After peripubertal stress	Aggressive behaviors	**Low**	↗	Walker et al., 2018	
		Inter	Not reported		
		High	↔		
Forced-swim-test (FST)	Passive floating time	**Low**	↓	Walker et al., 2017 Walker et al., 2018	
		Inter	↓		
		High	↑		
Restraint stress	[CORT] in blood plasma	**Low**	↓	Walker et al., 2017	Endocrine profile
		Inter	↔		
		High	↑		
Basal HPA axis (diurnal peak and trough)	[CORT] in blood plasma	**Low**	↔	Walker et al., 2017	
		Inter	↔		
		High	↔		
Adrenal glands (P100)	Weight (normalized by body-weight)	**Low**	↔	Walker et al., 2017	
		Inter	↔		
		High	↔		

Table 1. Biobehavioral phenotype of the three lines at adulthood (Walker et al., 2017; Walker and Sandi, 2018).

Aims and objectives

Aims of the thesis

Stressful experiences at early ages are an important risk factor for the development of psychopathologies. Moreover, as outlined in the introduction, the stress responsiveness and habituation are two major components of the stress response that might influence the way individuals deal with environmental challenges. Moreover, in order to study the gene x environment interactions in the development of psychopathologies, our lab developed an animal model in order to investigate the long-term biobehavioral phenotypic alterations related to differences in stress responsiveness. Such research showed an important involvement of differential HPA axis responsiveness and the development of biobehavioral outcomes, specifically socio-affective and aggressive behaviors in rats responding to stress with a high HPA activity.

As described in the introduction, the stress system is closely related to key regulators of homeostasis throughout life, namely the autonomic nervous system, the energy metabolism and the aging process. Any of those systems can be dysregulated by stress exposure, which leads to detrimental effects on organism health and survival. The cardiovascular, metabolic and age-related disorders are among the principal sources of mortality and constitute a major economic burden to society. Therefore, providing an animal model, allowing to simultaneously analyzing the effects of stress on those key biological systems, is of major interest. Such a model could offer a better understanding of the underlying mechanisms as well as being a potent tool for the development of pharmacological treatments by including important biobehavioral readouts.

The goal of this thesis is to investigate the effects of stress responsiveness on main biological systems by using a unique animal model in order to understand better the biobehavioral consequences of stress.

Objective 1

The stress system and the autonomic nervous system are closely related and are responsible for the stress response. The heart rate variability extracted from electrocardiogram recordings can be used as a marker of cardiovascular health. Both extremes in HPA axis activity have been related to the development of psychopathologies and individuals showing psychopathologies have been showed to display an autonomic imbalance in cardiovascular regulation. However, there are no models showing a link between HPA axis activity and cardiac autonomic regulation and our first objective was to measure the autonomic cardiac regulation of rats with different stress responsiveness. Therefore, with our model of rats with differential stress responsiveness and radiotelemetric implants we recorded electrocardiogram in order to assess heart rate variability in basal conditions and in response to stress. We also applied pharmacological treatments to block the sympathetic and parasympathetic influences on the heart. The findings of this study are presented in chapter 1.

Objective 2

Individuals with different stress adaptation have different behavioral responses to threat and may present differences in susceptibility to stress. These differences might be related to differences in energy mechanisms during stress response. Indeed, in order to mount a proper stress response an organism as to properly distribute the energy required to adapt to the situation. Thus, the interrelation between stress responsiveness and energy metabolism are of major interest and our second objective was to determine an energetic and metabolic profile of rats with different stress responsiveness. We applied indirect calorimetry recording on rats from the different lines, in order to measure energy expenditure and the main source of fuel used be the organism. The findings of this study are presented in chapter 2.

Objective 3

Responses to stress differ on the context in which it is delivered and not all the stressors have the same valence in stress effects induced. For example, one of the stronger stressor in social species derives from social situations (aggression, social defeat and subordination). As previously mentioned, individuals might have different susceptibilities to stress outcomes depending on their response to stress and socio-emotional behaviors. Our third objective was therefore to analyze social behaviors of rats from the different lines when housed together (in mixed-line dyads) and to establish the long-term outcome of these housing situations on general and central metabolism. After evaluating, social behaviors during their first encounter, rats from the different lines were left undisturbed for one month and then, we used indirect calorimetry and mitochondrial respirometry techniques to assess metabolic states. We compared the difference in energy expenditure and energy fuel used between rats from each line but housed in difference mixed-line dyads in order to assess whether pairing with high stress responsive animals could have long-term metabolic influences. The findings of this study are presented in chapter 2.

Objective 4

Throughout life, stress has fundamental influences on organisms, by affecting cardiac (Chapter 1) and metabolic (Chapter 2) outputs, as well as affecting aging processes. Aging and stress are related in a sense that stress accelerates aging and aging makes the organism vulnerable to stress. Our fourth objective was to investigate the stability of the biobehavioral phenotype of the rats from the different lines throughout life. We applied a number of behavioral tests between mid-life (12 months) and early-aging (18 months) and we exposed the rats to stress at 16 months of age. We were able to describe the evolution of anxiety-like behaviors and coping strategies as well as the HPA axis activity during aging. The findings of this study are presented in chapter 3.

Objective 5

Aging is also related to cognitive decline by affecting brain regions predominantly important in learning and memory. Stress has been showed to increase hippocampal damage and to decrease cognitive abilities. It was suggested that excess exposure to glucocorticoids increased toxicity in the brain, leading to neuronal damage subsequently affecting cognition throughout aging. Therefore, our fifth objective was to study cognitive abilities of early-aged rats from the three lines. We used a Morris water-maze to study learning and memory retention two weeks after training as well as reversal learning capacities. We also determine the swimming strategies used to escape the maze, reflecting more in details the learning strategies of the rats. The findings of this study are presented in chapter 3.

Chapter 1

Low vagal modulation of heart rate in rats with low or high corticosterone responsiveness to stress

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Abstract

Cardiovascular disease (CVD), a leading cause of morbidity and mortality worldwide, is highly associated with or exacerbated by stress exposure. Growing evidence suggests that sympathovagal imbalance such as, reduced vagal modulation of resting heart rate (HR) plays a key role in the development of CVD. While individual differences in stress responsiveness and stress adaptation, either hyper- or hypo-regulated, have been highly linked with sympathovagal imbalance, the links between dysregulated stress adaptation and impaired vagal modulation of resting HR remain poorly understood. Here, we investigated the autonomic nervous system modulation of HR in lines of rats that differ largely in their responsiveness to stress. Electrocardiographic recordings were performed on rats exhibiting low, intermediate or high stress responsiveness (classified based on their plasma glucocorticoid responses), at rest, during stressful stimuli and under autonomic pharmacological manipulations. Rats with intermediate reactivity to stress displayed a reduced resting HR and a higher parasympathetic (vagal) modulation compared to rats with low or high HPA-axis activity in response to stress. Additionally, those differences in basal conditions were associated with increased HR and lower vagal tone in response to stress. Furthermore, muscarinic-receptor pharmacological antagonism induced a higher vagal suppression in intermediate animals. Collectively, our data point to several key physiological changes that contribute to impaired autonomic modulation of cardiac activity because of altered stress adaptations. We provide for the first time an animal model in which extreme HPA-axis responsiveness to stress in term of hypo- and hyper-secretion of corticosterone is associated with impaired autonomic modulation of cardiac activity.

Keywords

Stress responsiveness/adaptation, heart rate variability, vagal, parasympathetic tone, autonomic nervous system

Introduction

Cardiovascular diseases (CVDs) represent the leading cause of disability and death worldwide (Mathers and Loncar, 2006). Of particular concern is the observation that cardiovascular functions are severely influenced or worsened by stress (Kivimäki and Steptoe, 2017), and affective disorders such as depression, post-traumatic stress disorders (PTSD) and anxiety are highly comorbid with CVDs (Cohen et al., 2015; Coughlin, 2011). Several studies have reported that individuals experiencing depressive illness show cardiovascular complications including autonomic nervous system (ANS) imbalance, reduced heart rate variability (HRV), and altered baroreflex sensitivity (Lett et al., 2004; Carnevali et al., 2017). Similarly, CVD predisposes individuals to develop depression and other mental illnesses (Rudisch and Nemeroff, 2003).

The physiological response to stress involves rapid activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenocortical (HPA) axis. In the early onset of the stress response, SNS activity induces release of norepinephrine in the sinoatrial node leading to increase in heart rate (HR). The HPA-axis response, constituted of a neuroendocrine cascade, is slower than the SNS (Ulrich-Lai and Herman, 2009). Glucocorticoids, mainly corticosterone (CORT) in rodents, the final product of the activated HPA-axis released from the adrenal glands, peaks tens of minutes after stress exposure (Herman et al., 2016). The SNS and HPA-axis systems have complementary actions in the organism from energy mobilization to maintenance of blood pressure during stress (Ulrich-Lai and Herman, 2009).

Determination of vagal modulation of HR in individuals with stress-related diseases has become an important clinical tool to determine susceptibility to develop CVDs (Billman et al., 2015) or psychopathologies (Flórez et al., 2017). Many studies have recently validated the use of HRV as a diagnostic tool of certain psychological disorders (Chang, 2015; Hage et al., 2017a, 2017b). For example, electrocardiographic (ECG) recordings reveal that reduced vagal tone could serve as a key tool to distinguish bipolar patients from depressed individuals (Hage et al., 2017a). In addition, the spectral component of HRV has been found to correlate with various psychopathological conditions including conduct disorder (Beauchaine et al., 2000; Koenig et al., 2016; Beauchaine et al., 2007), depression and psychopathy (Hansen et al., 2007; Beauchaine and Thayer, 2015). The vagal index of HRV was also shown to be effective in detecting subjects with high psychopathic scores among a representative sample of inmates (Flórez et al., 2017). Indeed, elevated resting HR and decreased parasympathetic nervous system (PNS) regulation of cardiac activity have been found in people with PTSD (Meyer et al., 2016). In contrast, increased vagal modulation and high resting vagal tone have been found in healthy individuals (Porges, 2007; Thayer et al., 2009) and in association with resilience to stress (Smeets, 2010; Souza et al., 2013).

Recently, rodent studies have identified critical links between effectors of vagal modulation and stress physiology, cardiac functions and behavioral reactivity (Carnevali and Sgoifo, 2014; Sgoifo et al., 2015). Under resting conditions, a decrease in vagal modulation of HR has been related to vulnerabilities to arrhythmias and deficits in cardiac functions (Esler, 1992; Schwartz et al., 1988; Volders, 2010). This was partly due to an inability in counteracting the stress-induced sympathetic stimulation and consequently leading to long-term impairment in cardiovascular functions (Sloan et al., 1994; Lucini et al., 2005; Sabbah et al., 2011). Exposure to repeated stressors, such as restraint or footshocks, was shown to increase resting vagal tone for several days, which was interpreted as an adaptive response to overcome stress-induced hyperactivity of the SNS (Trombini et al., 2012; Carnevali et al., 2011). However, this adaptation phenomenon might in time fail and lead to vagal withdrawal and sympathetic dominance (Björkqvist, 2001; Sgoifo et al., 2014) and, eventually, to related cardiovascular disorders. Moreover, rats with increased anxiety- and depression-like behaviors have been shown to have lower vagal modulation of resting HR (Sévoz-Couche et al., 2013; Carnevali et al., 2014), higher basal HR and reduced HRV indicating an imbalance between SNS and PNS

regulation of HR (Grippo et al., 2006, 2004; Wood et al., 2012). A rat model of PTSD induced by predator exposure, displayed cardiovascular abnormalities (Zoladz et al., 2008; Zoladz and Diamond, 2016) along with lower basal corticosterone levels (Zoladz et al., 2012). Another rodent model of PTSD demonstrated that "maladapted animals" exhibit diminished vagal tone and high plasma CORT during stress exposure (Cohen et al., 2003). Collectively, the above studies suggest the existence of a close interaction between stress-related disorders and alterations of the vagal modulation of cardiovascular functions. However, to our knowledge, there is currently no animal model that simultaneously involve low or high corticosterone responsiveness in relation to impaired parasympathetic activity (e.g. resting vagal tone). Here, using rats selected for their differential corticosterone adaptation to repeated stress exposure, we have investigated the link between glucocorticoid stress responsiveness and the autonomic control of HR.

Our laboratory has performed selective breeding of rats differing in stress responsiveness and habituation to stress during juvenile period (Walker et al., 2017). Using a 'corticosterone-adaptation-stress-test' (CAST) protocol (Figure 1A), breeder rats were selected, for several generations, based on their CORT responses to stress during the juvenile and pre-puberty periods (Figure 1B and 1C). Following exposure to the CAST protocol, CORT responses were measured and rats were classified as high, intermediate and low (called 'High', 'Inter' and 'Low' -lines respectively). Rats from the High-line showed a lack of habituation to stress in term of CORT secretion while rats from the Inter- and Low-line habituated, with Low-animals showing significantly lower CORT at P30 (Figure 1C). Progeny of High-line animals were tested at adulthood and showed increased secretion of CORT in reaction to restraint stress, increased aggressive behaviors in a resident-intruder setting, increased anxiety-like behaviors in the elevated-plus-maze (EPM) and used a passive floating strategy during a forced swim test (Walker et al., 2017).

Based on previous observations that anxiety-like behaviors, passive coping style and aggressive behaviors are associated with autonomic dysregulations (Carnevali and Sgoifo, 2014), we hypothesized that rats from the High-line will exhibit increased stress reactivity and autonomic imbalance, such as reduced vagal modulation. On the other hand, based on reports on PTSD subjects linking blunted HPA-axis, autonomic imbalance and decreased vagal regulation of heart rate (Zoladz et al., 2008; Zoladz and Diamond, 2016b), we hypothesized that animals showing low corticosterone responsiveness will exhibit high stress reactivity and decreased HRV and parasympathetic nervous system influence. To test these hypotheses, we performed a detailed characterization of the autonomic neural modulation of the heart rate of three groups of rats that differed in CORT responses to stress, and assessed the autonomic influences of heart rate via time- and frequency-domain analyses of HRV at rest and in response to different stress situations. In order, to understand the mechanisms associated with the differential cardiovascular responses, we assessed the relative contribution of sympathetic and parasympathetic control over heart rate by means of pharmacological manipulations.

We show that rats exhibiting low or high CORT responsiveness to stress differ in autonomic regulation of heart rate in comparison to animals having an intermediate stress phenotype. Both Low- and High-line rats have higher resting heart rate and lower basal vagal tone. When tested in different stressful situations, rats from the intermediate line showed lower maximal heart rate and higher HRV response than Low- and High-line rats. Finally, pharmacological manipulations confirmed a relative higher vagal modulation in Inter-line rats compared to both Low- and High-line animals with no difference in the sympathetic regulation of heart rate. To our knowledge, this is the first study that presents an animal model in which a clear 'U-shape' relationship between stress responsiveness and the parasympathetic regulation of heart rate is delineated.

Material and Methods

i. Animals

Experiments were performed using male Wistar-Han offspring from the breeding of three lines of differentially stress reactive rats (Walker et al., 2017) developed in our animal facility (EPFL, Lausanne, Switzerland) as described below (See *Protocol for selective breeding* paragraph). The experimental rats were not exposed to the CAST procedure and were briefly handled on P28-30 and then left undisturbed, except for weekly cage changes, until experimental procedures at adulthood (P90). Male Wistar-Han rats were used as instigators or intruders in the social-instigation and in the resident-intruder (RI) tests and were purchased from a commercial breeder (Charles River, L'Arbresles, France). These interacting animals were used only once. Animals were housed in a 12:12 h light-dark cycle (lights ON at 07:00) in a temperature- and humidity-controlled environment (22 ± 1 °C; 55% humidity ± 5%). Rats had *ad-libitum* access to food and water unless otherwise stated. All procedures were conducted in accordance with the Swiss National Institutional Guidelines on Animal Experimentation and were approved by the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

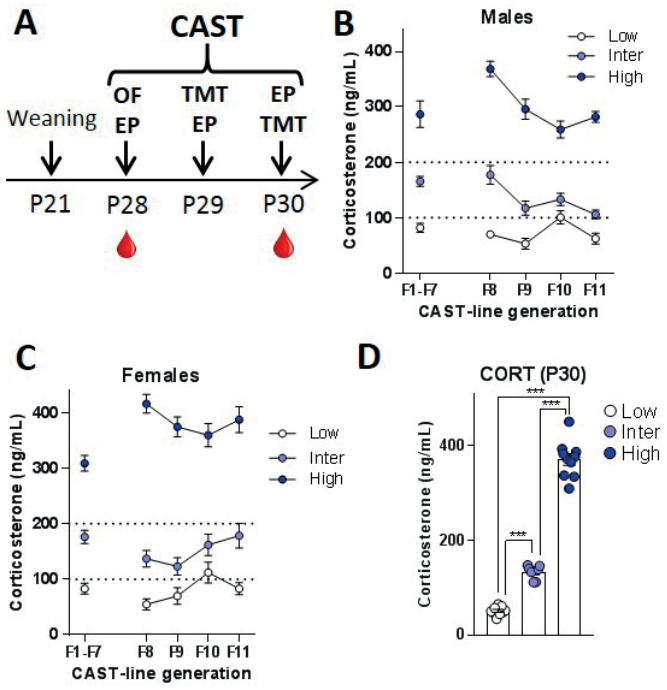

Figure 1: The corticosterone-adaptation-stress-test (CAST) and selection of 'Low', 'Inter' and 'High'-line animals according to CORT responsiveness at P30. A, Experimental timeline of the CAST protocol at per-pubertal juvenile ages. After weaning (P21), rats were submitted to 3 days of stress (P28-30) with tail-blood sampled at P28 and P30. After assessment of basal activity with and open-field (OF), stressors included EP (Elevated-platforms) and TMT (predator scent) for 25 min each. **B**, **C,** Representations of plasma CORT from eleven generations of the lines in males (**B**) and female breeders (**C**). The selection of rats from lines was based on CORT concentration at P30 and classified as `Low' ([CORT] < 100ng/ml), `Inter' ([CORT] between 100-200 ng/ml) and `High'-line ([CORT] > 200ng/ml). **D,** Averaged CORT response at P30 of the breeding pairs selected to generate the 12th generation of CAST animals.

ii. Protocol for selective breeding

The breeding procedure of selected rats was performed as described previously (Walker et al., 2017). Breeders were selected following a CAST procedure that involved exposure to different stressors over 3 days-period during the juvenile period (P28-P30; Figure 1A). Rats were exposed to fear-inducing situations following a protocol previously developed in our lab to study the impact of peripubertal stress in neurobehavioral development (Tzanoulinou et al., 2014). Notably, as opposed to the peripubertal stress protocol that involves exposing animals to the stressors for7 days, the CAST procedure only reproduces its first 3 days. On P28, prior to any stress experience, animals were exposed to a novel open field stress (5 min) followed by exposure to an elevated-platform (EP) for 25 min. On P29,

rats were put in a new environment with synthetic predator scent ('fox odor': trimethylthiazoline, TMT, for 25 min) followed by exposure to the EP (25 min). On P30, the same stressors as on P29 were used but in a reverse order. Blood was sampled from tail incision immediately following exposure to stressors on P28 and P30 and after 30 min of recovery.

As can be seen in Figure 1B and 1C, male and female rats from the three lines differed in CORT responses at P30. The CAST selection process was applied to 11 generations in the lab in order to produce experimental animals. Figure 1D illustrates the plasma CORT concentration of breeder rats from the three lines, issued from the 12th generation of CAST selection, in response to stress exposure at P30.

iii. Radiotelemetric transmitters for ECG recordings

Different experiments, with implanted radiotelemetric transmitters for recording electrocardiogram (ECG), core body temperature and locomotor activity, were performed as described below.

Surgery: radiotransmitter implantation

Following a procedure adapted from previously validated techniques (Sgoifo et al., 1996; Adeyemi et al., 2009), rats were implanted with radiotelemetric transmitters (TA11CTA-F40, Data Sciences International (DSI), St. Paul, MN, USA). Briefly, rats were anaesthetized by inhalation of Isoflurane, the transmitter body was inserted in the abdominal cavity and sutured to the abdominal wall for stability, one electrode was sutured to the xiphoid process and the other electrode was sutured between the sternomastoid and sternohyoid muscles (Adeyemi et al., 2009). Rats were individually housed after surgery and given 1 week to recover before baseline ECG recording.

Radiotelemetric data recording

The radiotelemetric transmitters allowed recording of electrocardiogram (ECG, 1000Hz), body temperature (T°, 256Hz) and locomotor activity (LOC expressed in counts/min, 256Hz). In order to record data, implants had to be in close proximity to specific receivers (RPC-1, DSI) and recorded by specific software (Dataquest ART-platinum, DSI). During baseline recordings, receivers were individually placed below the home-cages of the subjects. During behavioral testing, cages or apparatus were placed on top of similar receivers in the experimental rooms.

Baseline recording

During baseline recording, ECG, T° and LOC were measured for 4 minutes every hour and data were averaged over the 12 h-light and 12 h-dark phases for statistical analyses.

Quantification of HRV

HR as well as time- and frequency-domain parameters of HRV were quantified using LabChart 8.0 software (AD Instruments, Sydney, Australia). ECG signals were visually inspected to ensure that all R-waves were properly detected. In the time-domain, we obtained the square root of the mean squared differences of successive RR intervals (RMSSD, ms), which quantifies short-term, high-frequency variations of RR and therefore estimates the activity of the parasympathetic nervous system (Stein et al., 1994). For spectral (frequency-domain) analysis of HRV, the power spectrum was computed with a fast Fourier transform-based method. We considered the total power of the spectrum (ms^2), which reflects all the cyclic components responsible for variability, as well as the power of the low frequency (LF power: 0.2-0.75 Hz) and high frequency (HF power: 0.75-2.5 Hz) bands in absolute values (ms^2). The power of LF band is a non-specific index as it contains contributions of both the sympathetic and parasympathetic influences (Eckberg, 1997; Reyes del Paso et al., 2013). The power of HF band is due to the activity of the parasympathetic nervous system and includes respiration-linked oscillations of HR (Berntson et al., 1997).

The relevance of the low over high frequency ratio (LF/HF) is a matter of debate (Reyes del Paso et al., 2013; Hage et al., 2017b). It has been used as a synthetic measure of sympathovagal balance (Task Force, 1996) but it has also been suggested to be an indicator of baroreflex sensitivity instead of sympathetic innervation (La Rovere et al., 1998; Moak et al., 2007). For this reason, LF/HF ratio were reported in supplementary methods, but no conclusions concerning the sympathovagal balance or baroreflex sensitivity will be drawn.

iv. Radiotelemetric experiments

The first experiment aimed at comparing the autonomic reaction to social (social instigation and resident-intruder) and non-social stressors (reaction to novelty and restraint stress). The second experiment used specific autonomic pharmacological modulators to assess the autonomic influences on cardiac activity of the three lines.

Behavioral and psychosocial challenges

The experimental timeline is represented in supplementary Figure 1A. Rats were bred from the 8th generation of the lines submitted to the CAST protocol. Reaction to novelty and anxiety-like behaviors were assessed at adulthood (on P90 and P93 respectively). Rats were then separated into three cohorts of 10 animals for ECG experiments. After implantation of ECG implants, 1 week of recovery and 1 week of baseline recording, rats were tested on four behavioral tests: light/dark-box, social instigation, resident-intruder and restraint stress (see below for experimental details on these challenges). For each of the behavioral test, ECG of the rats were recorded during a habituation period of 20-30 min (c.f., specific experiments), during the test and during a recovery period.

Pharmacological autonomic treatments

In order to assess the relative contribution of sympathetic and parasympathetic components on HR modulation, beta-adrenoceptor and muscarinic receptor antagonists were injected respectively.

On the first day, a subcutaneous injection of saline solution was applied as a control manipulation. Then, after a 24 h-washout period, sympathetic blockade was achieved with injection of atenolol (2 mg/kg, SC; Sigma, St Louis, MO, USA), a β-1 adrenergic receptor antagonist. Rats were given 24 h before the third injection and the muscarinic receptor antagonist, methylscopolamine bromide (0.05 mg/kg, SC; Sigma, St Louis, MO, USA), was applied in order to block the vagal component of the heart rate. Drug doses were selected based on previous studies (Carnevali et al., 2013, 2014).

The ECG recordings were started 30 min prior injection as 'habituation period' and were performed until 50 min post-injections.

v. Corticosterone response to Novelty

We used a novelty test to measure plasma corticosterone reactivity, as previously described (Veenit et al., 2013). Immediately after 25 min exposure to a novel environment (a circular plastic container; Ø = 40 cm, height = 50 cm), blood samples were obtained by tail-nick and rats were returned to their home-cage. Animals from the same home-cage were simultaneously tested in adjacent containers. The containers were cleaned with 5% ethanol and dried properly before placing the animals.

vi. Corticosterone analysis

Blood samples were collected into heparin-coated capillary tubes (Sarsted, Switzerland), kept on ice until centrifugation (4 min, 4 °C and 10000 rpm), and stored at −20 ° C. Plasma corticosterone levels were measured using a highly sensitive ELISA kit (ADI-900-097, Enzo Life Sciences, Switzerland). Blood plasma samples were diluted 20

times and the ELISA was performed according to manufacturer's instructions. Concentration values of CORT were calculated using a 4-parameter logistic fit (www.myassays.com).

vii. Light-Dark box (LD box)

In order to assess cardiovascular changes during exposure to the natural aversion of rats against bright environments we used a LD box apparatus. The maze (30 x 60 x 40 cm) consisted of 2 connected equal-sized chambers, one covered and dark (approx. 0 lux) and one open and bright (approx. 100 lux). During the test, the rat was placed in the open chamber facing the wall and allowed to explore for 10 min. Continuous ECG, T° and LOC recordings were collected under baseline conditions (20 min, prior to the test), during the LD-box (10 min) and throughout the recovery period (20 min).

viii. Social instigation

Social instigation was used to allow visual, olfactory and auditory interactions with a conspecific while preventing physical interactions. To do so, a holed translucent separator was added in the home-cage of and the subjects and an instigator was introduced on the other side of the separator for 30 min. Continuous ECG, T° and LOC recordings were collected under baseline conditions (30 min, prior to the test), during social-instigation (30 min) and throughout the recovery period (30 min).

ix. Resident-intruder (RI)

The test was performed during the beginning of the dark phase (between 19:00 and 22:00). After one week of social isolation, resident animals remained in their homecage, while an unfamiliar and smaller intruder (approx. 10% lighter) was inserted for 30 min in the cage (without separator). Continuous ECG, T° and LOC recordings were collected under baseline conditions (30 min, prior to RI), during the RI encounter (30 min) and throughout the recovery period (30 min).

x. Restraint stress

Each animal was introduced for 15 min into a restrainer and returned to their homecages after the test. Continuous ECG, T° and LOC recordings were collected under baseline conditions (30 min, prior to restraint), during the restraint test (15 min) and throughout the recovery period (30 min).

xi. Statistics

Data are presented as mean ± standard error of the mean (SEM) in all tables and figures. Statistics and graphs were performed using the GraphPad Prism software (version 7). Prior statistical analysis, data distribution were checked for normality and outliers were removed using the robust regression and outlier removal (ROUT) method (Motulsky and Brown, 2006). The number of subjects used and the number of rats excluded by ROUT method are reported in the corresponding figure captions. Two-way ANOVA with repeated measures with line as between-subject factor (three levels: Low-, Inter- and High-lines) and with time as within-subject factor (number of levels depend on individual cases) was applied when data followed a repeated design. All other variables were analyzed using One-way ANOVA with the line as between-subject factor. Posthoc analysis was performed with a Fischer's LSD test or multiple comparisons. A previous report illustrated a HR difference specific to the onset of the stress response with restraint stress (Carnevali and Sgoifo, 2014). Thus, data from HR and RMSSD during the immediate response to stress (first block after the onset of stress) following the different behavioral tests were analyzed with Student's t-tests. Linear regression was applied in order to determine correlation between CORT and RMSSD values. The area-under-the-curve (AUC) was calculated as the change in HR and RMSSD by taking the habituation values as "zero". Statistical significance was set for $p < 0.05$.

Results

i. Differences in resting HR and vagal tone

Difference in CORT responsiveness at adulthood between lines

First, we verified that rats from the different lines differed in their CORT responses to stress at adulthood. Indeed, plasma CORT levels in response to novelty (i.e., bucket exposure during 25 min) were significantly different between the lines (Figure 2A; $F_{2,55} = 23.25$, $p < 0.001$). As expected, High-line rats displayed higher CORT response than animals from the Low- ($t = 6.71$, $p < 0.001$) and Inter-lines ($t = 4.23$, $p < 0.001$). In addition, rats from the Inter-line had significantly higher CORT response to novelty than Low-line rats ($t = 2.17$, $p = 0.034$).

Lower resting HR and higher vagal tone in the Inter-line

Basal HR was averaged separately for the 12 h-light and 12 h-dark phases (Figure 2B). There was a main effect of day period on HR ($F_{3,138} = 164.9$, $p < 0.001$) with higher HR during the active, dark phase. There was a line effect ($F_{2,66} = 7.49$, $p = 0.002$) with Inter-line rats having lower basal HR than both Low- ($t_{66} = 5.1$, $p = 0.002$) and High-lines ($t_{66} = 4.96$, $p = 0.003$). There was no interaction between light/dark-phases and lines ($p = 0.32$).

Resting parasympathetic activity was determined with HRV parameters, RMSSD and HF-power (Figure 2C & 2D). First, RMSSD values (Figure 2C) fluctuated greatly according to the light/dark-phases ($F_{3,198} = 72.64$, $p < 0.001$) with higher RMSSD during the non-active light-phases. Moreover, there was a highly significant line effect on RMSSD ($F_{2,66} = 10.94$, $p < 0.001$) with higher RMSSD values in Inter-line rats than Low- ($t_{66} = 5.1$, $p = 0.002$) and High-line animals ($t_{66} = 6.44$, $p < 0.0001$). There was no difference between Low- and High-line rats in RMSSD values during baseline recording ($t_{66} = 1.4$, $p = 0.586$).

In parallel to RMSSD, the HF-power values (Figure 2D) were higher during light-phases ($F_{3,198} = 42.59$, $p < 0.001$). There was also a line effect on the amplitude of HF ($F_{2,66} = 10.1$, $p < 0.001$) with Inter-line rats having higher HF than Low- ($t_{66} = 5.17$, $p = 0.0015$) and High-line animals ($t_{66} = 6.07$, $p < 0.001$). There was no difference between the Low- and High-lines in power of the HF band during baseline recording ($t_{66} = 0.919$, $p = 0.793$).

Daily rhythms parameters of HR, HRV, T° and LOC measured during baseline are summarized in Table 1. Inter-line rats exhibited higher total power than Low- ($p < 0.008$) and High-lines ($p < 0.027$) during light- and dark-phases. During the dark-phase, there was a tendency for a higher LF power in rats from the Inter-line compared to Low- ($p = 0.05$) and High-lines ($p = 0.033$). There were no differences in the LF/HF ratio, T° and LOC between the lines.

Negative linear correlation between CORT response to novelty and resting vagal tone

As illustrated in Figure 2E, when considering data from all animals, there was a general negative correlation between the CORT response to novelty stress at adulthood and the resting RMSSD value ($r = 0.343$, $p = 0.003$). The High-line exhibited a comparable negative correlation ($r = 0.545$, $p = 0.009$) whereas the Low and Interlines did not show a correlation between CORT and RMSSD ($p = 0.48$ and $p = 0.75$ respectively).

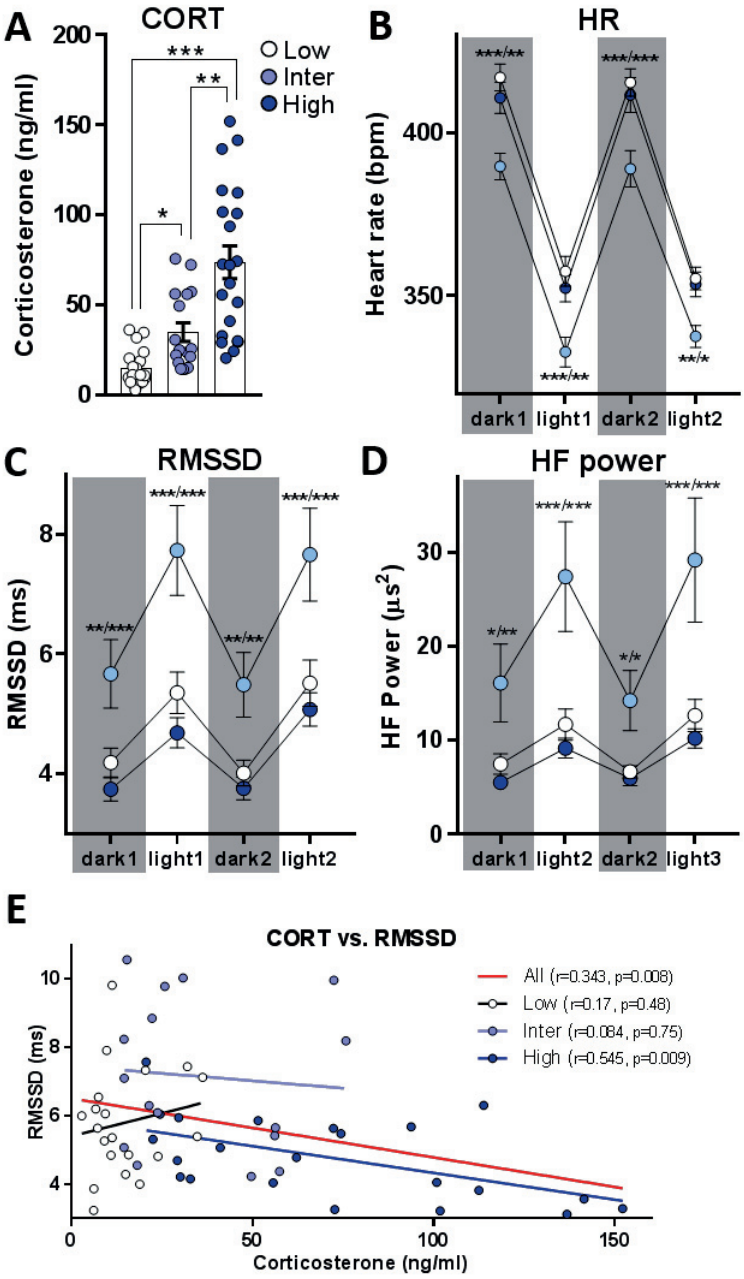

Figure 2: Rats with high and low CORT responsiveness had higher resting HR and lower vagal tone. **A,** At adulthood, after 25 min of novelty stress, rats from the lines displayed different CORT responses. High-line rats had higher CORT response and Low-line animals had a blunted CORT response. **B,** HR was measured and averaged over two 12h-dark and two 12h-light phases. Inter-line animals had lower basal HR ($p = 0.005$) than both Low- and High-line rats. **C,D,** Differences in vagal tone are shown by higher RMSSD values (**C,** $p = 0.014$) and higher HF power (**D,** $p=0.002$) in Inter-line rats compared to Low- and High-lines. **E,** Negative linear correlation for the High-line animals between corticosterone levels in response to a novelty-stress and basal RMSSD. Values are reported as means ± SEM. ECG data were obtained by averaging 4 min segments acquired every hour. Figure **A**: Low (n = 20), Inter (n = 17) and High (n = 21). For figures **B, C, D** and **E**: Low (n = 23), Inter (n = 16) and High (n = 26). In **B, C** and **D,** asterisks indicate comparisons between Inter- and Low-/High-line rats. $^{\#}p<0.1$ (trend), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

parameters	Light/dark phase	Lines			Statistics (Fischer's LSD p-values)		
		Low (n=22)	Inter (n=13)	High (n=21)	Low vs. Inter	High vs. Inter	Low vs. High
Heart-Rate (bpm)	Dark	414.7 ± 3.1	392.4 ± 4.1	411.4 ± 5.2	**.001**	**.006**	.56
	Light	356 ± 3.9	336.1 ± 4.4	353 ± 4.3	**.003**	**.012**	.059
RMSSD (ms)	Dark	4.2 ± 0.2	5.3 ± 0.7	4 ± 0.2	**.035**	.014	.65
	Light	5.6 ± 0.4	7.4 ± 0.9	5.1 ± 0.3	**.019**	**.003**	.44
Total Power (ms^2)	Dark	69.2 ± 4.3	97.2 ± 8.6	67.6 ± 5	**.002**	**.001**	.83
	Light	99.5 ± 6.5	142.7 ± 15	107 ± 10.9	**.008**	**.027**	.58
HF Power (ms^2)	Dark	7.2 ± 0.8	14.1 ± 4.5	6.4 ± 0.7	**.021**	**.011**	.75
	Light	13.5 ± 1.8	26.7 ± 7.8	10.8 ± 1.3	**.009**	**.002**	.53
LF Power (ms^2)	Dark	7.9 ± 0.9	8.7 ± 0.7	6.1 ± 0.6	**.050**	**.033**	.086
	Light	13.4 ± 1.5	13.8 ± 1.1	10.6 ± 1.3	.85	.14	.135
LF/HF	Dark	1.26 ± 0.09	1.11 ± 0.13	1.16 ± 0.08	.3	.73	.43
	Light	1.24 ± 0.1	1.01 ± 0.17	1.2 ± 0.09	.18	.27	.79
Temperature (°C)	Dark	37.9 ± 0.06	37.9 ± 0.05	37.9 ± 0.045	.99	.99	.99
	Light	37.3 ± 0.07	37.3 ± 0.09	37.2 ± 0.04	.99	.32	.25
Locomotion (count)	Dark	3.63 ± 0.33	4.04 ± 0.41	3.69 ± 0.19	.38	.45	.88
	Light	1.66 ± 0.16	1.26 ± 0.11	1.33 ± 0.15	.09	.76	.11

Table 1: Daily rhythms of radiotelemetric and HRV parameters during basal recording. For the 12 hours of two dark- and two light-phases, values of 4min segments recorded every hour, were averaged and are reported as 'means + SEM' for Low- (n=23), Inter- (n=16) and High- (n=26) line rats. Abbreviations: HRV = heart rate variability; RMSSD = square root of the mean squared differences of successive RR intervals; HF = high frequency; LF = low frequency; Statistical comparisons are represented as p-values of posthoc LSD's Fischer comparisons.

ii. Differences in HR and RMSSD responses to behavioral challenges

Increased anxiety-like behaviors in the High-line

To determine anxiety-like behaviors, rats from the different lines were tested in an EPM prior to the telemetry surgery and in a LD box test after recovery from surgery (Supplementary Figure 1). During both timepoints (before and after surgery), rats from the High-line displayed increased anxiety-like behaviors than Low- and Inter-lines (Supplementary Figure 1A and 1B).

No differences in locomotion in reaction to the different tests

As reported in supplementary tables (1, 2, 3, and 4), there were no differences in LOC between rats from the three lines during behavioral testing. The T° of the animals differed only during the habituation period before LD-box (supplementary table 1) and before restraint stress (supplementary table 4) with Inter-line rats having higher T° than Low- and High-lines. However, there were no differences in T° during behavioral testing and recovery periods.

Low- and High-lines showed higher maximal HR and lower RMSSD in response to stress

The HR and HRV responses to the different behavioral and psychosocial challenges are reported in Figure 3 as well as in supplementary tables 1-4. Figure 3 illustrates the habituation values as well as the immediate and late responses to the different tests. The supplementary tables present HR and HRV parameters during habituation, behavioral challenges and during recovery phases (in blocks of 15 minutes).

As represented in Figure 3, there was a significant effect of the challenges on HR and RMSSD values. Indeed, in response to the different stressors rats from all the lines exhibited an immediate increase in HR (Figure 3B, D, F, H) associated with a decrease in RMSSD values (Figure 3C, E, G, I).

During the LD box test (Figure 3B), there was no line effect on HR values ($F_{2,23}$ = 2.32, p = 0.121). There was an interaction between time-bins and the lines ($F_{4,46}$ = 3.21, p = 0.021) with Inter-line rats having lower maximal HR (Figure 3B, left panel), at onset of behavioral test, than Low- (t_{69} = 2.05, p = 0.044) and High-lines (t_{69} = 2.24, p = 0.028). The relative increase in HR ($F_{2,23}$ = 3.12, p = 0.063) illustrated by the AUC (figure 3B, right panel) showed that Low-line rats had a significantly higher HR response than Inter- (t_{23} = 2.21, p = 0.037) and High-lines (t_{69} = 1.93, p = 0.53). The RMSSD values during LD box recording (Figure 3C, left panel) did not show any difference between the lines ($F_{2,23}$ = 1.32, p = 0.286) or interaction between time-bins and lines ($F_{4,46}$ = 1.29, p = 0.288). Importantly, when looking specifically at the immediate response to stress, during the first 180 s following LD box, there was a line effect ($F_{2,23}$ = 2.14, p = 0.046) with Inter-line rats having higher RMSSD than Low- (t_{23} = 2.53, p = 0.019) and High-line animals (t_{23} = 2.3, p = 0.031). There was no line effect on the AUC of RMSSD changes ($F_{2,23}$ = 0.54, p = 0.591).

Analyses of HR and HRV changes during social instigation are represented in Figure 3D, 3E. There was a line effect on HR ($F_{2,25}$ = 4.85, p = 0.017) as well as an interaction between time-bins and lines ($F_{4,50}$ = 2.58, p = 0.049). The interaction was illustrated (Figure 3D, left panel) by a higher HR during habituation for High-line rats than Low- (p = 0.018) and Inter-lines (p = 0.012). In response to the introduction of an unfamiliar rat in the home-cage, Inter-line rats had a lower maximal HR than Low- (t_{75} = 2.63, p = 0.01) and High-line rats (t_{75} = 3.41, p = 0.001). High-line rats exhibited higher HR during late response to instigation than Inter-line rats (t_{75} = 2.39, p = 0.019). The relative change in HR, shown by AUC values (Figure 3D, right panel), did not differ between the lines ($F_{2,24}$ = 0.168, p = 0.847). The RMSSD values during social instigation (Figure 3E, left panel) did not show a line effect ($F_{2,25}$ = 1.21, p = 0.316) or an interaction between time-bins and lines ($F_{4,50}$ = 1.74, p = 0.156). When looking specifically at the immediate 5 first min in response to stress there was no line effect ($F_{2,25}$ = 2.25, p = 0.126) but t-tests showed that Inter-line rats had significantly higher RMSSD than High-line rats (t_{25} = 2.1, p = 0.046) but not than Low-line animals (t_{25} =1.59, p = 0.124). The AUC of RMSSD did not show differences between the lines ($F_{2,25}$ = 1.95, p = 0.164).

Analysis of HR and RMSSD changes during RI (Figure 3F, 3G) led to comparable differences. There was a line effect on HR values ($F_{2,24}$ = 4.22, p = 0.009) as well as a significant interaction between time-bins and lines ($F_{4,48}$ = 2.74, p = 0.039). High-line rats had higher HR during habituation than Low- (p = 0.050) and Inter-line rats (p = 0.005; Figure 3F, left panel). Inter-line rats had lower maximal HR than Low- (t_{72} = 3.41, p = 0.001) and High-line rats (t_{72} = 2.59, p = 0.012). Inter-line animals had lower HR during the entire RI session than Low- (t_{72} = 2.64, p = 0.01) and High-line rats (t_{72} = 2.33, p = 0.023). The relative change in HR, illustrated by the AUC values (Figure 3F, right panel), differed between the lines ($F_{2,24}$ = 3.99, p = 0.032) and Low-line rats had a higher increase in HR than the High-line animals (t_{24} = 2.74, p = 0.011) and a tendency when compared to Inter-line rats (t_{24} = 1.74, p = 0.094). The RMSSD values during RI (Figure 3G, left panel) did not show a line effect ($F_{2,24}$ = 1.21, p = 0.316). There was a tendency for an interaction between time-bins and lines ($F_{4,48}$ = 2.13, p = 0.091). When looking specifically at the early response to stress (5 first min of RI) there were differences according to the lines of the rats ($F_{2,24}$ = 5.39, p = 0.012). It was found that Inter-line rats had higher RMSSD than Low-line rats (t_{24} = 2.264, p = 0.027) and there was a tendency for a difference between the Low- and High-lines (t_{24} = 1.89, p = 0.07). There was no difference in the AUC of RMSSD (Figure 3G, right panel) between the lines ($F_{2,24}$ = 0.86, p = 0.437).

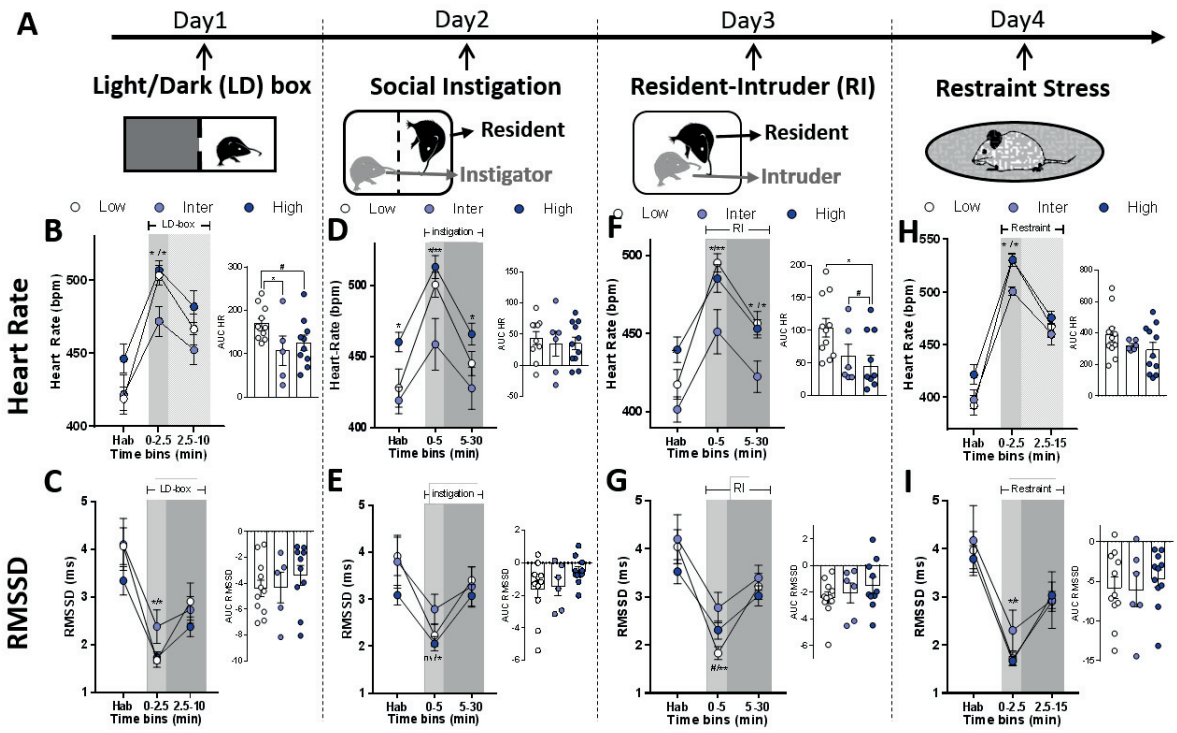

Figure 3: Inter-line rats had lower heart rate response during early exposure to different behavioral tests. A, Schematic showing experimental timeline of 4 days of behavioral and psychosocial stressors. **B, C,** During the LD-box, Inter-line rats had lower HR **(B, left)** and higher RMSSD **(C, left)** immediately after the onset of the test. Low-line rats showed a higher relative increase in HR (**B, right**). There were no differences in AUC of RMSSD (**C, right**). **D, E,** During the social instigation period, Inter-line rats had a lower HR increase **(D, left),** coupled with a higher RMSSD **(E, left)** at early onset of stress. During habituation, High-line rats had higher HR **(D, left)** but no differences in RMSSD **(E, left).** The AUC of HR (**D, right**) and RMSSD (**E, right**) did not differ between CAST-lines. **F, G,** During the entire RI recording Inter rats had lower heart rate (**F, left**). However, higher RMSSD (**G, left**) was only observed during the first 5 min of social interactions (). High-line rats had a lower relative increase in heart rate (**F, right**) than the Low- and Inter-lines (trend). There were no differences in AUC for RMSSD (**G, right**). **H, I,** During the first 180s of restraint, Inter-line rats had lower maximal HR **(H, left)**, and higher RMSSD (**I, left**). There were no differences in AUC in HR (**H, right**) and RMSSD (**I, right**). For graphs **B, C**: Low (n = 11), Inter (n = 5) and High (n = 10). For graphs **D, E, H, I**: Low (n = 11), Inter (n = 6) and High (n = 11). For graphs **F, G**: Low (n = 11), Inter (n = 6) and High (n = 10). Symbols represented on the graphs show comparisons between Inter and Low/High rats with: #p<0.1 (trend), *p<0.05, **p<0.01, ***p<0.001

During restraint stress (Figure 3H, 3I), there was a tendency for a line effect on HR values ($F_{2,25}$ = 2.61, p = 0.094), and a tendency for an interaction between time-bins and lines ($F_{4,50}$ = 2.43, p = 0.059). High rats (Figure 3H, left panel) had higher HR during habituation than Low- (p = 0.076) and Inter-line rats (p = 0.01). Inter-line animals had a lower maximal HR than Low- (t_{75} = 2.24, p = 0.028) and High-line rats (t_{75} = 2.28, p = 0.025). There were no difference in HR between the three lines during the late response to restraint stress (t_{75} < 1.18, p > 0.24). There were no differences in the AUC of HR in response to restraint (Figure 3F, right panel) between the lines ($F_{2,25}$ = 1.39, p = 0.267). There was no line effect ($F_{2,25}$ = 0.31, p = 0.74) on the RMSSD values during restraint stress (Figure 3I, left panel) and no interaction between time-bins and lines ($F_{4,50}$ = 0.64, p = 0.63). However, when looking specifically at the immediate response to restraint stress (first 180 s following onset of restraint) there was a tendency for a line-related difference in RMSSD ($F_{2,25}$ = 2.66, p = 0.089). In particular, t-tests showed that Inter-line rats had a tendency for a higher RMSSD than Low- (t_{25} = 2.01, p = 0.056) and High-line animals (t_{25} = 2.17, p = 0.040). The AUC of RMSSD (Figure 3I, right panel) did not show differences between lines ($F_{2,25}$ = 0.29, p = 0.748).

Collectively, the results indicate that the Inter-line rats had a lower maximal immediate-response to different stressors in comparison to the Low- and High-lines. Inter-line rats had a higher vagal tone in response to stress. The

High-line and Low-line rats had similar HR and RMSSD responses (with few exceptions). Interestingly, when Low-line and High-line rats were repeatedly challenged with mild- or acute-stressors (three consecutive days of handling and restraint respectively), Low-line animals displayed lower HR responses than High-line rats to handling stress but not to restraint stress (supplementary figure 2).

iii. Pharmacological confirmation of higher vagal tone in Inter-line rats

No difference in HR and HRV responses among the three lines after saline injection

As a control experiment, rats were injected with a saline solution and subsequent HR and HRV indexes analyzed (Figure 4A and 4B). There were important changes in HR ($F_{9, 54}$= 17.96, $p < 0.001$) and RMSSD ($F_{9, 54}$= 6.66, $p < 0.001$) immediately following saline injection. There was no line effect ($F_{2, 6} = 0.20$, $p = 0.822$) on the HR response to saline injection (Figure 4A, left panel). This was confirmed by the AUC for the HR response (Figure 4A, right panel) that showed a similar change in HR in response to saline for the three lines ($F_{2, 6} = 0.056$, $p = 0.94$). There was a significant line effect ($F_{2, 6} = 5.19$, $p = 0.049$) in RMSSD changes in response to saline injection (Figure 4B). Inter-line animals had higher RMSSD values throughout the entire recording (Figure 4B, left panel) than Low- ($t_6 = 2.64$, $p = 0.039$) and High-lines ($t_6 = 2.92$, $p = 0.027$). There was no difference in the AUC of RMSSD (Figure 4C, right panel) between the lines ($F_{2, 6} = 1.1$, $p = 0.391$). Therefore, in reaction to saline injection, the three lines showed a similar cardiac response.

Similar HR and RMSSD changes after sympathetic blockade in the three lines

Injection of atenolol induces a fast blockade of sympathetic influences to the heart (Distler et al., 1978). Maximal HR response to atenolol was significantly lower than the HR response to saline injection in the three lines ($p < 0.001$). There was no line effect ($F_{2, 6} = 2.76$, $p = 0.142$) on HR values in the overall recording and no interaction between time-bins and lines ($F_{22, 66} = 0.86$, $p = 0.639$). The relative changes in HR, illustrated by the AUC of the HR response (Figure 4D, right panel) did not differ between lines ($F_{2, 6} = 1.01$, $p = 0.417$). The RMSSD response to atenolol (Figure 4E, left panel) showed an important decline following injection for the three lines ($F_{11,66} = 7.03$, $p < 0.0001$). There was a line effect on HR values ($F_{2, 6} = 8.81$, $p = 0.016$) as well as an interaction between time-bins and lines ($F_{22, 66} = 2.5$, $p = 0.002$). During habituation, Inter-line rats exhibited a higher RMSSD values than Low- ($t_6 = 5.9$, $p = 0.001$) and High-lines animals ($t_6 = 7.25$, $p < 0.001$). In addition, during the entire post-injection period Inter-line rats maintained a higher RMSSD than Low- ($t_6 = 2.85$, $p = 0.029$) and High-lines rats ($t_6 = 3.89$, $p = 0.008$). The relative difference in RMSSD, illustrated in Figure 4E (right panel) by the AUC of the RMSSD response to atenolol, did not show difference in vagal modulation after sympathetic blockade ($F_{2, 6} = 3.16$, $p = 0.115$). The early differences in HR between the lines might be explained by the higher vagal activity of Inter-line rats at basal levels as well as in response to stress (Figure 4E). Altogether, these results suggest that all three lines have similar activation of the sympathetic nervous system in response to a stressor exposure.

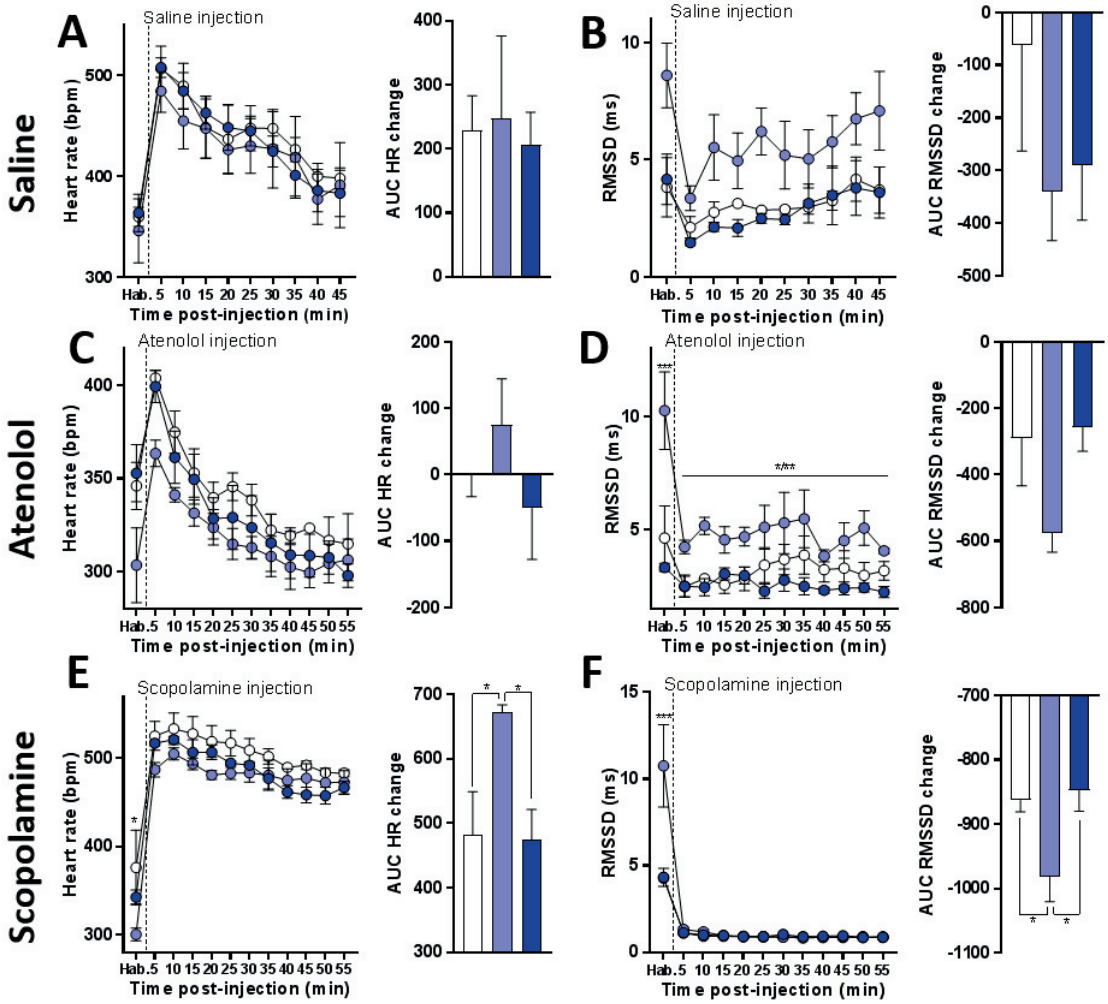

Figure 4: Inter-line rats showed higher parasympathetic modulation of heart rate in response to pharmacological treatments. **A, B,** HR and RMSSD values following saline injection as control manipulation. There was no difference in HR increase (**A, left**) and no difference in relative changes in HR (**A, right**) in response to saline injection. Inter-line rats had higher RMSSD throughout recording (**B, left**). There were no significant differences in AUC of RMSSD values (**B, right**). **C, D,** Injection of atenolol blocked sympathetic activity and induced a lower maximal HR (**C, left**). There was no line effect on HR (**C, left**). There was no difference in AUC of HR (**C, right**). There was a consistent difference in RMSSD (**D, left**) with Inter-line rats having higher RMSSD values. There was no significant difference in AUC of RMSSD (**D, right**). **E, F,** Scopolamine injection blocked the parasympathetic inputs to the heart. There was no difference in HR after scopolamine injection (**E, left**), but there was a relative higher increase in HR for Inter-line rats, in comparison to Low- and High-line animals (**E, right**). Scopolamine decreased RMSSD value in the three lines (**F, left**) but Inter-line rats had bigger relative decrease in RMSSD than both Low- and High-line animals (**F, right**).

Larger HR increase and RMSSD decrease in Inter-line rats after vagal blockade

The HR and HRV responses to vagal blockade by scopolamine injection are shown in Figure 4F and 4G. The HR response to scopolamine injection (Figure 4F, left panel) showed an important increase ($F_{11,66} = 83$, $p < 0.001$) for the three lines, but no difference between lines ($F_{2,6} = 2.45$, $p = 0.166$). There was a tendency for an interaction between time-bins and lines ($F_{22,66} = 1.61$, $p = 0.072$). Inter-line rats had a higher increase in HR ($F_{2,6} = 5.56$, $p = 0.043$) as shown by a higher AUC (Figure 4F, right panel) in comparison to Low- ($t_6 = 2.83$, $p = 0.03$) and High-lines ($t_6 = 2.94$, $p = 0.026$). When the PNS was blocked there was a remarkable decrease in RMSSD in the three lines ($F_{11,66} = 44.2$, $p < 0.0001$) which lasted for the entire recording session (Figure 4G, left panel). There was a line effect on RMSSD values

($F_{2, 6}$ = 10.9, p = 0.01) and an interaction between time-bins and lines ($F_{22, 66}$ = 6.65, p < 0.0001). The three lines had different RMSSD values during habituation (t_{72} > 11.1, p < 0.0001) but similar RMSSD values post-injection (t_{72} < 0.42, p > 0.67). Additionally, the AUC of the RMSSD changes (Figure 4G, right panel) differed between the lines ($F_{2, 6}$ = 5.72, p = 0.041) and Inter-line rats had a greater vagal decrease than Low- (t_6 = 2.73, p = 0.034) and High-line animals (t_6 = 3.09, p = 0.021). Taken together, the results following scopolamine injection reveal that blockade of the parasympathetic influence on cardiac activity elicited a higher HR increase (Figure 4F) and a RMSSD decrease (Figure 4G) in the Inter-line only and not in the Low- and High-lines. This confirmed that, as opposed to the other lines, the Inter-line is characterized by a larger vagal modulation at rest.

Discussion

Here, we characterized the cardiac autonomic regulation in a rat model with constitutive differential responsiveness to stress. We illustrated the interaction between two main mediators of the stress response, the HPA-axis and the ANS modulation, which are inter-related (Cohen et al., 2015a; Coughlin, 2011). Alterations in these systems constitute key risk factors for cardiovascular and psychiatric disorders. The results provided here show that selection for either low or high glucocorticoid reactivity to stress, measured by plasma CORT level, were associated with impaired autonomic modulation (low vagal tone) compared to intermediate stress reactivity.

Consistent with previous observations in early generations (Walker et al., 2017), we found that at adulthood, rats from the High-line exhibited higher CORT secretion in reaction to stress and displayed higher anxiety-like behaviors. Investigations of the autonomic correlates of rats differing in stress-responsiveness was performed with HRV analysis. Measures of HRV in the time and frequency domains are used as indexes of vagal modulation of HR (Reyes del Paso et al., 2013). Vagal tone is an indicator of cardiac health, representing behavioral and physiological flexibility of an organism, as well as its ability to adapt in response to stress (Porges, 1995).

In order to perform a basal characterization of cardiac autonomic regulation, we measured HR and HRV parameters during baseline electrocardiogram recordings under resting conditions. We found that rats from the three lines differed in resting HR values. Both Low- and High-line rats had higher HR at rest compared to Inter-line animals. This difference was not due to differences in locomotor activity between the lines. Furthermore, we found that HRV was significantly lower in rats from the Low- and High-lines compared to Inter-lines animals. This was indicated by lower vagal modulation of HR, as indexed by RMSSD and HF power values.

Interestingly in our vagal blockade studies, we observed a smaller increase in HR in Low- and High-lines compared to Inter-line animals following methylscopolamine administration. This suggests that in both Low- and High-lines have lower contribution of the vagal control over resting HR. Vagal blockade induced a more important decrease in RMSSD in Inter-line animals compared to the two other lines due to significant differences in baseline values of vagal index, which were lower in Low- and High-lines. However, it cannot be excluded that the differences in HR observed in response to vagal blockade were due to the baseline differences recorded. Indeed, after vagal injection, Inter-line rats showed a lower HR response, but 25 min post-injection the HR difference vanished while the RMSSD remained shut down. One possible explanation could be a floor effect in response to this dose of methylscopolamine and future experiments may want to determine the dose-response curve to vagal blockade. In order to assess cardiac sympathetic influences, body temperature analysis and pharmacological sympathetic blockade were performed. Thermogenesis is sympathetically regulated (Himms-Hagen, 1984), and there was no difference between the lines in basal body temperature. Moreover, following saline control injections and sympathetic blockade by atenolol, there were no differences in HR changes and Inter-line rats, which constantly exhibited higher RMSSD. This indicates that the three lines did not differ in sympathetic modulation of heart rate. Taken together, HRV analyses revealed the

autonomic determinant underlying the differences in resting HR between the three lines. Low- and High-lines had similar SNS activity than Inter-line animals but lower parasympathetic drive and thus a lower 'cardiac vagal brake', leading to a higher resting HR.

Low vagal tone is a marker of reduced behavioral, physiological and stress response flexibility (Porges, 1995), thus our findings suggests that cardiac responsiveness and HR sensitivity to stress might be impaired in the High- and Low-lines (Goldberger, 1991; Carnevali et al., 2014). In this with idea, we exposed rats from the lines to different behavioral challenges. Results from ECG recordings performed during the behavioral tests showed similar differences in HR and RMSSD values as found at rest. Consistently, rats from the Low- and High-lines had higher HR values than Inter-line rats and lower vagal tone (lower RMSSD). There were no differences in vagal withdrawal (i.e. the decrease in parasympathetic influence induced by stress) between the three lines, as indicated by similar area under the curve of RMSSD values in response to stress. However, as previously reported (Walker et al., 2017; Walker and Sandi, 2018), rats from the three lines displayed important differences in CORT secretion in response to stress. Taken together, our findings indicate that differences in glucocorticoid levels during stress did not affect cardiac regulation. This is in accordance with previous studies showing that CORT treatment had no significant effect on arterial pressure and HR, while moderate elevation in CORT for several days induced pressure-independent modulation of baroreflex control of HR (Scheuer and Bechtold, 2002). The HPA axis and the ANS are extremely complementary (Ulrich-Lai and Herman, 2009) with autonomic control of HR from brain regions involved in the HPA-axis regulation and glucocorticoids acting on those regions as well as the adrenal gland being involved in CORT and norepinephrine production. Therefore, we suggest here that the inherent differences in HPA axis functioning between the lines had restricted outcome on HR responsiveness to acute stressors.

Constitutive differences in glucocorticoid responsiveness to stressors in these lines of rats were shown to be involved in the expression of psychopathology-like behaviors at adulthood (Walker et al., 2017). Rats from the High-line displayed increased anxiety-like behaviors, aggressive behaviors and passive coping relative to Inter-line rats (Walker et al., 2017). Here, we showed increased resting HR and reduced vagal modulation of cardiac activity in High-line rats compared to Inter-line animals. This is in line with previous studies showing a link between anxiety-like and aggressive behaviors with impaired vagal tone (Carnevali and Sgoifo, 2014). Indeed, previous studies reported sympathovagal imbalance in pre-clinical models of increased anxiety and aggressive behaviors. Rats with increased anxiety- and depression-like behaviors have been shown to have lower vagal modulation of resting HR (Sévoz-Couche et al., 2013; Carnevali et al., 2014) , higher basal HR and reduced HRV indicating an imbalance between SNS and PNS regulation of HR (Grippo et al., 2006, 2004; Wood et al., 2012). In accordance with our previous report from the three corticosterone rat lines (Walker et al., 2017) and previous reports linking psychopathologies and low vagal modulation and deficiencies in emotion regulation (Beauchaine et al., 2007), we suggest that rats from the High-line might be a relevant model to investigate the development of psychopathologies associated to cardiovascular dysfunction. Specifically, High-line animals, showing high CORT, high anxiety and more passive coping could be used as a model for depressive disorders but further study would be required to validate it.

In addition, we showed here that rats from the Low-line have blunted corticosterone responsiveness, high resting HR and low resting parasympathetic nervous system influence on cardiac activity. Similarly, PTSD is often associated with a blunted basal HPA-axis (Cohen et al., 2006). Even if CORT levels alone do not conclusively identify a PTSD state, the current literature supports a role for HPA axis hormones as biomarkers of PTSD (Zoladz and Diamond, 2013). Moreover, a recent study showed that a blunted basal CORT pulsatility predicted susceptibility to develop PTSD (Danan et al., 2018). PTSD patients exhibit enhanced negative feedback inhibition of the HPA axis and an exaggerated suppression of CORT as well as enhanced CORT sensitivity (Goenjian et al., 1996; Grossman et al., 2003; Yehuda et al., 2004). In our study, Low-line animals had reduced HPA-axis response to stress and previous report showed a

slightly lower mRNA expression of *Fkbp5* in paraventricular nucleus (PVN) of the hypothalamus of Low- as compared to High-line rats (Walker et al., 2017). FKBP5 regulates the sensitivity of the glucocorticoid receptor to its ligand and reduces its binding affinity (Binder, 2009). Thus, a reduction in FKBP5 could enhance GR activity in PVN and increase the negative feedback of Low-line animals (Walker et al., 2017) compared to the High-line. Future studies aiming at assessing changes in mRNA expression and protein levels in following generations of the lines would be required for further validation.

The literature regarding baseline autonomic activity in PTSD patients is still emerging. Previous studies indicated that Individuals with PTSD tend to display greater baseline HR and BP (Zoladz and Diamond, 2013) and significantly reduced HRV with lower parasympathetic influences (Sahar et al., 2001; Zoladz and Diamond, 2013; Shah et al., 2013). Similarly, animal models of PTSD displayed cardiovascular abnormalities (Zoladz et al., 2008; Zoladz and Diamond, 2016b) and diminished vagal tone (Cohen et al., 2003). Accordingly, Low-line animals from our study displayed higher resting HR and lower resting vagal tone. Altogether, our data seem to coincide with previous literature on PTSD and suggest that Low-line animals may serve as an important tool for the analyses of PTSD like symptoms. Moreover, stress applied during peripuberty selectively enhanced aggression in the Low-line, but not in the High-line (Walker and Sandi, 2018), suggests that the low lines show enhanced susceptibility if they had experienced prior adversity. A previous animal model of PTSD showed that a blunted HPA axis response to stress influences susceptibility to develop post-traumatic stress response in rats (Cohen et al., 2006). Our work highlights a new rodent model of PTSD that, in addition, shows autonomic dysfunctions resembling those described in PTSD patients.

Therefore, our studies show that animals from the two extreme lines with dissimilar glucocorticoid responsiveness profile but have similar HR and similar impaired cardiac vagal modulation. During behavioral challenges, though, they showed differences in cardiovascular responses when exposed to the LD box and resident-intruder tests. Low-line rats exhibited a higher increase in HR, illustrated by the AUC of heart rate, compared to High-line animals, indicating an increased HR stress responsiveness. However, both lines had similar maximal HR in response to the four different stressors applied. Additionally, in response to handling (Axelrod and Reisine, 1984; Koolhaas et al., 2011), High-line rats mounted higher HR stress responses immediately after handling. However, no differences were observed following exposure to restraint stress. These results indicate that, even if Low- and High-lines appeared to have similar cardiac autonomic regulation, there might be underlying differences in the way they respond to different stressful situations. For example, High-line animals seem to react more against mild handling stress, showing a high default responsiveness state, in accordance with their high CORT responses.

Conclusion

This study provides a detailed investigation of cardiac autonomic regulation in a rat model of differential stress responsiveness. For the first time, we provide two animal models relating hyper- and hypo-responsiveness of the HPA-axis with impaired parasympathetic modulation of heart rate. We show that rats selected for their high or low constitutive glucocorticoid responsiveness are characterized by low vagally mediated HRV. This deficiency in tonic vagal modulation was validated by pharmacological manipulations. Altogether, our results demonstrate that our animal model link two key risk factors for psychiatric and cardiovascular disorders by exhibiting dysregulations in the HPA-axis and the PNS. Finally, we suggest that the two extreme lines are potentially attractive preclinical models for further testing for psychopathologies, like depression and PTSD.

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Disclosure of interests

The authors report no conflicts of interest.

Supplementary Material

i. Material and methods

Elevated plus maze (EPM)

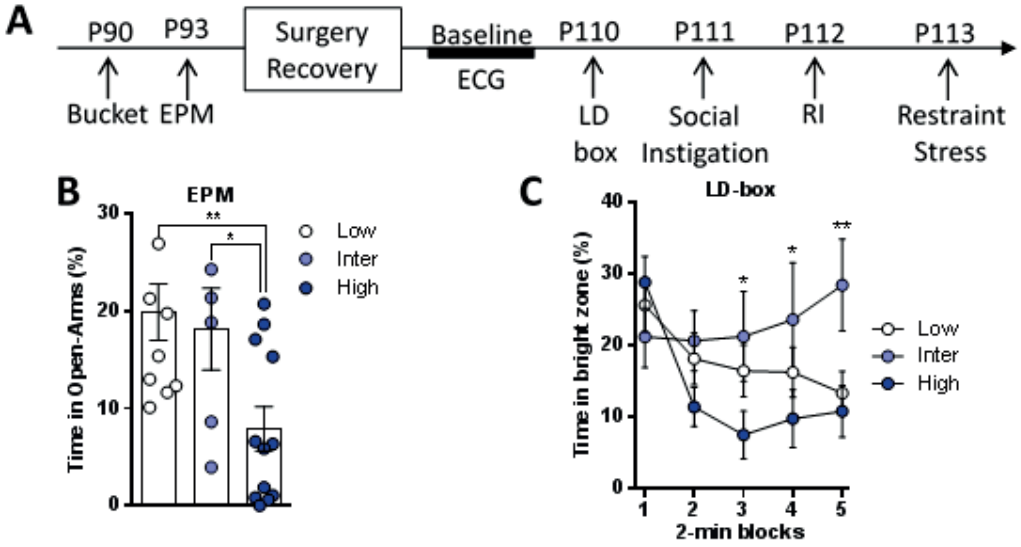
Anxiety-like behaviors were evaluated using the EPM test (Walf and Frye, 2007) . The apparatus consisted of two opposite open-arms (50 x 10 cm) perpendicular to two closed-arms (50 x 10 x 50 cm), extending from a central platform (10 x 10 cm) and elevated 65 cm above the floor. Light levels were maintained between 14-16 lx on the open-arms and 5-7 lx in the closed-arms. At the start of the test, the rat was placed on the central platform facing a closed-arm and allowed to explore the maze for 5 minutes. Behavior was monitored using a ceiling mounted video camera and analyzed with a computerized tracking system (Ethovision 10; Noldus IT, Netherlands). The distance traveled, the time spent in the open- and closed-arms were analyzed. In between animals, the apparatus was cleaned with 5% ethanol solution and dried.

Light-Dark box (LD box)

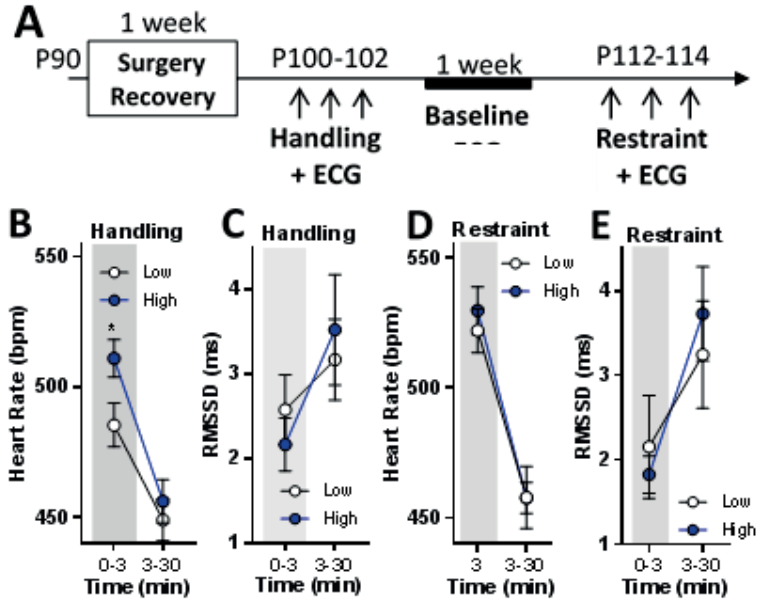
In order to assess anxiety-like behaviors, the natural aversion of rats against bright environments was used in the LD-box. The maze (30 x 60 x 40 cm) consisted of 2 connected equal-sized chambers, one covered and dark (approx. 0 lux) and one open and bright (approx. 100 lux). During the test, the rat was placed in the open chamber facing the wall and allowed to explore for 10 min. Behavior of the rat in the bright compartment was video-recorded. An experimenter blind to group assignment, scored the videos and determined the time spent in the dark or bright chambers. The more the rats spent time in the lit chamber was used as an index of lower anxiety-like behaviors. Continuous ECG, T° and LOC recordings were collected under baseline conditions (20 min, prior to the test), during the LD-box (10 min) and throughout the recovery period (20 min).

ii. Results

Before (P93) and after (P110) radiotelemetric implants surgery, anxiety-like behaviors of the rats were assessed rats were in and EPM and LD box respectively (Supplementary figure 1A). During EPM (Supplementary figure 1B), there was a line effect ($p = 0.012$) on the time spent in the open arms. High-line rats showed higher anxiety-like behaviors than the Low- ($p = 0.003$) and Inter-lines ($p = 0.046$). During the LD box (Supplementary figure 1C), there was a line effect ($p = 0.003$) on the time spent in the bright part. High-line rats showed higher anxiety-like behaviors than the Inter-line ($p = 0.005$), but there were no differences with Low-line rats ($p > 0.151$) in the time spent in the bright part.



Supplementary Figure 1: Increased anxiety-like behaviors in the High-rats. A, experimental timeline of Experiment 2. B, During 5 minutes on the EPM, there was a line effect on the time spent on the open arms ($F_{2,26}$ = 5.3, p = 0.012). High-line rats spent less time in the open arms than Low- (p = 0.003) and Inter-line rats (p = 0.046). C, During 10 minutes in the LD-box test, results were analyzed using 2-min blocks and a repeated measure ANOVA. There was a line effect on the time spent in the bright part ($F_{2,115}$ = 6.12, p = 0.003). High-line rats spent less time in the bright part compared to Inter-line rats (t_{92} = 2.89, p = 0.005), but there were no differences between Low-line rats and the other lines (p > 0.151). During the two first blocks all rats had similar exploration of the bright part (blocks 1 & 2: p > 0.152), but then, High-line rats spent less time than Inter-line rats in the light (blocks 3, 4 & 5: p < 0.035). Due to technical issues, two animals (1 Inter and 1 High) were not video-recorded during LD-box testing.



Supplementary Figure 2: Cardiovascular differences during early reaction to mild stress (handling) between Low- (n=5) and High-line (n=5) animals. A, Experimental timeline of three days of handling and three days of restraint stress. **B, C, D, E,** Averaged results of the 3 days of ECG recording following handling (**B, C**) and restraint (**D, E**) Analyzed separately for early (grey background) and late response of heart rate (**B, D**) and RMSSD values (**C, E**). Rats from the High-line have higher immediate HR response to handling (**B,** t_5=2.258, p = 0.038) but not to acute restraint (**D**) and not during the late response (**B,** t_5=0.63, p = 0.537). There were no differences RMSSD after stress (**C, E**).

parameters	Line	Habituation	LD box Test	Recovery	Fischer's LSD (p-values)
Heart rate (bpm)	Low	439.7 ± 6.45	476 ± 9.1	421.3 ± 8.7	Hab (LvH .063, IvH .056) LD box (IvH .047)
	Inter	432.9 ± 14.8	456.1 ± 10.2	408.6 ± 10.8	
	High	462.5 ± 8.9	488 ± 9.5	435 ± 8.6	
RMSSD (ms)	Low	3.81 ± 0.36	2.84 ± 0.34	3.84 ± 0.42	N.S.
	Inter	3.88 ± 0.46	2.76 ± 0.26	3.99 ± 0.56	
	High	3.08 ± 0.25	2.35 ± 0.19	3.53 ± 0.26	
Total Power (ms^2)	Low	78.2 ± 12.3	40.3 ± 9.2	49.4 ± 10.2	N.S.
	Inter	53.7 ± 7.9	32.7 ± 5.8	86.3 ± 21.5	
	High	47 ± 5.9	25.1 ±5	43.6 ± 6.4	
HF power (ms^2)	Low	5.07 ± 1.05	3.19 ± 0.83	5.68 ± 1.72	N.S.
	Inter	5.69 ± 1.47	2.80 ± 0.48	5.69 ± 1.5	
	High	3.15 ± 0.39	1.72 ± 0.33	3.91 ± 0.55	
LF Power (ms^2)	Low	10.4 ± 2.1	5.2 ± 1.5	7.4 ± 2.1	Hab (LvH .035)
	Inter	8 ± 0.6	3.8 ± 0.6	7.3 ± 1	
	High	5.7 ± 0.8	2.6 ±0.6	5 ± 0.6	
LF/HF	Low	2.16 ± 0.28	1.65 ± 0.17	1.64 ± 0.34	N.S.
	Inter	1.68 ± 0.3	1.43 ± 0.17	1.63 ± 0.38	
	High	1.92 ± 0.22	1.42 ± 0.12	1.37 ± 0.13	
Temperature (°C)	Low	37.9 ± 0.14	38.3 ± 0.1	38.5 ± 0.08	Hab (LvI .017, IvH .016)
	Inter	38.3 ± 0.18	38.57 ± 0.1	38.6 ± 0.1	
	High	37.89 ± 0.1	38.35 ± 0.08	38.5 ± 0.07	
Locomotion (count)	Low	8.71 ± 0.77	25.3± 2.1	4.6 ± 0.76	LD box (IvH .047)
	Inter	11.75 ± 2.29	28.9 ± 2.2	5.5 ± 0.99	
	High	10.1 ± 0.94	23.8 ± 2.4	4.8 ± 0.43	

Supplementary Table 1: Radiotelemetric and HRV parameters during the light-dark box test. Values are reported as means ± SEM of data obtained by averaging multiple 180s segments acquired during habituation (20min), during the LD box (10min) and the recovery phase (30min), in Low- (n = 11), Inter- (n = 5) and High-line (n = 10) animals. Abbreviations: HRV = heart rate variability, LD box = Light-Dark box, bpm = beat-per-minute, Hab = Habituation, LvH = Low vs. High, LvI = Low vs. Inter, HvI = High vs. Inter, N.S. = not significant, RMSSD = square root of the mean squared differences of successive RR intervals, LF = Low-Frequency, HF = High-frequency.

parameters	Line	Habituation	Social Instigation	Recovery (0-15min)	Recovery (15-30min)	Fischer's LSD (p-values)
Heart rate (bpm)	**Low**	439.5 ± 11.9	454.5 ± 8.2	456.4 ± 8.7	432.2 ± 10.1	Hab (LvH .03, IvH .008)
	Inter	425.5 ± 9.6	432.9 ± 15.4	425.3 ± 12.2	409.8 ± 14	Instig (IvH .011)
	High	468.2 ± 7.5	473.8 ± 7.7	466.5 ± 8.4	453.3 ± 10.8	Recov 1 (LvI .051, IvH .01)
RMSSD (ms)	**Low**	3.82 ± 0.41	3.21 ± 0.26	3.09 ± 0.22	3.83 ± 0.29	
	Inter	3.76 ± 0.54	3.19 ± 0.39	3.40 ± 0.42	4.12 ± 0.44	Hab (LvH .03, IvH .09)
	High	2.95 ± 0.15	2.90 ± 0.22	2.99 ± 0.18	3.41 ± 0.28	
Total Power (ms^2)	**Low**	90.5 ± 14.3	62.4 ± 8.5	58.7 ± 8.3	75.9 ± 9.8	
	Inter	62.9 ± 13.5	67.4 ± 12.2	51.8 ± 12.1	54.8 ± 8.9	Hab (LvI .077, LvH .001)
	High	47.8 ± 5	49.3 ± 77.7	52.5 ± 9.7	53.4 ± 8	
HF power (ms^2)	**Low**	8.71 ± 1.85	5.72 ± 1.06	5.83 ± 1.18	7.04 ± 0.87	
	Inter	8.46 ± 2.1	6.78 ± 1.61	7.49 ± 1.92	7.41 ± 1.48	Hab (LvH .003, IvH .018)
	High	4 ± 0.53	3.99 ± 0.69	4.74 ± 0.75	5.25 ± 0.75	
LF Power (ms^2)	**Low**	14 ± 2.67	10.75 ± 1.88	10.65 ± 2.28	10.55 ± 1.29	
	Inter	9.41 ± 1.8	7.87 ± 1.85	10.31 ± 3.22	7.58 ± 1.09	Hab (LvH .005)
	High	5.51 ± 0.6	6.79 ± 1.2	7.31 ± 1.6	7.03 ± 1.15	
LF/HF	**Low**	1.81 ± 0.13	1.96 ± 0.18	1.96 ± 0.21	1.60 ± 0.11	Hab (LvI .029, LvH .066)
	Inter	1.27 ± 0.16	1.27 ± 0.14	1.46 ± 0.21	1.16 ± 0.23	Restraint (LvI .006, IvH .055)
	High	1.43 ± 0.09	1.75 ± 0.14	1.56 ± 0.1	1.4 ± 0.16	Recov1 (LvI .042, LvH .051)
Temperature (°C)	**Low**	38.73 ± 0.15	38.6 ± 0.09	38.64 ± 0.1	38.46 ± 0.1	
	Inter	39.04 ± 0.28	38.92 ± 0.14	38.88 ± 0.15	38.7 ± 0.18	Hab (LvH .07)
	High	39.02 ± 0.14	38.85 ± 0.07	38.76 ± 0.07	38.53 ± 0.07	
Locomotion (count)	**Low**	9.28 ± 0.96	11.43 ± 0.64	8.17 ± 1.03	4.92 ± 0.79	
	Inter	10.24 ± 0.76	12.05 ± 0.96	9.07 ± 1.86	6.53 ± 1.47	Recov1 (LvH .042)
	High	10.35 ± 1.3	12.66 ± 1.00	11.11 ± 1.22	6.3 ± 0.98	

Supplementary Table 2: Radiotelemetric and HRV parameters during the social-instigation test. Values are reported as means ± SEM of data obtained by averaging multiple 180s segments acquired during habituation (30min), during the social instigation (30min) and the recovery phase (0-15min and 15-30min), in Low-line (n = 11), Inter-line (n = 6) and High-line (n = 11) animals. Abbreviations: HRV = heart rate variability, bpm = beat-per-minute, Hab = Habituation, Instig = instigation, Recov = recovery, LvH = Low vs. High, LvI = Low vs. Inter, HvI = High vs. Inter, RMSSD = square root of the mean squared differences of successive RR intervals, LF = Low-Frequency, HF = High-frequency.

parameters	Line	Habituation	Resident Intruder	Recovery (0-15min)	Recovery (15-30min)	Fischer's LSD (p-values)
Heart rate (bpm)	Low	436.3 ± 8.1	463.2 ± 6.7	429.7 ± 8.1	406.9 ± 11	Hab (LvI .045, IvH .004)
	Inter	409.4 ± 8.7	427.3 ± 10.2	396 ± 8.3	378.8 ± 8.1	RI (LvI .008, IvH .02)
	High	449.5 ± 9.1	458.7 ± 5.8	441.7 ± 5.8	402.4 ± 10.6	Recov 1&2 (LvI .001, IvH .001)
RMSSD (ms)	Low	3.57 ± 0.33	2.99 ± 0.23	3.66 ± 0.29	4.05 ± 0.3	
	Inter	3.94 ± 0.5	3.29 ± 0.27	3.57 ± 0.39	4.32 ± 0.53	N.S.
	High	3.28 ± 0.19	2.9 ± 0.18	3.08 ± 0.27	4.26 ± 0.34	
Total Power (ms^2)	Low	61.2 ± 7	48.6 ± 4.7	60.7 ± 9.4	62.9 ± 6.4	
	Inter	60.4 ± 8	49.2 ± 4.9	44.5 ± 4.5	63.7 ± 8.1	Recov1 (LvH .03)
	High	50.7 ± 4.4	52.7 ± 6.6	39.3 ± 4.8	72 ± 12.1	
HF power (ms^2)	Low	6.97 ± 1.25	4.8 ± 0.96	6.52 ± 1.2	6.39 ± 1	
	Inter	7.16 ± 1.67	4.57 ± 0.6	5.22 ± 1.25	7.87 ± 2.64	N.S.
	High	4.58 ± 0.66	4.13 ± 0.63	4.07 ± 0.76	7.01 ± 1.25	
LF Power (ms^2)	Low	10.05 ± 1.49	8.43 ± 1.46	10.44 ± 1.9	8.78 ± 1.42	
	Inter	8.87 ± 0.92	6.12 ± 0.87	5.35 ± 0.46	7.09 ± 1.08	Hab (LvH .078)
	High	6.57 ± 0.76	8.26 ± 1.79	6.13 ± 1.04	8.93 ± 1.81	Recov 1 (LvI .027, LvH .03)
LF/HF	Low	1.68 ± 0.16	1.91 ± 0.16	1.76 ± 0.21	1.53 ± 0.16	
	Inter	1.5 ± 0.21	1.41 ± 0.19	1.34 ± 0.22	1.28 ± 0.22	RI (LvI .09, IvH .07)
	High	1.57 ± 0.15	1.96 ± 0.22	1.51 ± 0.09	1.45 ± 0.28	
Temperature (°C)	Low	38.39 ± 0.11	38.76 ± 0.09	38.7 ± 0.08	38.29 ± 0.08	
	Inter	38.59 ± 0.12	38.85 ± 0.19	38.77 ± 0.22	38.38 ± 0.15	N.S.
	High	38.43 ± 0.15	38.8 ± 0.09	38.7 ± 0.08	38.6 ± 0.21	
Locomotion (count)	Low	8.51 ± 0.76	17.53 ± 1.05	6.03 ± 0.97	3.42 ± 0.84	
	Inter	9.78 ± 1.76	15.79 ± 1.84	6.28 ± 2.38	4.1 ± 1.6	N.S.
	High	8.49 ± 1.01	18.81 ± 1.62	8.09 ± 0.92	4.4 ± 0.81	

Supplementary Table 3: Radiotelemetric and HRV parameters during the resident-intruder test. Values are reported as means ± SEM of data obtained by averaging multiple 180s segments acquired during habituation (30min), during the resident-intruder (30min) and the recovery phase (0-15min and 15-30min), in Low-line (n = 11), Inter-line (n = 6) and High-line (n = 11) animals. Abbreviations: HRV = heart rate variability, bpm = beat-per-minute, Hab = Habituation, RI = resident-intruder, Recov = recovery, LvH = Low vs. High, LvI = Low vs. Inter, HvI = High vs. Inter, N.S. = not significant, RMSSD = square root of the mean squared differences of successive RR intervals, LF = Low-Frequency, HF = High-frequency.

parameters	Line	Habituation	Restraint Stress	Recovery (0-15min)	Recovery (15-30min)	Fischer's LSD (p-values)
Heart rate (bpm)	**Low**	409.1 ± 9.8	478.2 ± 9.5	431.3 ± 9.4	396.3 ± 8.9	Hab (LvH .02, IvH .088)
	Inter	412.9 ± 9.8	468.7 ± 9.8	423.3 ± 8	399.7 ± 8.7	
	High	437.7 ± 9.9	485.1 ± 5.8	438.4 ± 8.6	408.4 ± 9.2	
RMSSD (ms)	**Low**	3.8 ± 0.39	2.71 ± 0.18	3.71 ± 0.40	4.3 ± 0.38	N.S.
	Inter	3.86 ± 0.59	2.83 ± 0.55	2.97 ± 0.32	3.94 ± 0.6	
	High	3.44 ± 0.25	2.81 ± 0.23	3.21 ± 0.19	3.66 ± 0.24	
Total Power (ms^2)	**Low**	61.6 ± 7.9	34.0 ± 3.7	54 ± 8.2	62.8 ± 8	Recovery 1 (LvI .048) Recovery 2 (LvI .069)
	Inter	51.2 ± 8.7	27.7 ± 2.1	34.3 ± 4.9	44.8 ± 6.1	
	High	53.4 ± 5.8	37.1 ± 4.9	44.3 ± 3.6	55.0 ± 4.9	
HF power (ms^2)	**Low**	5.71 ± 1.16	2.81 ± 0.33	5.12 ± 1.06	6.73 ± 1.11	N.S.
	Inter	5.91 ± 2.1	3.42 ± 1.47	3.22 ± 0.72	6.25 ± 1.95	
	High	4.2 ± 0.57	3.14 ± 0.75	3.77 ± 0.48	4.75 ± 0.76	
LF Power (ms^2)	**Low**	9.2 ± 1.68	4.2 ± 0.73	7.8 ± 1.52	10.3 ± 1.69	Recovery 1 (LvI .038) Recovery 2 (LvI .037, LvH .024)
	Inter	6.35 ± 0.86	2.26 ± 0.36	4.1 ± 0.34	6.54 ± 0.6	
	High	6.49 ± 0.64	2.96 ± 0.46	5.42 ± 0.84	6.87 ± 0.86	
LF/HF	**Low**	1.84 ± 0.17	1.32 ± 0.10	1.71 ± 0.12	1.69 ± 0.17	N.S.
	Inter	1.46 ± 0.22	0.98 ± 0.18	1.53 ± 0.2	1.41 ± 0.24	
	High	1.83 ± 0.25	1.16 ± 0.13	1.68 ± 0.24	1.91 ± 0.32	
Temperature (°C)	**Low**	38.3 ± 0.12	38.71 ± 0.09	38.84 ± 0.06	38.53 ± 0.065	Hab (LvI .07, IvH .02)
	Inter	38.57 ± 0.08	38.82 ± 0.08	38.73 ± 0.1	38.34 ± 0.08	
	High	38.22 ± 01	38.7 ± 0.09	38.84 ± 0.09	38.43 ± 0.11	
Locomotion (count)	**Low**	7.94 ± 0.74	3.27 ± 0.38	7.85 ± 0.88	3.54 ± 0.59	N.S.
	Inter	8.69 ± 1.16	3.14 ± 1.02	8.6 ± 0.83	4.13 ± 0.91	
	High	8.18 ± 1.07	2.4 ± 0.55	7.91 ± 1.11	3.77 ± 0.88	

Supplementary Table 4: Radiotelemetric and HRV parameters during the restraint-stress. Values are reported as means ± SEM of data obtained by averaging multiple 180s segments acquired during habituation (30min), during the restraint stress (30min) and the recovery phases (0-15min and 15-30min), in Low-line (n = 11), Inter-line (n = 6) and High-line (n = 10) animals. Abbreviations: HRV = heart rate variability, bpm = beat-per-minute, Hab = Habituation, LvH = Low vs. High, LvI = Low vs. Inter, HvI = High vs. Inter, N.S. = not significant, RMSSD = square root of the mean squared differences of successive RR intervals, LF = Low-Frequency, HF = High-frequency.

Chapter 2

Social interactions between rats of differential stress responsiveness: effects on general and central metabolism

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Abstract

In social species, including humans and rats, stress, anxiety and social interactions are closely related. Indeed, stress can affect social interactions and anxiety, but social interactions can be the source of critical stress in return. Furthermore, the stress system, which main goal is to provide proper energetic distribution in one's organism in order to offer the most appropriate response to environmental challenges, is closely related to key metabolic mediators. Here, we investigated the general metabolism of rats with different biobehavioral phenotypes derived from lines of rats selected for their differences in their corticosterone responsiveness to stress. Moreover, by pairing rats from the different lines into mixed-line dyads we evaluated the differences in social interactions as well as the effect of such social pairing on general and central metabolism after one-month. Indirect calorimetry and mitochondrial respirometry techniques were applied for the assessment of energy expenditure and mitochondrial function respectively. Results indicated that the selection for differences in glucocorticoid responsiveness induced constitutive differences in energy expenditure and fuel use. Moreover, results showed that the diverse biobehavioral phenotypes of the line affected the social interactions and that long-term mixed-line pairing affected global and central metabolism of the rats, with Low- and Inter-line animals being more susceptible to social pairing with the High-line. Taken together, those results illustrate that social interactions between rats of differential biobehavioral phenotype might affect metabolic processes of some individuals and it emphasizes that particular phenotypes might present susceptibility or resilience to such changes.

Keywords

Stress responsiveness, social behaviors, aggressive behaviors, calorimetry, metabolism, mitochondria

Introduction

Social interactions frequently induce stress in individuals (Sapolsky, 2005) and, conversely, stress and anxiety influence social behaviors, the outcome of the social interactions and the susceptibility to social status (Beery and Kaufer, 2015). For example, in humans, high-anxious individuals are more prone to be subordinate (Gilbert et al., 2008) and their competitiveness decreases under stress (Goette et al., 2015). Importantly, low social status has been related to increased morbidity (Boyce, 2004) and to the development of psychopathologies (Allan and Gilbert, 1997). Moreover, groups of individuals often form dominance hierarchies, leading to inequality in resource access (Sapolsky, 2005; Clutton-Brock and Huchard, 2013). Importantly, decades of work have demonstrated that position in the social hierarchy has profound implications for health and well-being (Adler et al., 1994; Sapolsky, 2005; Neumann et al., 2010). In semi-natural colonies, subordinate animals show differences in behavioral and physiological parameters depending on their corticosterone (CORT) responsiveness to restraint stress (Blanchard et al., 2001). This finding suggests that variation in stress responsiveness might be indicative of a differential susceptibility to position in social hierarchy. Studies showed that rats submitted to prenatal stress display resilience to social stress possibly due to a facilitated adaptation to subordinate status (Scott et al., 2017) and male rats showing more active burying coping experienced higher allostatic load (having bigger adrenals and higher stress response) than passive copers, while it did not affect social status (Boersma et al., 2017). In addition, high anxiety levels are associated with alterations in social behaviors (Scott, 2011) and anxious individuals frequently become subordinate (Hollis et al., 2015; van der Kooij et al., 2017; Gilbert et al., 2009). A recent study reported that dominant mice exhibited higher trait anxiety than subordinates and were more vulnerable to social defeat stress (Larrieu et al., 2017).

During the stress response, the hypothalamic-pituitary-adrenal (HPA) axis is activated and a hormonal cascade is initiated. The corticotropin-releasing hormone (CRH) is secreted from the paraventricular nucleus (PVN) of the hypothalamus leading to production of the adrenocorticotropic hormone (ACTH) from the anterior pituitary that acts on the adrenal cortex to promote CORT production. The stress response facilitates energy distribution in the organism in order to cope with challenges, which is driven by the secretion of different regulators of energy metabolism and appetite (e.g. Leptin, ghrelin, Insulin, peptide YY, etc.). For example, stress increases ghrelin production that, in turn, acts on the arcuate nucleus (ARC) of the hypothalamus (on NPY/AgRP neurons) and increases appetite and food intake (Bali and Jaggi, 2016). However, chronic activation of the HPA axis leads to pathological effects including psychopathologies and metabolic disorders, such as diabetes, obesity and metabolic syndrome (Patterson et al., 2013). Additionally, links between stress, anxiety and depressive behaviors have been related to central action of metabolic hormones, like ghrelin (Bali and Jaggi, 2016) and differences in coping strategies or anxiety-like behaviors have been related to several metabolic and physiological biomarkers, in a range of different species. It was recently shown that fish exhibiting passive coping had higher oxygen consumption (Martins et al., 2011). Furthermore, active fish exhibited higher aggressive behaviors (Øverli et al., 2004) and had lower basal CORT (Silva et al., 2010) and lower parasympathetic reactivity (Verbeek et al., 2008). In a recent rodent study, high anxiety was found to be a predisposing factor to subordination, along with lower mitochondrial respiratory function in the nucleus accumbens (NAc), a brain region relevant for motivation and depression (Hollis et al., 2015). In addition, dominant mice were reported to have higher levels of energy metabolites in the NAc than subordinates (Larrieu et al., 2017).

Our laboratory has developed three lines of rats genetically selected for their glucocorticoid responsiveness to repeated stress exposure during the juvenile period (Walker et al., 2017). During a 'corticosterone adaptation-stress-test' (CAST) protocol, CORT responses were measured and rats having high, intermediate or low CORT concentration in blood plasma on the third day of stress exposure were selected as 'High-', 'Inter-' and 'Low-' lines, respectively. Recent studies showed major biobehavioral differences between rats from the three lines (Walker et al., 2017;

Walker and Sandi, 2018, Huzard et al. Chapter 1). Indeed, at adulthood, progeny from the High-line show increased CORT secretion in reaction to novelty and restraint stress, increased anxiety-like behaviors in the elevated plus maze (EPM), increased aggressive behaviors in a resident-intruder setting, and increased passive floating during a forced swim test (Walker et al., 2017; Walker and Sandi, 2018). These results suggest the High-line rats as more prone to develop psychopathologies linked with increased basal aggression and emotionality. Interestingly, stress, applied during peripuberty enhanced aggression specifically in the Low-line animals (Walker and Sandi, 2018), describing them as more susceptible to develop psychopathology-like phenotype following early-life stress.

Taken together, the literature shows a relationship between anxiety-like, aggressive behaviors, coping style, social interactions and energy metabolism. Therefore, our model of differential stress responsiveness offers a potent tool for the investigation of the correlations between biobehavioral phenotypes and metabolism. We hypothesized that our selection for stress responsiveness might be related to differences in energy metabolism, that social interactions between rats from the different lines might affect the lines differently, and that an overall shift in metabolism could be observed. However, the current literature linking responsiveness to stress, social behaviors and metabolism is limited. Thus, we decided to adopt an explorative approach in order to obtain descriptive information on the metabolism and social interactions between rats from the different lines as well as the effects of long-term effects of mixed-line social housing on metabolism. We first investigated calorimetric differences between the three lines in same-line dyads, and then we housed rats from the different lines together in order to analyze social interactions and long-term effects of mixed-line dyads on central and brain metabolism. We hypothesized that, rats' cohabitation with individuals that display high stress responsiveness will affect physiological and metabolic parameters and that, Low-line individuals might display an increased biobehavioral vulnerability. To test this, after validation of the phenotypic background of the lines, rats were house in mixed-line dyads, during one month and, we assessed their energy metabolism by indirect calorimetry, mitochondrial respiration in the NAc and medial prefrontal cortex (mPFC) and a number of physiological markers (adrenals, testis and gonadal fat).

We first report that rats from the different lines display differential basal metabolism. Then we show that mixed-line pairing induces differences in social behaviors. Finally, we report that long-term cohabitation with High-line rats alters the metabolism of the cagemate. In addition, rats from the high-responsive line exhibited lower mitochondrial respiration in the NAc and pairing with High-line animals increased mitochondrial respiration in Low- and Inter-lines.

Materials and Methods

i. Animals and experimental timeline

Experiments were performed on Wistar-Han rats derived from the breeding of three lines with differential responsiveness to stress (Walker et al., 2017). Those genetically selected lines were selected for low, intermediate or high glucocorticoid responsiveness to repeated stress during juvenile period (called 'Low-', 'Inter-' and 'High-line' rats respectively). Experimental rats were not exposed to the 'corticosterone-adaptation stress test' (CAST) procedure but were briefly handled during the juvenile period (P28-30) and then left undisturbed until experimental procedures at adulthood (P90). Animals were housed in a 12:12 h light-dark cycle (lights ON at 07:00) in a temperature- and humidity-controlled environment (22 ± 1 °C; 55% humidity ± 5%). Rats had *ad-libitum* access to food and water unless otherwise stated. All procedures were conducted in accordance with the Swiss National Institutional Guidelines on Animal Experimentation and were approved by the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

The same-line pairing experiment to assess calorimetry in normal housing condition used 24 males (n = 8 per line). After weaning rats were pair-housed in same-line dyads. Rats were tested in the indirect calorimetry cage at adulthood.

For the mixed-line pairing experiment 64 male rats were used (n = 16 Low-, 32 Inter- and 16 High-line rats). After weaning at postnatal day 21 (P21) rats were pair-housed in same-line dyads. Rats remained undisturbed in homecages until adulthood. Anxiety-like behaviors were assessed on the elevated plus maze (EPM) at P90, following by a reaction to novelty test at P96. In order to establish social competition between rats from the different lines, four mixed-line pairing were created: Low- vs Inter-line ('LvI'), Low- vs. High-line ('LvH'), Inter- vs. High-line ('IvH') and Inter- vs. Inter-line ('IvI'). The IvI pairs were used as control housing situation. At P112, for the territory-competition-test (TCT), rats were simultaneously introduced in a neutral novel cage and social interactions were recorded for 20 minutes. Rats were then left cohabitating in these mixed-line dyads for one month before indirect calorimetry (P150-160) and between P160-169 rats were sacrificed for mitochondrial respirometry. A timeline of the experimental procedures is illustrated in Figure 1.

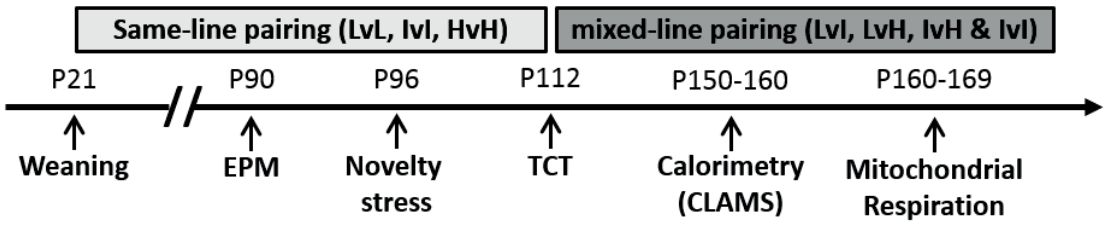

Figure 1: Experimental timeline for the mixed-line pairing experiment. After weaning (P21), rats were pair-housed in dyads with rats from the same genetic line (but not littermates). During same-line pairing anxiety-like behaviors and reaction to stress were tested with an EPM (P90) and a reaction to novelty test (P96), respectively. Rats were then paired with animals from different lines for territory competition test (TCT, P112). Rats were left in mixed-line dyads for one month of cohabitation before calorimetric measurement with the CLAMS system at P150-160 and mitochondrial respirometry of mPFC and NAc (P160-169). Dyads abbreviations during mixed-line pairing: "LvI" = Low- vs. Inter-line, "LvH" = Low- vs. High-line, "IvH" = Inter- vs. High-line, "IvI" = Inter- vs. Inter-line.

ii. Elevated plus maze (EPM)

When rats reached adulthood, anxiety-like behaviors were assessed in the EPM. This apparatus consists of an elevated (60 cm above the floor) plus-like maze with two (opposite) enclosed and two open arms (10 × 50 cm). The enclosed arms were surrounded by walls (50 cm high). Each animal was placed in the center of the maze facing a closed arm and allowed to explore for 5 min with the cumulative time spent in the open and closed arms measured. A low amount of time spent into the open arms was interpreted as an anxiety-like response (Pellow et al., 1985). The lighting was maintained between 14-16 lx on the open arms and 4-6 lx in the closed arms. The maze was cleaned with a 5% ethanol solution and dried between animals.

iii. Corticosterone response to novelty

We used a reaction to novelty test to measure plasma CORT reactivity as previously described (Veenit et al., 2013; Huzard et al., Chapter 1). Immediately after 25 min exposure to a novel environment (a circular plastic bucket: Ø = 40 cm, height = 50 cm), tail blood was sampled and rats were returned to their homecage. Animals from the same homecage were simultaneously tested in adjacent buckets and buckers were cleaned with a 5% ethanol solution and dried before placing the animals.

iv. Territory competition test (TCT)

Rats from the different lines were weight-matched in order to avoid facilitation of dominance based on body weight (Cordero and Sandi, 2007; Hollis et al., 2015). Animals were marked on their fur, using a permanent marker, for identification and placed, in pairs, in a neutral and clean cage without food or water, for 20 minutes. During TCT both rats spontaneously displayed offensive behaviors (Benus et al., 1992). Social encounters were video recorded and analyzed for exploratory, social, grooming, aggressive (van der Kooij et al., 2017) and defensive behaviors (rat in submissive posture without active aggression from the opponent). The total duration of offensive behaviors from each rat was calculated as the sum of the different agonistic behaviors expressed (offensive upright, lateral-threat and keeping-down) as previously described (Koolhaas et al., 2013).

v. Indirect calorimetry

In the control experiment with same-line pairing animals, 24 animals were tested (n = 8 per line) and 48 animals (n = 48: 13 Low-, 22 Inter-, 13 High-line rats) from mixed-line dyads were tested for indirect calorimetry. Metabolic rates of oxygen (O_2) consumption and carbon dioxide (CO_2) production were measured by indirect calorimetry, using the Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbia Instruments, Columbus, Ohio). This technique relies on the fact that all the O_2 consumed and CO_2 produced are due to the oxidation of three major energy substrates: fats, carbohydrates and proteins (Jéquier et al., 1987). The combination of an oxygen (Zirconia Oxide based) and carbon dioxide (high-speed single beam NDIR) sensors allowed precise assessment of the volume of oxygen (VO_2) inhaled and the volume of carbon dioxide (VCO_2) exhaled. Rats were acclimatized to individual cages for 24 h before recording, and then underwent 24 h of recording. The heat production (energy expenditure (EE)) was calculated using the following equation: $EE = 3.815 \times VO_2 + 1.232 \times VCO_2$ (Elia and Livesey, 1992) where heat is measured in $Kcal.h^{-1}$, VO_2 and VCO_2 are expressed in $l.kg^{-1}.h^{-1}$ and body weight is measured in kg. The respiratory-exchange-ratio (RER), an estimate of the respiratory quotient (RQ), was calculated as follow: $RER = VCO_2 / VO_2$. RER changes depending on the energy source the animal is using. When carbohydrates (CHO) are the only substrate being oxidized, the RER is 1.0, and when only fatty acids are used as fuel the RER is 0.7. Activity was measured on the horizontal and vertical directions by infrared beams counting infrared beam breaks using an Opto-Varimetrix-3 sensor system (Columbus Instruments, Columbus, OH). Indirect calorimetry was performed with a computer-controlled calorimetry system (Oxymax; Columbus Instruments). CLAMS data were extracted using CLAX software (Columbus Instruments) and data were extracted for further statistical analyses.

vi. Mitochondrial respirometry

After recovery from the calorimetric recording, 4 dyads from each mixed-line pairing combination were by rapid decapitation, and the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) were dissected out, weighed, and processed as previously described (Hollis et al., 2015). Brain samples were placed in a petri-dish on ice with 2 mL of relaxing solution (2.8 mM Ca_2K_2 EGTA, 7.2 mM K_2 EGTA, 5.8 mM ATP, 6.6 mM $MgCl_2$, 20 mM taurine, 15 mM sodium phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol and 50 mM MES, pH = 7.1) until further processing. Tissue samples were then gently homogenized in ice-cold respirometry medium (MiR05: 0.5 mM EGTA, 3mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose and 0.1% (w/v) BSA, pH=7.1) with an Eppendorf pestle. Then, 2 mg of tissue were used to measure mitochondrial respiration rates at 37°C using high-resolution respirometry (Oroboros Oxygraph 2K, Oroboros Instruments, Innsbruck, Austria). A multi-substrate protocol was sequentially used to explore the numerous components of mitochondrial respiratory capacity. To measure the respiration due to oxidative phosphorylation, we added substrates for the activation of specific complexes. Thus, oxygen flux due to complex I activity (CI) was quantified by the addition of ADP (5 mM) to a mixture of malate (2mM), pyruvate (10mM) and glutamate (20mM), followed by the

addition of succinate (10 mM) to subsequently stimulate complex II (CI+II). We then uncoupled respiration to examine the maximal capacity of the electron-transport-system (ETS) using the protonophore, carbonylcyanide 4 (trifluoromethoxy) phenylhydrazone (FCCP; 0.2 µM). We then examined consumption in the uncoupled state due solely to the activity of complex II by inhibiting complex I with the addition of rotenone (0.1µM; ETS-CII). Finally, electron transport through complex III was inhibited by adding antimycin (2 µM) to obtain the level of residual oxygen consumption (ROX) due to oxidating side reactions outside of mitochondrial respiration. The O_2 flux obtained in each step of the protocol was normalized by the wet weight of the tissue sample used for the analysis and corrected for ROX. All respiration experiments were performed in the morning over 2 weeks.

vii. Organ collection

Four dyads from each mixed-line pairing were decapitated, and dissection took place immediately afterwards. The brain was removed and snap frozen in isopentane kept on dry ice. Further dissection was performed to collect and weigh the adrenal glands, the testes as well as gonadal fat. All tissues were immediately frozen in liquid nitrogen and subsequently stored at -80 °C.

viii. Plasma corticosterone concentration measurement

Blood samples were collected in heparinized test tubes (Sarsted, Nümbrecht, Germany), and the blood was separated by centrifugation (4 °C, 4 min 10000 rpm). The plasma was collected and stored at -20 °C until corticosterone levels were assayed by ELISA (Enzo Life Sciences) following manufacturer's instructions.

ix. Statistics

Data were analyzed using Graphpad Prism (version 7.02, San Diego) for one and two-way ANOVAs and paired t-test and we used SPSS (version 21, Chicago, USA) for mixed-model and ANCOVAs analyses. Results are presented as the mean ± standard error of the mean (SEM). Prior to statistical testing, data were checked for the presence of outliers with the 'robust regression and outlier removal method' (ROUT, Q = 1%) and outliers were removed. Samples sizes (n) and the number of rats excluded by the ROUT method are indicated in figure captions. One-level comparisons (e.g. body weight, food intake and RER) were performed by one-way ANOVAs with the lines as between-subjects factor (testing line effect). Activity during calorimetry was analyzed with 2-way repeated-measures ANOVAs with line as between-subjects factor. Activity measures in the CLAMS were analyzed with repeated-measure two-way ANOVA with line as between-subjects' factor and time-blocks as within-subject factor. Comparison of parameters for mixed-line pairing effects were performed with individual t-tests (and one-way ANOVA for Inter-line animals) between rats from the same line in different dyads as well as t-tests against the control (IvI) pairing condition. Since mitochondrial respirometry experiments were performed in blocks across days, a mixed linear model was created, including blocks as a random effect and the lines as fixed effects. Energy expenditure was analyzed with ANCOVAs statistics in order to control for body weight (BW) differences (regression-based adjustment for BW) as recommended by Tschöp and colleagues (2011). Here, the first step consisted of controlling that the main variable (the genetic lines) does not interact with the covariate (body weight). In other words, the slopes of the linear regressions between BW and EE of the different lines have to be similar (parallel lines). If the interaction term 'lines x BW' was significant the ANCOVA analysis was not applicable. If the interaction term 'lines x BW' was not significant, the full factorial model was applied to compute the ANCOVA statistics and determine the line effect. ANCOVA analysis centered the data on the mean BW and computed the 'estimated marginal means' of the variable. The estimated marginal means (with SEM obtained from the mixed-model) of mitochondrial respirometry and energy expenditure are represented in Figure 5F & 6.. Statistical significance level was set at $p < 0.05$.

Results

i. Same-line dyads:

RER and EE differences between the three lines

First, using control pairing conditions (same-line dyads), we established the energy expenditure of rats from the three lines during 24 h of indirect calorimetry. Body weight was measured before initiating calorimetry recordings (Figure 2A) and there was no line effect ($F_{2,21} = 0.17$, $p = 0.847$). Food consumption during 24 h in the CLAMS system (Figure 2B) did not differ between the lines ($F_{2,21} = 0.31$, $p = 0.738$).

Activity measures were analyzed in blocks of 6 hours with two dark- and two light-phase blocks (Figure 2C). There was a significant line effect on the total activity ($F_{2,21} = 4.58$, $p = 0.022$) and a significant interaction between 6h-blocks and lines ($F_{6,63} = 6.49$, $p < 0.001$). During the first light-phase block, Low-line rats were less active than High-line animals ($t_{84} = 2.03$, $p = 0.046$). During the first 6 h of the dark-phase, Inter-line animals were more active than Low- ($t_{84} = 4.08$, $p < 0.001$) and High-lines($t_{84} = 3.43$, $p < 0.001$). During the last 6 h of the dark-phase, High-line animals were more active than Low- ($t_{84} = 4.06$, $p < 0.001$) and Inter-line rats ($t_{84} = 3.8$, $p < 0.001$). There were no differences in activity during the last light-phase block ($p > 0.233$). RER, an indirect measure of metabolic fuel selection (Figure 2D), was significantly different between the lines ($F_{2,21} = 4.58$, $p = 0.022$). Low-line animals had higher RER than Inter- ($t_{21} = 2.22$, $p = 0.038$) and High-lines ($t_{21} = 2.89$, $p = 0.009$).

Energy expenditure is known to depend on body composition and BW (Tschöp et al., 2011). As shown in Figure 2E, there was a positive linear correlation between BW and EE (red line; $r = 0.78$, $p < 0.001$). Analysis of EE was performed with an ANCOVA with BW as covariate (Tschöp et al., 2011). The interaction between lines x BW was significant ($p = 0.021$) meaning that the ANCOVA analysis was not applicable. In order to analyze the differences in energy expenditure, Low- and High-lines were compared separately with Inter-line rats (Figure 2F and 2G). ANCOVA analyses were applied and the line x BW interaction were not significant (LvI: $p = 0.0829$, IvH: $p = 0.473$). Full-factorial ANCOVA model was used to determine the differences between the lines when corrected for BW differences. In the Low vs. Inter comparison (Figure 2F), ANCOVA was applied on data centered on the mean BW (dash line, BW = 390.6) and the estimated marginal means were calculated (Low = 2.867 + 0.037; Inter = 2.770 ± 0.037). There was a tendency for a line effect ($p = 0.094$) between Low- and Inter-line animals. In the Inter vs High comparison (Figure 2G) data were centered on the mean BW value (dash line, BW = 384.6) and the estimated marginal means were computed (Inter = 2.756 ± 0.028, High = 2.868 + 0.027). There was a significant line effect ($p = 0.014$) between Inter- and High-lines, with High-line rats having higher energy expenditure.

Taken together, those results showed that in same-line pairing conditions, rats had similar BW and food intake, but differences in locomotor activity during the active phase. Low-line rats had higher RER values, indicative of a higher use of carbohydrates as fuel than Inter- and High-lines. Moreover, High-line rats had a higher energy expenditure than Inter-line rats.

Rats from the three line in control same-line pairing conditions were tested for active/passive coping in a shock-probe burying test (details in Supplementary methods). It was observed in a shock-probe-burying test that rats from the Low-line had a tendency to do more active burying than the Inter- and High-line rats (Supplementary Figure 1A). However, there was no difference in passive freezing time during this test (supplementary Figure 1B).

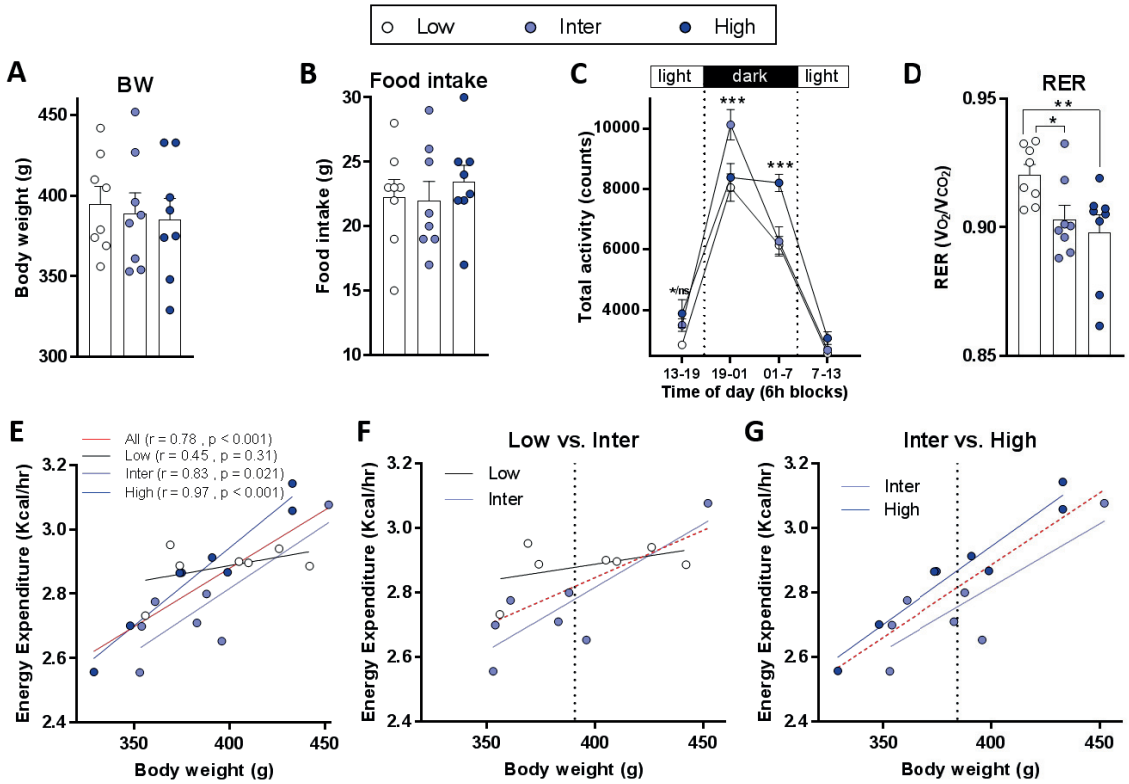

Figure 2: Differences in RER and EE between rats from the different lines in same-line pairing conditions. A, Rats from the three lines had similar BW at the beginning of the indirect calorimetry session (p = 0.847). **B,** The three lines had similar food intake during 24 h or calorimetry recording (p = 0.738). **C,** Rats from the three lines had different pattern of activity during the dark-phase. Inter-line animals were more active during the first half of the dark-phase while High-line rats were more active during the second half. In the block '13-19': */ns indicates comparisons between High-/Inter- vs. Low-line animals. In blocks '19-01' and '01-7', comparisons are made between Inter- or High-lines and the 2 other lines respectively. **D,** During the 12 h dark-phase, Low-line rats had higher RER than Inter- and High-line rats. **E,** BW and EE were linearly correlated (red line: r = 0.78). When EE was analyzed with BW as a covariate, there was an interaction between lines and BW (as seen by different slopes between the linear regressions of Low- and High-lines). 2 outliers were excluded: 1 Low-, 1 Inter-line rats. **F,** ANCOVA analysis (with BW as covariate) applied to LvI pairs showed a tendency for higher EE in Low-animals. **G,** ANCOVA analysis (with BW as covariate) applied to IvH pairs showed that High-line rats had higher EE than Inter-line animals. Number of animals was n = 8 for each line. Statistical p-values (*** p < 0.001, ** p < 0.01, * p < 0.05 and # p < 0.1).

ii. Mixed-line dyads

In the second experiment, we first validated the biobehavioral phenotype of the three line under same-line pairing by assessing anxiety-like behaviors and CORT response to novelty. Then rats were paired in mixed-line dyads for metabolic assessment with indirect calorimetry and mitochondrial respirometry. The results are first reported for the three lines and then each line is analyzed according to their mixed-line pairing.

High-line rats were more anxious and had higher CORT response to novelty

Before studying the consequences of mixed-line pairing on the rats, we first validated the phenotype of the rats from the different lines in term of anxiety-like behaviors and CORT response to novelty, as previously shown (Walker et al., 2017; Huzard et al., chapter 1). Rats were tested on an EPM to assess anxiety-like behaviors (Figure 3A) and there was a line effect on the amount of time spent in the open arms ($F_{2,60}$ = 5.17, p = 0.0085). Rats from the High-line spent less time in the open arms than Low- (p = 0.002) and Inter-line rats (p = 0.028). There was no difference in open arms time between Low- and Inter-line rats (LvI, p = 0.169). In order to assess stress response to novelty at adulthood,

rats were introduced 25 min in a bucket and blood was sampled for CORT measurements (Figure 3B). There was a significant line effect on the production of CORT ($F_{2,61}$ = 22.7, p < 0.001), and High-line rats had higher CORT production than Low- (p < 0.001) and Inter-line animals (p < 0.001). There was no difference in CORT between the Low- and Inter-lines (p = 0.5).

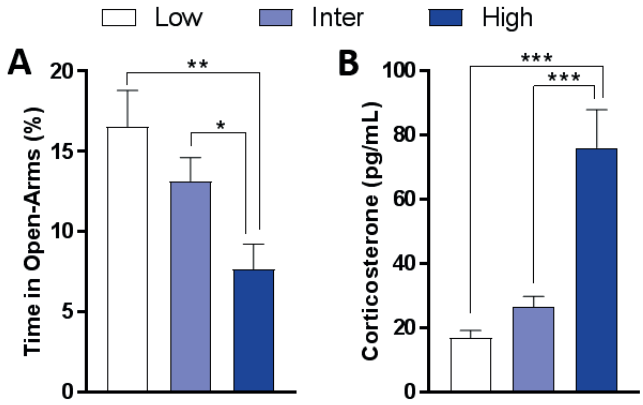

Figure 3: Rats from the High-line were more anxious and had higher CORT response to novelty. A, Rats from the High-line spent less time in the open arms than the Low- (p = 0.002) and Inter-lines (p = 0.028). One rat from the Inter-line was excluded by outlier analysis. **B,** High-line rats had higher CORT production than Low- (p < 0.001) and Inter-line animals (p < 0.001). Low (n = 16), Inter (n = 32) and High (n = 16). Statistical p-values (*** p < 0.001, ** p < 0.01, * p < 0.05).

Behavioral differences during competition for territory between the three lines

The territory competition test was used to assess behavioral and social interactions between rats from the different lines. During 20 min of TCT, affiliative (social investigation and aggression) as well as non-affiliative (cage exploration and self-grooming) behaviors were determined (Figure 4A-B) and analyzed according to the mixed-line dyads (Figure 4C-E). In order to determined mixed-line pairing effects, data from each line was compared to rats from the same line but housed in other dyads. All groups were also compared to the control IvI dyads.

During the TCT, there was a line effect ($F_{2,61}$ = 3.32, p = 0.043) in the amount of time rats spent exploring the neutral novel cage/territory (Figure 4A), with Inter-line rats exploring more than Low-line rats (p = 0.02). There was a tendency for a difference between Inter- and High-lines (p = 0.093). There was no difference in self-grooming between the lines (Figure 4A: $F_{2,60}$ = 0.63, p = 0.54). Social investigation of cagemates during TCT was calculated using sum of social sniffing, grooming and anogenital exploration (Figure 4B). There was no line effect on social investigation ($F_{2,61}$ = 1.44, p = 0.244). The different agonistic behaviors (offensive upright, keeping-down and lateral threats) of the animals were summed and analyzed as an aggressive behavior parameter. There was a line effect ($F_{2,56}$ = 5.79, p = 0.005) on the amount of aggressive behaviors (Figure 4B). High-line animals were more aggressive than the Low- (p = 0.005) and Inter-lines (p = 0.033) independently of pairing. Finally, there was no main differences in self-grooming (Figure 4A) between the lines ($F_{2,60}$ = 0.63, p = 0.54).

Mixed-line pairing altered behaviors during social competition for territory

Behaviors expressed during TCT from each line were analyzed according to their mixed-line pairing. We observed that there were effects of social pairing on social investigation time (Figure 4C), with Low-line animals from LvH pairs exhibiting less social investigation than High-line cagemates (t_{13} = 2.83, p = 0.014) and Low-line rats from LvI pairs (t_{13} = 5.94, p < 0.001). Within LvI pairs with Low-line rats exhibited more social-behaviors than Inter-line rats (t_{14} = 2.13, p = 0.051). High-line rats were more social in LvH pairs than in IvH pairs (t_{13} = 2.67, p = 0.019). A one-way ANOVA between Inter-line rats from mixed-line pairings, did not show any differences in social behaviors ($F_{2,29}$ = 0.2, p =

0.82). Data from aggressive behaviors during TCT (Figure 4D) showed that there was an effect of pairing ($F_{2,55}$ = 3.49, p = 0.022).

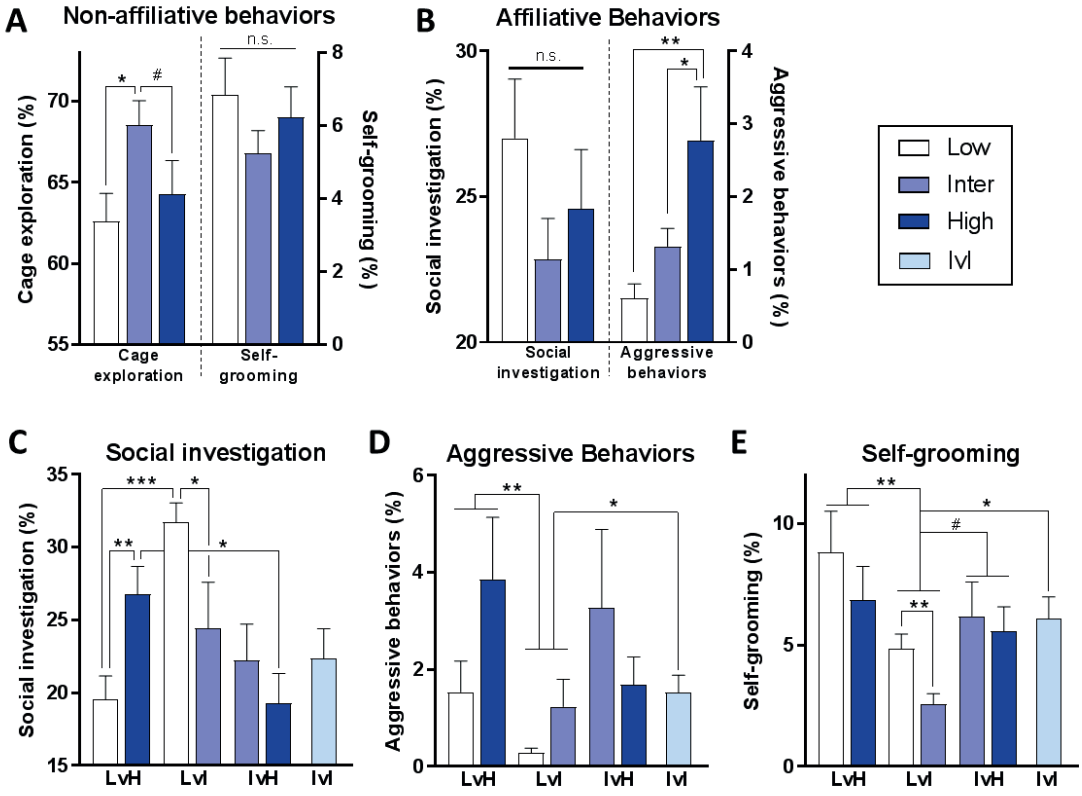

Figure 4: Differences in affiliative and non-affiliative behaviors during social competition according to social housing context. **A,** Inter-line rats explored the cage more than both Low- (p = 0.02) and High-lines (p = 0.093). There was no main difference in self-grooming. One Low-line animal was excluded by outlier analysis. **B,** There was no line effect (p = 0.244) on social interactions. High-line animals were more aggressive than Low- (p = 0.005) and Inter-lines (p = 0.033). Outlier analysis removed 2 Low- and 3 High-line rats. **C,** In LvH pairs, Low-line rats did less social investigation than High-line rats (p = 0.014). Low-line rats investigated their cagemate less in LvH than in Lvl pairs (p < 0.001). Within Lvl pairs: Low-line rats exhibited more social-behaviors than Inter-line rats (p = 0.051). High-line rats were more social in LvH pairs than in IvH pairs (p = 0.019). One Low- and one High-line rats were removed by outlier analysis. **D,** Lvl pairs were less aggressive than Ivl (p = 0.042) and LvH pairs (p = 0.003). Additionally, LvH pairs had a tendency for more aggression than the IvH pairs (p = 0.071). **E,** Pairs with Low- and Inter-line animals self-groomed less than LvH, IvH and Ivl pairs (p = 0.006 and p = 0.025, p = 0.057 respectively). There was a difference within Lvl pairs with Low-line rats self-grooming more than Inter-line rats (p = 0.009). Except when outliers were excluded, there were 16 Low- (8 Lvl, 8 LvH), 32 Inter- (8 Lvl, 8 IvH and 16 Ivl) and 16 High-line rats (8 LvH, 8 IvH). Statistical p-values (*** p < 0.001, ** p < 0.01, * p < 0.05 and # p < 0.1).

Rats from Lvl pairs were less aggressive than Ivl (p = 0.042) and LvH pairs (p = 0.003). Additionally, LvH had a tendency for higher aggression than the IvH pairs (p = 0.071). Analysis of self-grooming (Figure 3E) showed that there were differences between mixed-line pairings ($F_{3,60}$ = 3.07, p = 0.035). Rats in Lvl pairs self-groomed less than rats from LvH (Lvl vs. LvH, p = 0.006), IvH (Lvl vs. IvH, p = 0.025) and Ivl pairs (Lvl vs. Ivl, p = 0.057). There was a difference within Lvl pairs with Low-line rats self-grooming more than Inter-line rats (t_{14} = 3.05, p = 0.009).

Taken together, these results showed increased activity in Inter-line rats and higher aggressiveness in High-line animals. Pairing with High-line individuals led to higher aggressive behaviors and self-grooming while Low-line rats displayed reduced social-investigation.

Based on aggressive behaviors displayed during TCT and amount of time drinking in a water-competition test (WCT), we assessed social status of the rats from the differences dyads (see Supplementary Materials). Due to low number of animals, Chi-square statistics were not significant (Supplementary Figure 2). Briefly, there was no difference in social status between Low- and Inter-lines animals, there was a tendency for High-line rats to become dominant over Low-line rats and Inter-line rats had a tendency to dominated High-line rats.

Differences in RER and EE between the three lines

Then, energy expenditure and metabolic fuel, measured by RER, were determined for rats from the three lines following one month of social pairing with rats from the other lines. After 24 h of habituation, indirect calorimetric recording was performed for 24 h.

The BW was measured before and after the 24 h CLAMS session. There was no line effect on basal BW before recording (Supplementary figure 3A) or on food intake (Supplementary Figure 3B). However, there was a difference in BW evolution (Figure 5A) between the lines ($F_{2,45} = 4.92$, $p = 0.012$). High-line rats lost more BW than Low- ($t_{45} = 3.02$, $p = 0.004$) and Inter-lines ($t_{45} = 2.4$, $p = 0.021$). One-sample t-tests were performed to test the BW evolution against zero (no change in BW) and it appeared that both Inter- ($t_{21} = 3.1$, $p = 0.006$) and High-line rats ($t_{12} = 4.75$, $p < 0.001$) had a significant decrease in BW, but not Low-line rats ($t_{12} = 0.63$, $p = 0.54$).

Then, we established which fuel was used by the organism with the RER parameter. There was a tendency for a line effect on RER values (Supplementary figure 3E: $F_{2,45} = 2.83$, $p = 0.069$) with High-line animals having lower RER than both Low- ($t_{45} = 2.31$, $p = 0.026$) and Inter-line rats ($t_{45} = 1.77$, $p = 0.083$).

In order to analyze energy expenditure values, the influence of body weight was corrected by ANCOVA analysis. There was a linear positive correlation between BW and EE (Figure 5D: red line; $r = 0.35$, $p = 0.016$). In order to compare the energy expenditure between the lines, an ANCOVA analysis was performed with BW as covariate. There was no interaction between lines and BW ($p = 0.295$) and there was a significant line effect on the EE values ($p = 0.018$). Comparison of the estimated marginal means controlled for BW (dashed line, BW = 403.4) showed significant differences between Low- and Inter-line animals (Low = 2.829 ± 0.062 vs. Inter = 2.665 ± 0.058: $p = 0.005$). There was no difference between High-line rats and the two other lines (High = 2.73 ± 0.06; $p > 0.115$).

Mixed-line pairing affected RER and EE

In order to assess the effects of social pairing with rats from other lines, data were separated according to their mixed-line pairing. There were differences in the BW evolution during 24 h of calorimetry (Figure 5B). The Low- and High-line rats from LvH pairs lost significantly more BW than their conspecifics paired with Inter-line rats ($p = 0.022$ and $p = 0.044$ respectively). In LvH pairs, Low-line rats lost less BW than High-line rats ($p = 0.058$). There were no effects of the mixed-line pairing on the BW of the animals except that rats in IvI pairing had higher BW than all other groups (Supplementary Figure 3C). There was no effect of mixed-line pairing on the food intake of the animals (Supplementary Figure 3D).

Then, RER values, analyzed according to mixed-line pairing (Figure 5C), showed that Low-line animals had higher RER in LvI pairs than in LvH dyads ($p = 0.037$). Moreover, both Low- and Inter-line rats paired together had higher RER than the control IvI dyads ($p = 0.003$ and $p = 0.017$ respectively). A two-way ANOVA showed that housing with High-line animals significantly decreased the RER of Low- and Inter-lines (Supplementary Figure 3F)

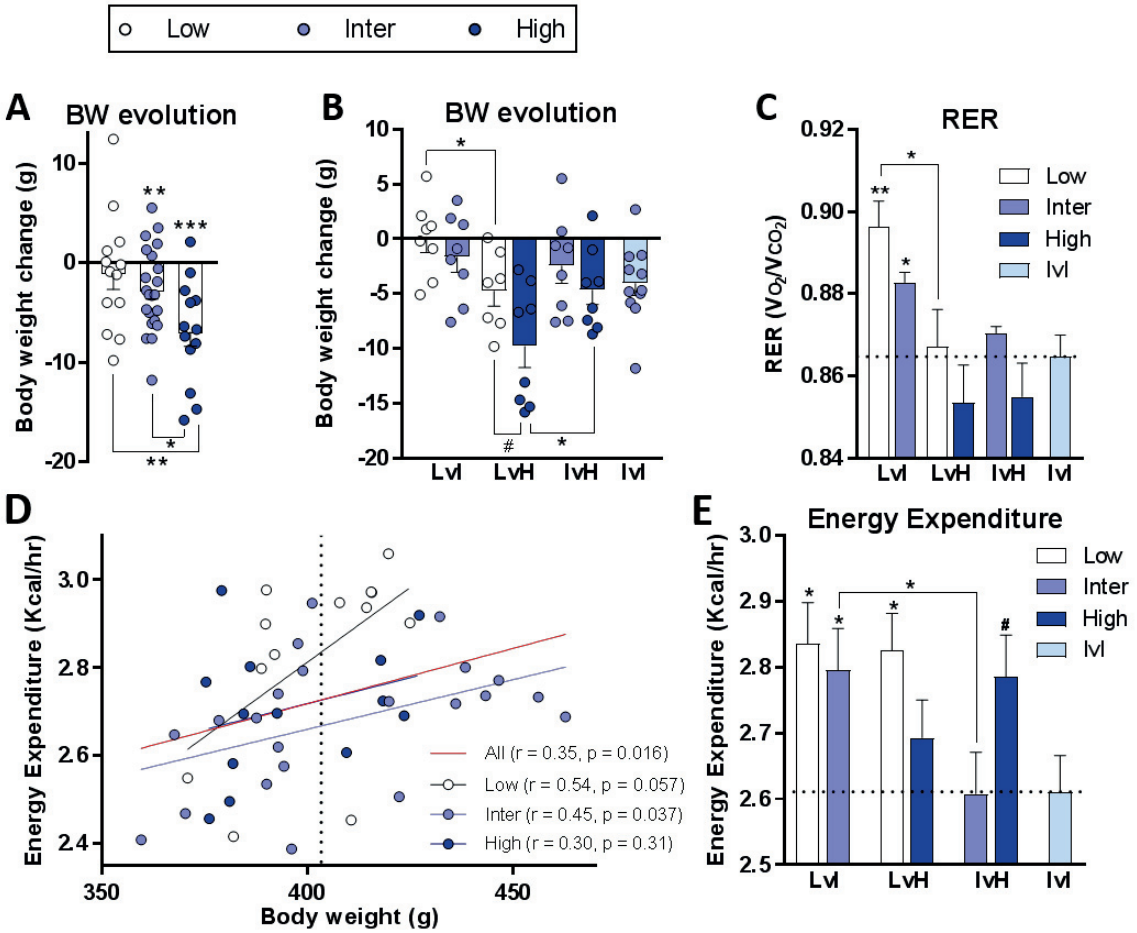

Figure 5. Differences in RER and EE according to lines and depending on social pairing during CLAMS recording. A, There was a higher body weight loss in High-line animals ($p < 0.05$). Symbols above Inter- and High-lines data represent significant differences from one-sample t-tests against 0: $p = 0.006$ and $p < 0.001$ respectively. **B,** Low- and High-line rats from the LvH pair had more important decrease in BW during calorimetry. (Low (LvH) vs. Low (LvI): $p = 0.022$; High (LvH) vs High (IvH): $p = 0.044$; Low vs High (LvH): $p = 0.058$). **C,** Low-line animals in LvI pairs RER than Low-line rats in LvH pairs ($p < 0.05$). IvI was compared to all other groups: there were significant differences with Low from LvI (**) and with Inter from LvI (*). **D,** When body weight is used as a covariate, Low-line rats have higher energy expenditure then Inter-line animals (see 'results' section for more details). **E,** Energy expenditure values estimated by ANCOVA (at BW = 403.4) were compared between mixed-line dyads. Inter-line rats have higher EE when paired with Low-line rats. IvI was compared to all other groups: there were significant differences with Low from LvI (*), Inter from LvI (*), L from LvH (*) and H from IvH (#).

The means of EE estimated by BW correction were compared for rats in mixed-line dyads (Figure 5E). Inter-line rats paired with Low-line animals showed higher EE than Inter-line animals in IvH ($p < 0.05$) and IvI dyads ($p < 0.05$). Low-line rats from both LvI and LvH pairs had higher EE than IvI animals ($p < 0.05$).

Altogether, those data showed that mixed-line pairing affected individuals in their respective behaviors and in their calorimetry measurements. Rats in LvH pairs were the ones that lost body weight at a highest level. Pairing with High-line rats reduced RER of Low-line animals and pairing with low-line increased RER of Inter-line rats. Energy expenditure of Inter-line rats was lower when paired with High- than with Low-line animals.

High-line rats had lower mitochondrial respiration in NAc and pairing with High-line animals increased mitochondrial respiration

The mitochondrial respirometry method allowed the analysis of O_2 consumption by mitochondria from freshly extracted mPFC and NAc from the three lines (Figure 6). There was no line effect on the mitochondrial respiration in the mPFC (Figure 6A; $F_{2,130} = 1.32$, $p = 0.271$). There was a significant line effect on the mitochondrial respiration in the NAc (Figure 6C; $F_{2,130} = 24.5$, $p < 0.001$). High-line rats had lower mitochondrial respiration than Low- ($t_{130} = 6.38$, $p < 0.001$) and Inter-line rats ($t_{130} = 5.89$, $p < 0.001$).

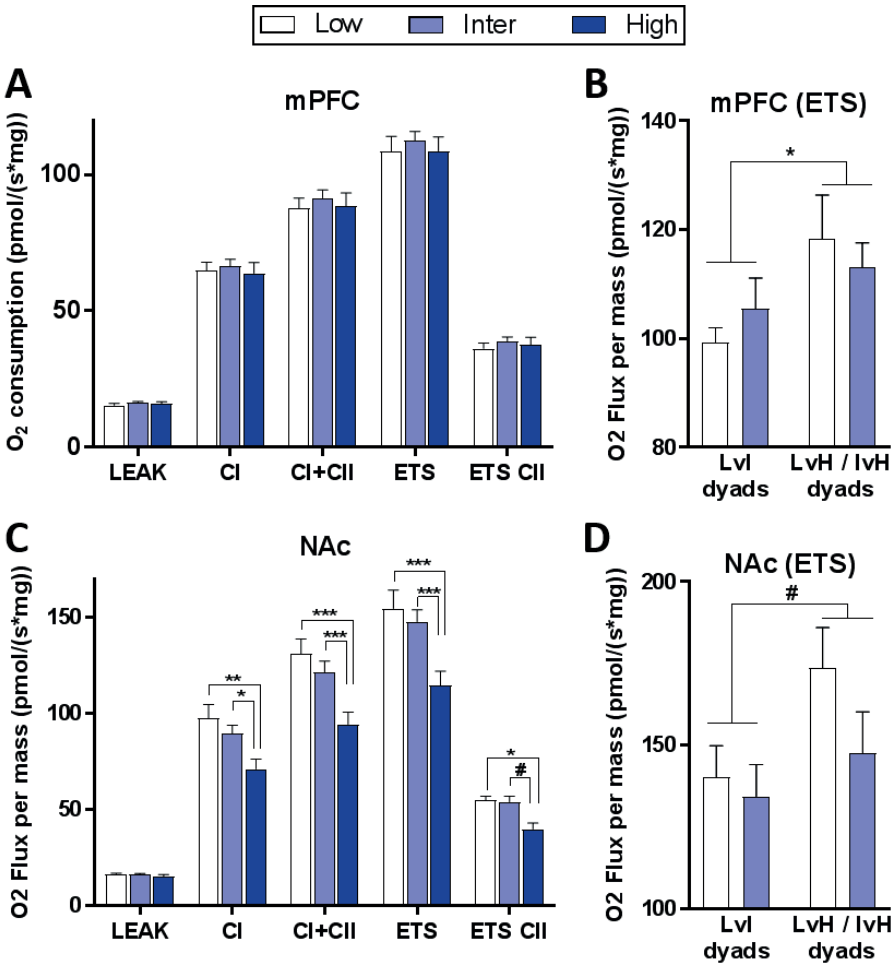

Figure 6. High-line animals had lower mitochondrial respiration in the NAc and pairing with High-line rats increased mitochondrial respiration. A, There was no difference in mitochondrial respiration in the mPFC of rats from the three lines ($F_{2,130} = 1.3$, $p = 0.271$). Low- (n = 8), Inter- (n = 14) and High-lines (n = 7). **B,** ETS value of mitochondrial respiration in mPFC was higher in Low- and Inter- rats when paired with High-line animals ($F_{1,12} = 5.69$, $p = 0.034$). Low- (n = 4 & 4), Inter-line (n = 4 & 4). **C,** There was a line effect on the mitochondrial respiration measured from NAc homogenates ($F_{2,130} = 24.5$, $p < 0.001$) with high-line animals having significantly lower respiration than Low- ($t_{40} = 6.38$, $p < 0.001$) and Inter-line rats ($t_{40} = 5.88$, $p < 0.001$). Low- (n = 7), Inter- (n = 14) and High-lines (n = 8). **D,** There was a tendency for a higher ETS value of mitochondrial respiration in the NAc in Low- and Inter-line rats when paired with High-line animals ($F_{1,11} = 4.44$, $p = 0.059$). Low- (n = 4 & 3) and Inter-lines (n = 4 & 4).

Then, data were analyzed according to mixed-line pairing condition and focusing on the effect on Low- and Inter-line animals when paired together (LvI pairs) or with High-line rats (LvH and IvH pairs respectively). There was a significant increase in mPFC ($F_{1,12}$ = 5.689, p = 0.034) and a statistical tendency in NAc ($F_{1,11}$ = 2.07, p = 0.059) mitochondrial respiration (Figure 6B & D respectively) when Low- and Inter-line rats were paired with High-line rats compared to LvI pairing. There was a tendency for an increase in mitochondrial respiration in the mPFC (Supplementary Figure 3G) of Low-line rats paired with High-line in comparison to Low-line rats in LvI pairs (p = 0.067). Low-lines rats in LvH pairs had a tendency for an increase in mitochondrial respiration in the NAc (Supplementary Figure 3H) in comparison to Low-line rats in LvI pairs (p = 0.081).

Taken together, those results showed that High-line rats had lower mitochondrial respiration in the NAc than Low- and Inter-lines. Moreover, Low- and Inter-line rats paired with High-line rats had a moderate increase in mitochondrial respiration in both mPFC and NAc.

Pairing with High-line animals reduced testis and gonadal fat weight of Low-line rats

In order to establish long-term physiological effects of the mixed-line pairing; gonadal fat, adrenal glands and testes were weighed and data were normalized to body weight of the animals (Figure 7). There were no differences in normalized weight of adrenals (Figure 7A, p = 0.22).

The normalized weight of the testes (Figure 7B) of Low-line rats differed according to their pairing condition. Low-line rats paired with High-line animals had lighter testes than Low-line animals in LvI pairs (t_6 = 4.77, p = 0.003). In LvH pairs, Low-line rats had lighter testes than their High-line cage-mates (t_6 = 5.58, p = 0.002). The analysis of gonadal fat results (Figure 7C) gave similar results. Low-line rats in LvH pairs had less gonadal fat than Low-line animals in LvI pairs (t_6 = 3.99, p = 0.007). In LvH pairs, Low-line rats had relatively less gonadal fat than their High-line cagemates (t_6 = 5.88, p = 0.001).

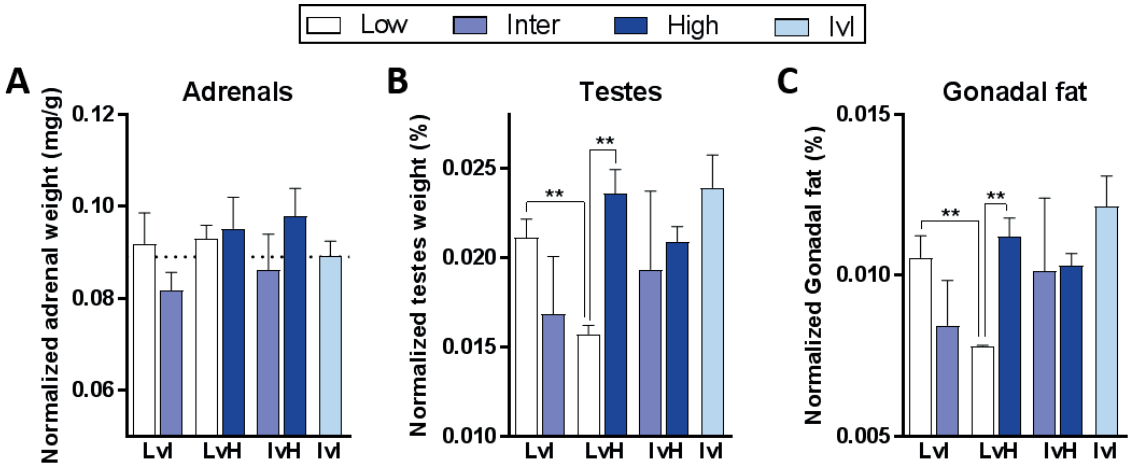

Figure 7. Long-term effects of social pairing with High-line animals decreased testes and gonadal fat of Low-line animals. A, There was no difference in the weight of adrenal glands, normalized to BW, between the three lines (t-tests, p > 0.221). **B,** Testes weight differed between Low- and High-line animals from LvH pairs (t_6 = 5.58, p = 0.001). Interestingly, Low-line rats had lower testes after LvH pairing than after LvI pairing (t_6 = 4.77, p = 0.003). **C,** Weight of gonadal fat, normalized to BW, differed between Low- and High-line animals from LvH pairs (t_6 = 5.88, p = 0.001). Interestingly, Low-line rats had less gonadal fat after pairing with high- than with Inter-line rats (t_6 = 3.98, p = 0.007).

Discussion

Here, we characterized a number of metabolic markers of rats with constitutive differential responsiveness to stress and we demonstrated that social housing between cagemates with different biobehavioral phenotypes had effects on their social behaviors and energy metabolism. We first showed, in same-line pairing conditions, that the selection for differential stress responsiveness led to differences in central energy metabolism. Then, using mixed-line pairing, we revealed that the biobehavioral background of the rats affected their social interactions on a first encounter and that Low-line rats were more susceptible to change social behaviors when paired with High-line rats. Moreover, we showed that after one month of mixed-line pairing with High-line animals, central and brain metabolic parameters (calorimetry and mitochondrial respiration) were affected in Low- and Inter-lines. Finally, Low-line rats also exhibited change in physiological markers (testis and gonadal fat) after social housing with High-line animals.

Rats from the three lines in same-line dyads had differences in metabolism

First, as a control situation, we determined the metabolism of rats housed in same-line dyads with indirect calorimetry. Low-line rats had higher RER than the two other lines, illustrating a higher use of carbohydrates as fuel. The hormones released during the stress response are interacting with major regulators of energy metabolism and appetite. For example, adrenalectomy of rats attenuated weight gain, food intake and decreased RER, indicating a higher use of fatty acids in the absence of CORT (Schiffer and Wertheimer, 1947; Hamelink et al., 1994) and another study showed that the release of CORT was followed by secretion of neuropeptide Y and ghrelin, increasing food intake and consumption of carbohydrates over fat (Patterson et al., 2013). Taken together, our findings do not support a decrease in RER related to low CORT production and although precise mechanisms are unknown, we suggest that the Low-line have a dysregulation in the control of central metabolism. The Low-line animals may have differences in metabolic processes used, potentially involving ghrelin pathways in the hypothalamus but further investigation would be required to identify the differences using brain mRNA screening (e.g. NPY and ghrelin receptors). Assessing tissue specific protein expression and circulating levels of these peptides would be also necessary before making solid inferences.

Indirect calorimetry results showed that Inter-line rats displayed lower energy expenditure than the other lines, although the difference between Inter- and Low-lines was a statistical tendency. The differences in metabolic rate cannot be accounted for differences in food intake or locomotor activity since there was no difference in food intake and Low-line rats had lower activity. A previous report suggested that mice having increased heat production without activity changes might have differences in leptin or CRHR2 receptors in the VMH leading to differences in sympathetic control of thermogenesis and heart rate (Kuperman et al., 2010). However, no differences in sympathetic nervous system or temperature were observed between the lines, Inter-line rats had significantly lower resting heart rate than the two other lines and no differences in activity (Huzard et al., Chapter 1). The heart rate has been showed to be an estimate of energy expenditure (Keytel et al., 2005), thus, the lower energy expenditure in Inter-line animals might be due to their higher vagal tone and lower resting heart rate.

Rats from the three lines displayed different social behaviors in the mixed-line dyads

Secondly, we analyzed rats from the mixed-line housing conditions. We established social behaviors during competition for a novel territory and, in line with previous reports (Walker et al., 2017; Walker and Sandi, 2018), High-line rats exhibited more aggressive behaviors than the other lines. Additionally, pairs including High-line rats displayed more aggressive behaviors and more self-grooming. In the literature, self-grooming was presented as a behavior expressed by dominant rats after attacking their opponent while subordinates did not exhibit any self-grooming (Miczek, 1974). Other studies reported that self-grooming followed an inverted U-shape relative to arousal and stress level (Song et al., 2016) and that it was a nonsocial and non-affiliative behavior (Govic et al., 2009).

Furthermore, during TCT, Low-line rats showed less social behaviors when paired with High-line rats. Taken together, we suggest that pairing with High-line rats increased social competition and aggressiveness in the dyads, leading to a higher, but not extreme, arousal state and subsequently inhibiting social behaviors. Thus, we emphasize that pairing with rats selected for high stress responsiveness modified social interactions and that Low-line rats were more susceptible to this social context. Previous studies showed that behavioral flexibility (changing behavioral response) is an important fitness factor that requires energy. Indeed, energy required to change one's phenotype might exceed adaptive advantages (Stamps and Groothuis, 2010) and saving energy by maintaining one's phenotype in a challenging environment might increase fitness (Réale et al., 2007). In that respect, pairing rats with different biobehavioral phenotype together, might affect their ability to adapt and long-term effects on energy metabolism might be observed. Finally, whether Low-line rats were more susceptible to changes in social pairing, and adopted a flexible strategy, might have been visible in behavioral changes and increased energy use.

Pairing in mixed-line dyads differently affected the metabolism of the three lines

After one month of housing in mixed-line dyads, during indirect calorimetry recording, we observed that Low-line rats did not lose body weight while animals from the High- and Inter-lines did. Interestingly, both Low- and High-line rats lost more BW than in LvH pairs than when paired with Inter-line animals. However, there were no differences in food intake or in initial body weight between the lines. The relation between stress and body weight is not consistent in the literature and a number environmental and metabolic processes have been showed to be involved. On the one hand, stress and glucocorticoids have been associated with increased food intake and body weight loss (Patterson et al., 2013), increased use of carbohydrates to meet rapid energetic challenges (Patterson et al., 2013) and increased preference for food rich of fats and highly palatable in rodents (Dallman et al., 2003, 2005; Pecoraro et al., 2005). On the other hand, repeated stress decreased body weight and energy efficiency in stressed animals, but not always linked to a decrease in food intake (van Leeuwen et al., 1997; Rybkin et al., 1997; Michel et al., 2005). Low-line rats are less responsive to stress which might have protected them from body weight loss induced by calorimetric recording stress. However, pairing with High-line rats increased susceptibility of Low-line rats to lose body weight and reduced their RER leading to higher use of fats as fuel. Pairing with Low-line rats increased RER, and carbohydrates metabolism, of Inter-line rats while RER from the High-line did not change with mixed-line housing. It seems that mixed-social pairing induced a normalization of the phenotypes on RER measurements, with animals from the Low- and Inter-lines being more susceptible to converge to their cagemate phenotype. A potential explanation might come from the social contagion phenomena, describing that the negative emotional consequences associated with life stress exposure in an individual can affect the emotional state of social partners (Carnevali et al., 2017). Indeed, they demonstrated that cohabitation with a rat that experienced chronic social defeat stress, substantially disrupted social behaviors and induced short-lasting cardiac autonomic activation and HPA axis hyperactivity (Carnevali et al., 2017). Taken together these results are of particular interest with our lines showing biobehavioral differences in emotional states, stress responsiveness and heart rate variability (Huzard et al., Chapter 1). Further investigation is required to test whether an emotional "state-matching" between cagemates of the different lines occurred (Meyza et al., 2017) and whether the normalization involved the entire biobehavioral phenotype, including anxiety-like behaviors, stress responsiveness and the autonomic modulation of heart rate.

Furthermore, the energy expenditure of rats in mixed-line dyads also showed alterations. Inter-line rats had lower EE when paired with High- than with Low-line animals. High-line rats were expected to have higher EE than IvI (same-line control condition), but there was no difference in EE when paired with Low-line rats. Finally, Low-line rats had stable EE independently on social pairing and, in line with same-line pairing results, they had higher EE values than IvI rats.

The mitochondrial function differed between the three lines and was affected by mixed-line pairing

In order to investigate differences in mitochondrial respirometry between the lines, data of rats from the same lines were first analyzed together. Overall, High-line rats had lower mitochondrial respiration in the NAc (but not in the mPFC) compared to Low- and Inter-lines. In recent rodent studies, high anxiety was a predisposing factor to subordination along with low mitochondrial respirometry in the NAc (Hollis et al., 2015) and dominants had high levels of energy metabolites in the NAc (Larrieu et al., 2017). Interestingly, within IvH and LvH pairs, Inter- and Low-line rats had lower anxiety-like behaviors and higher mitochondrial respirometry in the NAc than High-line rats, indicating a propensity to become dominant over High-line rats. An analysis of social status following TCT and a WCT (see supplementary material) showed that in IvH pairs, the Inter-line had a tendency to become dominants over the High-line, and in LvH pairs, the High-line had a tendency to dominate the Low-line. These observations would suggest that interactions between Inter- and High-lines are in lines with previous literature (Hollis et al., 2015), while the dominancy establishment between Low- and High-lines might not follow the same mechanisms. Indeed one can hypothesize that the high respirometry measured in the Low-line derived from their behavioral flexibility, induced by their susceptibility to the social contagion by High-line rats and, increasing energy demand and potentially increasing mitochondrial function. However, due to low number of subjects those results has to be interpreted with caution and further investigation is warranted in order to explain the higher NAc respirometry in the Low-line.

Mitochondrial respirometry data were also analyzed according to the mixed-line dyads. Low- and Inter-line rats paired with High-line rats had an increase in mitochondrial respirometry in both NAc and mPFC. The fact that those findings are not specific to the NAc are interesting and could be related to global changes in metabolism of Low- and Inter-line rats when paired with High-line rats. One potential explanation could be that coping with social interactions with High-line rats was challenging and stimulating a number brain circuits involved in behavioral, emotional and cognitive processes. In that respect, mitochondrion could have been more solicited when socially housed with high-line individuals and leading to a higher maximal mitochondrial function in the brain. This hypothesis, linking biobehavioral flexibility of Low- and Inter-lines when facing the High-line animals and increased maximal mitochondrial capacity in the brain, requires further investigation.

Long-term mixed-line pairing affected more rats from the Low-line

At sacrifice, adrenals, testes and gonadal fat were weighed. There were no differences in adrenals weight but Low-line rats paired with High-line animals had lighter testes and gonadal fat. Previous studies reported a reduction in plasma testosterone levels in subordinate males (Tamashiro et al., 2004; Hardy et al., 2002) and Low-line rats tended to become more subordinate than High-line rats. Moreover, it was suggested that weight of the gonadal fat might be used as an estimate of body fat (Rogers and Webb, 1980). In that respect, it suggests that Low-line rats in LvH pairs were more subordinate and had a decrease in body fat. Moreover, we showed that these animals also had a decrease in RER implicating higher use of fatty acids as fuel. Thus, higher use of fatty acid may have reduced the amount of body fat in Low-line animals paired with High-line rats, possibly due to social subordination.

Limitations and future studies

Few limitations from this study may be commented. In the same-line pairing experiment, rats were exposed twice to the calorimetric cages, since during the first session, a technical issue with ammonia filters in the air flow did not allow the recording of EE or RER. Indirect calorimetry results reported here were collected on a second exposure to the CLAMS system. During the second calorimetry session, data from body weight evolution were not available but the first calorimetry session High-line rats had lower BW evolution than the two other lines but not differences in food intake (data not shown), in line with results reported in the mixed-line experiment. Additionally, further experiments with indirect calorimetry on the lines might be considered by integrating highly palatable food, since

stress and ghrelin seem to have different effects depending on the type of the food available (Patterson et al., 2013b, 2013a). Another parameter that might have play a role in the differences obtained, in RER values between Low- and High-lines, is the time scale of the CORT secretion during calorimetry recording. Indeed, since the High-line show lower habituation, their CORT secretion might have been prolonged and an analysis of feces throughout recording might be a way to analyze CORT evolution and differences during indirect calorimetry.

Due to a low number of animals, it was not possible to assess difference in metabolic parameters according to the social status obtained during mixed-line pairing. However, future studies might give important insights on the difference on susceptibility and resilience to subordination according to stress responsiveness. Indeed, studies related specific energy balance changes and social status, with subordinate animals having a lower RER than dominant mice (Moles et al., 2006). Moreover, testing long-term effects of mixed-line pairing of anxiety- and coping-line behaviors and CORT response to stress would be valued.

Altogether, results presented in this study showed that selective breeding for stress responsiveness differences for several generations induced lines of rats with biobehavioral and emotional state differences which, when paired together, might show different social contagion (state matching). These social interactions might depend on the pro-social capacity of the rats (Meyza et al., 2017). Given that High-line rats had a lower sociability than Low-line in a three-chambered social test (data not shown), we hypothesize that pro-social behaviors differed between the lines leading to more intense social contagion on Low-line rats. Further studies would be needed to test empathy-like behaviors (Laviola et al., 2017; Bartal et al., 2011) of the rats from the different lines before concluding.

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Contributions

DH and CS, designed the experiment. DH, bred the animals, performed the behavioral experiments, analyzed the data and wrote the manuscript. CS, gave expert feedbacks and corrections on the manuscript.

Disclosure of interests

The authors report no conflicts of interest.

Supplementary material

i. Materials and methods

Shock-probe burying test. In the shock-probe-burying test rats were habituated for three days to the testing environment before being tested with the introduction of an unknown electrified cylindrical probe (Ø = 0.5 cm, Length = 7 cm) from a specific hole in the testing cage. Rats approached the probe a received an electric shock (0.7 mA) and electricity was turned off. After receiving electric shock, rats were video-recorded for 15 min and behaviors were subsequently scored by a blind experimenter. Time spent freezing and burying were extracted and analyzed as indicators or passive versus active burying, respectively (De Boer and Koolhaas, 2003).

Territory competition test (TCT). Rats from the different lines were weight-matched in order to avoid facilitation of dominance based on body weight (Cordero and Sandi, 2007; Hollis et al., 2015). Animals were marked on their fur, using a permanent marker, for identification and placed, in pairs, in a neutral and clean cage without food or water, for 20 minutes. During TCT both rats spontaneously displayed offensive behaviors (Benus et al., 1992). Social encounters were video recorded and analyzed for exploratory, social, grooming, aggressive (van der Kooij et al., 2017) and defensive behaviors (rat in submissive posture without active aggressive from the opponent). The total duration of offensive behaviors from each rat was calculated as the sum of the different agonistic behaviors expressed ("Agg-beh" = offensive upright + lateral-threat + keeping-down) as previously described (Koolhaas et al., 2013). In order to determine social dominance within a dyad, a dominancy index was created taking into account total aggressive behaviors and defensive behaviors. Within a dyad (rats A vs. B) the dominancy index was the sum of aggressive behaviors of one rats (Agg-beh(A)) with the defensive behaviors of his opponent (Def-beh(B)) divided by the total of aggressive and defensive behaviors from the pair. The formula of the dominancy-index of rat A was: Dom-index(A) = (Agg-beh(A) + Def-beh(B)) / ((Agg-beh(A) + Def-beh(B)) + (Agg-beh(B) + Def-beh(A))) x 100, and was expressed in %. Pairs of rats displaying less than 10 s in their sum of aggressive and defensive behaviors were considered 'non-fighting' and were excluded. Within a dyad when rats had more than 10% of difference in their dominancy ratio, the more aggressive one was designated "dominant (Dom)" (Dom-index(Dom) > 55%) and the other "subordinate (Sub)" (Dom-index(Sub) < 45%)). Pairs with less than 10% of difference between both animals were categorized as 'unstable'.

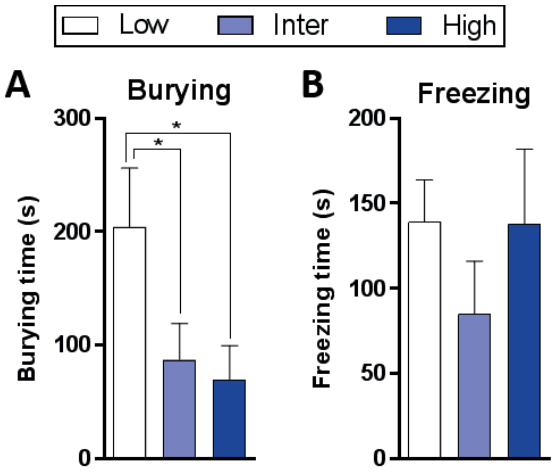
Water competition test (WCT). As previously validated (Lucion and Vogel, 1994), water-deprived rats competing for water quickly establish a firm relationship during which a Dom rat drinks consistently more than the Sub. One month after the first social-competition test and mixed-line cohabitation, rats were water deprived for 15 h (water bottles removed at 6pm and placed back at 9am the following morning).Interactions were video recorded for 10 min. Agonistic and drinking behaviors were determined for both rats in each dyad. As observed aggressive behaviors were minimal, dominancy was assessed based on the amount of time drinking during the first 5 min of water competition (Cordero and Sandi, 2007). A dominancy index was created as follows: Dom-index(A) = Drink(A) / Drink(A + B) x 100. Pairs with less than 10 % of differences were considered 'unstable' and among the other pairs, a dominant (Drink(Dom) > 55%) and subordinate (Drink(Sub) < 45%) rats were assessed.

Establishment of hierarchy in cohabitating pairs of rats. It was previously found that social dominance from TCT correlates with the outcome of WCT (Cordero and Sandi, 2007; Timmer et al., 2011). Here, by combining results from TCT and from the WCT, we established rank in rats' social hierarchy ("TCT & WCT" column in supplementary Figure 3). Rats being dominant/subordinate in both tests were considered dominant/subordinate in the long-term. Rats being in non-fighting pairs during TCT were considered non-fighting. Rats being in "unstable" pairs in both TCT and WCT were considered "unstable" in the long-term. Rats being in "unstable" pairs in one of the tests were attributed

the dominancy outcome obtained in the other test. When pairs had a different dominancy outcome in TCT and WCT they were considered “unstable”.

ii. Results

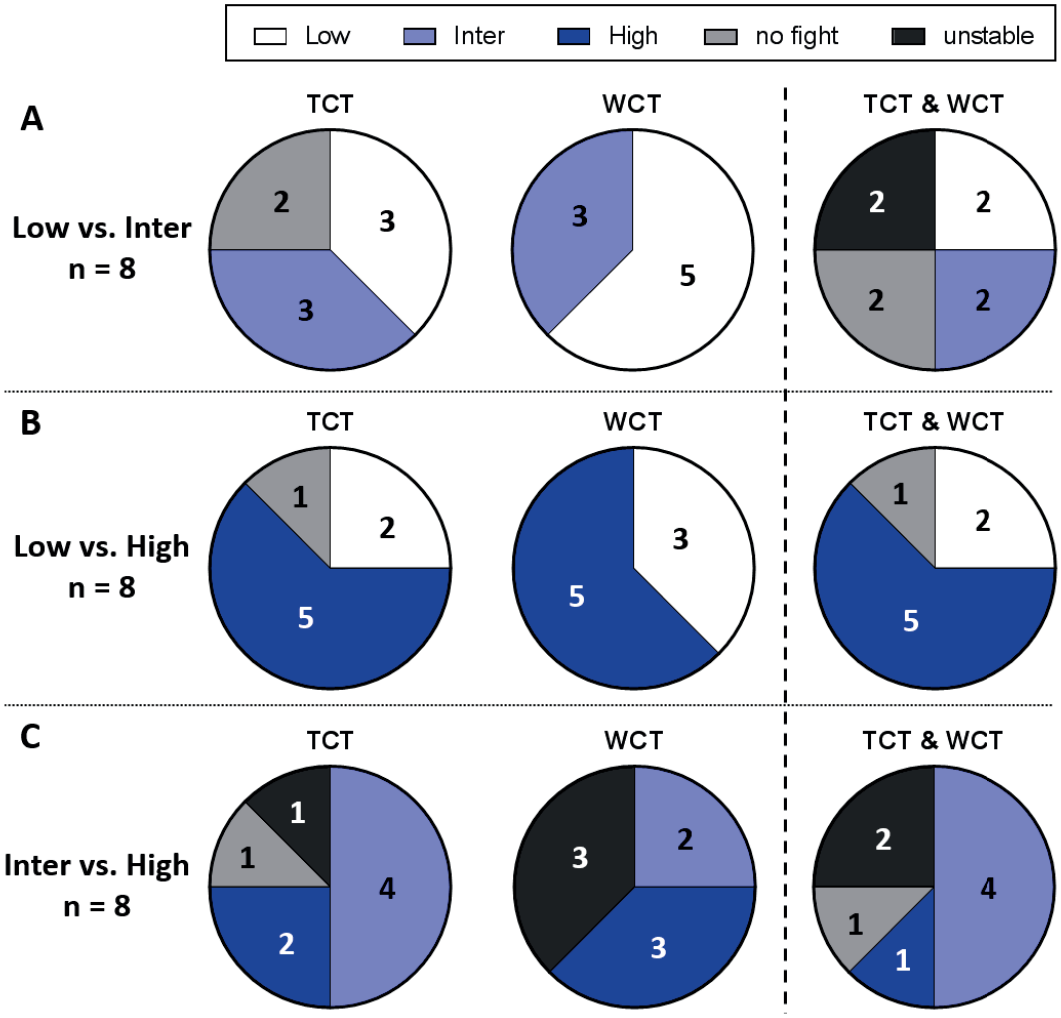
Low-line rats do more active burying than High-line rats. There was a tendency for a difference in active burying between the lines ($F_{2,20}$ = 3.39, p = 0.054) with Low-line rats burying more than Inter- (t_{20} = 0.047) and High-line rats (t_{20} = 0.029). There was no differences in the passive freezing time between the lines ($F_{2,20}$ = 0.8, p = 0.462).



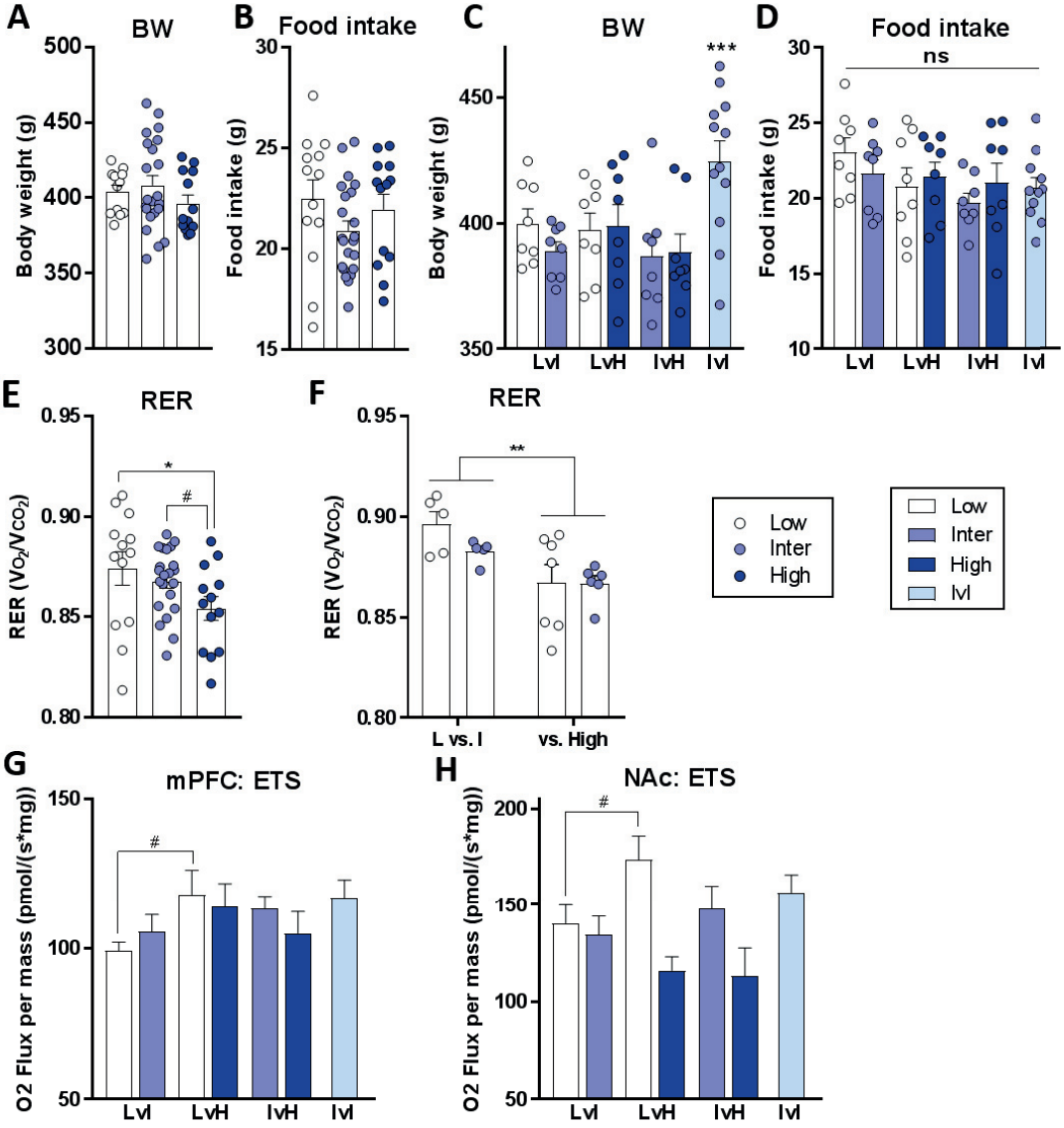
Supplementary Figure 1. Active burying and passive freezing during a shock-probe burying test.
A, There was a tendency for a difference in active burying between the lines ($F_{2,20}$ = 3.39, p = 0.054) with Low-line rats burying more than Inter- (t_{20} = 0.047) and High-line rats (t_{20} = 0.029). One High-line rats was excluded by ROUT outlier analysis **B,** There was no differences in the passive freezing time between the lines ($F_{2,20}$ = 0.8, p = 0.462). One High-line rats was excluded by ROUT outlier analysis

High-line rats were slightly dominant over Low-line animals and Inter-line tended to be dominant over High-line. The outcomes of TCT, WCT and the combination of both tests are summarized in Supplementary Figure 2. In the LvI pairs (Supplementary Figure 2A), there was no clear dominancy between Low- and Inter-line rats, with 2 pairs non-fighting, 2 unstable pairs and 2 pairs with Low- and 2 pairs with Inter-line rats as dominants. Within the LvH dyads (Supplementary Figure 2B), High-line animals were more dominant during the TCT and WCT (Chi-squared statistics = 2.57, p = 0.109). When TCT and WCT were combined, 62.5 % (5 out of 8) of High-line rats become Dom against 25 % (1 out of 8) in Low-line animals. In the IvH pairs (Supplementary Figure 2C), there was more instability in the dominancy between the tests with Inter-line rats being more dominant that High-line rats (Chi-squared statistics = 3.6 , p = 0.058). Combination of SC and WCT outcomes showed that 4 Inter-line rats became dominant while only 1 High-line animal did. There was 2 unstable pairs and 1 pair not fighting.

Effects of mixed-line pairing on calorimetry and mitochondrial respiration. There was no difference in BW (Supplementary Figure 3A) and IvI pairs had significantly higher BW than other housing conditions (Supplementary Figure 3A). There was no difference in food intake (Supplementary Figure 3B,D). There was a tendency for an effect of the lines on RER (Supplementary Figure 3E; p = 0.069) with High-line rats having lower RER than Low- (p = 0.026) and Inter-line rats (p = 0.083). RER of Low- and Inter-lines decreased when paired with High-line animals (Supplementary Figure 3F; p = 0.003). In the mPFC (Supplementary Figure 3G) and NAc (Supplementary Figure 3H) there was a tendency for an increase in ETS for Low-line animals when paired with High-responding animals (p = 0.067 and p = 0.081 respectively).



Supplementary Figure 2. Dominancy outcomes of SC and WCT in the different dyads. A, During SC, there was no clear dominancy between Low- and Inter-line rats with 3 Low- and 3 Inter- dominants and 3 pairs not fighting. In the WCT, 5 Low- and 3 Inter-line rats became dominants. Combination of SC and WCT outcomes showed that there was no clear dominancy: 2 pairs not fighting, 2 unstable, 2 Low- and 2 Inter-line rats were dominants. Chi-squared statistics = 0.0, p = 1.0. **B,** During SC, 5 High- and 2-Low-line rats became dominant and 1 pair did not fight. In the WCT, 5 High- and 3 Low-line rats became dominants. Combination of SC and WCT outcomes showed that High-line rats became dominant 5 times against 2 times for the Low-line rats. One pair was not fighting during SC. Chi-squared statistics = 2.57, p = 0.109. **C,** During SC, half of the pairs had an Inter-line rat as dominant. Two High-line rats were dominant, 1 pair did not fight and 1 pair did not show clear dominant. During the WCT, 2 Inter- and 3 High-line rats became dominants and 3 pairs did not show clear difference in drinking time. Combination of SC and WCT outcomes showed that 4 Inter-line rats became dominant while only 1 High-line animal did. There was 2 unstable pairs and 1 pair not fighting. Chi-squared statistics = 3.6, p = 0.058.



Supplementary Figure 3. Effects of mixed-line pairing on different parameters during calorimetry and mitochondrial respiration. A, There was no difference in body weight between the lines ($F_{2,44}$ = 1.01, p = 0.373). **B,** The food intake during the 24 h test did not show an effect of the lines ($F_{2,45}$ = 0.69, p = 0.223). **C,** Rats in IvI pairs had significantly higher BW than rats in the different housing conditions (*** indicates comparisons with all other groups with p < 0.001). **D,** There was no difference in food intake according to lines and pairing. **E,** There was a tendency for an effect of the lines on RER ($F_{2,45}$ = 2.83, p = 0.069) with High-line rats having lower RER than Low- (t_{13} = 2.31, p = 0.026) and Inter-line rats (t_{13} = 1.77, p = 0.083). **F,** RER of Low- and Inter-line animals decreased when paired with High-line animals ($F_{1,19}$ = 11.3, p = 0.003). **G,** The maximal mitochondrial respiration was measured with the Electron-Transfer-System (ETS) activation. When ETS values were compared according to different pairing situations in the mPFC there was a tendency for an increase in ETS for Low-line animals when paired with High-responding animals (t_6 = 2.23, p = 0.067). **H,** In the NAc, when ETS values were compared according to the pairing situations there was a tendency for an increase in ETS for Low-line animals when paired with High-responding animals (t_6 = 2.28, p = 0.081).

Chapter 3

Effects of aging on biobehavioral phenotype and cognition of rats with differential stress responsiveness

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Abstract

Changes associated with aging are related to an increased susceptibility to develop diseases. Stress is known to cause brain damages and dysfunctions of the HPA axis may contribute and facilitate aging processes, while aging impairs adaptation to stress. Glucocorticoids have neurotoxic effects on the brain and can induce long-term neuronal and cognitive alterations. Here, from mid-life (12 months old) to early-aging (18 months old), we investigated HPA axis indexes (corticosterone and adrenocorticotropic hormone concentrations, adrenal responsivity and adrenal weight) and the biobehavioral phenotype evolution in lines of rats selected for their differences in the corticosterone responsiveness to stress. Behavioral experiments, assessing anxiety-like behaviors, stress responsiveness, coping-style as well as cognitive functions in a Morris water-maze, were performed throughout aging. Results indicated that the line-related diverse behavioral and physiological traits were stable throughout aging. Furthermore, learning, swimming strategies, long-term memory and reversal ability in the Morris water-maze at early-aging were different between the three lines. Taken together, those results illustrate that the selection of rats for differential glucocorticoid secretion and adaptation to stress during juvenile period is related to stable biobehavioral phenotypes across aging and is linked with differences in performance in memory tasks at early-aging.

Keywords

Glucocorticoids, corticosterone responsiveness, aging, anxiety, coping style, cognition, learning, memory, Morris water-maze.

Introduction

Since slow degenerations of human organism are the major responsible of death in front of previous diseases, like tuberculosis, influenza or pneumonia (Levy and Moskowitz, 1982; Sapolsky et al., 1986), studying the underlying components of aging became of major interest. Stress may accelerate aging processes (Finkel and Holbrook, 2000; PARE, 1965) and aging may decrease adaptive abilities against stress (Sapolsky et al., 1986; Webb and Agnew, 1962). Stress triggers the activation of the hypothalamus-pituitary-adrenal (HPA) axis, culminating in the secretion of glucocorticoids by the adrenals (Lupien et al., 2009). The surviving ability of neurons can be altered by high levels of corticosterone (CORT) and this neuro-toxicity (e.g. in hippocampus) might be linked to CORT-induced exposure to "excitotoxins" (e.g. glutamate) leading to neural cell loss (McEwen and Sapolsky, 1995; Sapolsky, 1999). Hippocampal neuronal loss and memory impairments in old rats were reported to correlate with increased adrenal activity (Landfield et al., 1978). Indeed, low levels of CORT in middle-aged rats were shown to decrease aging damages to the hippocampus (Landfield et al., 1981) but, high levels of CORT accelerated brain aging (Arbel et al., 1994; Landfield and Eldridge, 1991; Sapolsky, 1986). In aging rodents, experimental studies showed that elevated basal corticosterone and extended release of CORT was due to a dysfunction of the negative feedback of the HPA axis (Sapolsky et al., 1983; Sapolsky, 1985b, 1985a; Segar et al., 2009). With aging, the adrenocortical axis has been reported to be desensitized to the inhibitory effects of glucocorticoids and progressive degeneration of the hippocampus might be responsible for a decrease in HPA axis negative feedback (Sapolsky et al., 1986). A delayed negative feedback of the HPA axis might lead to an accumulation of stress during aging, exacerbating the likelihood of neural damage (Wei et al., 2007). Both, impairment in termination of glucocorticoid secretion after aging and neural degeneration induced by chronic exposure to glucocorticoids may be detrimental to individuals (Sapolsky et al., 1986). The production of CORT is controlled by the adrenocorticotropic hormone (ACTH) released from the pituitary gland, that is controlled by corticotropic-releasing hormone (CRH) released from the PVN in reaction to stress (Ulrich-Lai and Herman, 2009).

Dysfunctions of the HPA axis may contribute to aging-related diseases like depression, cognitive deficits, and Alzheimer's disease. In addition to neuro-cognitive dysfunction, it has also been associated with declining physical performance. Different pathophysiology of aging might be due to HPA axis deteriorations with respect to increased ACTH or CORT secretion, decreased negative feedback and flattening of diurnal pattern of CORT release (Gupta and Morley, 2014). The measurements of CORT and ACTH values allowed an indirect assessment of adrenal responsivity of dogs and rats (Ulrich-Lai and Engeland, 2002).

Deleterious effects of aging on learning and memory are well documented in several species including humans (Bachevalier et al., 1991; Bergado and Almaguer, 2002; Gower and Lamberty, 1993). Some alterations in the neurotransmitter systems (acetylcholine, catecholamines and glutamate) have been revealed (Bartus, 2000; McEntee and Crook, 1993; Richter-Levin and Segal, 1993). In addition, plasticity processes (e.g. long-term potentiation) are impaired with aging (Barnes and McNaughton, 1985; Bergado et al., 1998). Synaptic plasticity and survival ability of neurons are compromised with increased CORT levels during aging (Sapolsky, 1999). Taken together, it was suggested that aging promotes anatomical and functional deficiencies at the level of amygdala, hippocampus and the neural systems linking them (Bergado et al., 2007, 2011; Frey and Frey, 2008). Those systems are central in learning and reinforcement of behavioral experiments. Cognitive decline of aged animals is classically tested with the Morris-water-maze (MWM) in brain aging research (Bergado et al., 2011; Baxter and Gallagher, 1996).

Accumulation of DNA damage might be involved in aging (Mitchell et al., 2015). Telomeres, the ends of eukaryotic chromosomes, are characterized by multiple repeats of a short DNA sequence and are protecting and maintaining the chromosomes (de Magalhães and Passos, 2017). The stress induced by environmental adversities induces

secretion of CORT that can increase oxidative damage (Agostinho et al., 2010). Oxidative stress is degrading telomere repeats (Houben et al., 2008; Zhang et al., 2014) which is then shortening and may lead to senescence or cell death (Blackburn, 2001; Campisi, 2003). It has been hypothesized that telomere length might be used as a predictor of the lifespan remaining in different species (Cawthon et al., 2003; Haussmann et al., 2005; Bize et al., 2009; Heidinger et al., 2012). Additionally, telomere loss has an important rate at early ages (Friedrich et al., 2001; Hall et al., 2004), making the development a critical period predicting telomere quality later in life (Price et al., 2013). In birds, embryonic exposure to CORT increased oxidative stress and decreased telomere length (Haussmann et al., 2012). In other chick species, a negative correlation was found between basal CORT and telomere length (Bauch et al., 2016). Additional literature expressed a link between adversities occurring early in life and long-term effects on the individual phenotype though telomere shortening and early telomere dynamics may centrally be related to survival of individuals (Quirici et al., 2016). Note, however, that telomere length analyses are ongoing and are not presented here.

Our laboratory has performed selective breeding of rats differing in stress responsiveness and habituation to stress during puberty (Walker et al., 2017; Walker and Sandi, 2018). Using a 'corticosterone-adaptation-stress-test' (CAST) protocol, breeder rats were selected, for several generations, based on their CORT response to stress during early-puberty. Following exposure to the CAST protocol, CORT responses were measured and rats were classified as high, intermediate and low (called 'High-', 'Inter-' and 'Low-line' respectively). Rats from the High-line showed a lack of habituation to stress in term of CORT secretion while rats from the Inter- and Low-line habituated, with Low-animals showing significantly lower CORT after 3 days of stress. Progeny of those animals, tested at 3-4 months of age, differed in stress response and anxiety-like behaviors in a way that Low-line animals produced less CORT in response to novelty stress and were less anxious in the elevated plus maze (Huzard et al., Chapter 2, 3) and High-line rats exhibited the opposite phenotype. Moreover, High-line rats were shown to perform more passive floating during a forced-swim test (FST) at four months old (Walker et al., 2017; Walker and Sandi, 2018) compared to Low- and Inter-lines. Overall, selection for differential stress responsiveness was linked to biobehavioral phenotypic differences at young-adulthood.

Based on the literature reported above and on the previous physiological and behavioral results obtained with rats from the three lines, at young-adulthood (3-4 months of age), our first goal was to investigate the effects of aging on those lines. Indeed, we explored whether early-aging would have a differential influences on CORT secretion in response to stress, anxiety-like behaviors and coping style on the lines. We also measured CORT and ACTH levels at resting conditions and in response to stress at 16 months of age in order to assess their adrenal responsivity. Furthermore, previous studies showed that the rodent hippocampus might be damaged by prolonged CORT exposure, whereas lower CORT levels during aging might protect the hippocampus from senescent neuron loss (Sapolsky, 1999, 1996; McEwen et al., 1992). Additionally, learning and memory deficits have been related to neuronal alterations in hippocampus that may arise from stress and glucocorticoids (Sapolsky and Goosens, 2007). Based on those observations, we hypothesized that rats from the High-line will display altered learning memory in a Morris water-maze (MWM) during aging. To test this hypothesis, we tested rats from the three lines, which differed in CORT response to stress, in a MWM during aging (at 17 months of age). We also assessed long-term retention of the platform's location and we tested the reversal flexibility of the rats in the MWM. Finally, we applied a detailed classification of swimming paths during MWM trials in order to assess differences in swimming strategies between the lines (Gehring et al., 2015; Vouros et al., 2017).

Here, we show that rats from the low-line have lower CORT responsiveness to stress from juvenile pre-puberty period (1 month of age) to early aging (19 months of age) in response to various stressors while High-line rats exhibited higher stress responses. Tests for anxiety-like behaviors confirmed higher anxiety profile in high-line animals and

showed neophobic behaviors in Low-and High-lines compared to Inter-line animals. Finally, during Morris water-maze testing, Inter-line animals used lower-level strategies, Low-line rats had memory retention and reversal learning deficits while High-line animals showed better platform location retention. This study emphasizes important long-lasting biobehavioral differences between rats selectively bred for their differential glucocorticoid responsiveness during juvenile period as well as differences in cognitive functions at early-aging.

Material and methods

i. Animals

All experimental animals were Wistar-Han rats obtained from in-house breeding (see *Experimental timeline* section). Rats from different litters were pair-housed under standard conditions in plastic standard cages (42 × 28 × 20 cm) with a 12 h light-dark cycle (lights on at 7 AM) and controlled temperature and humidity (22 ± 2 °C; 50 ± 20 %). Food and water were available *ad libitum*. All procedures were conducted according to the Swiss National Institutional Guidelines of Animal Experimentation and approved in a license issued by the Cantonal Veterinary Authorities (Vaud, Switzerland). All efforts were made to minimize animal suffering during the experiments.

ii. Experimental timeline: Behavioral Test Battery

Rats were behaviorally tested between 12 and 19 months of age when they are considered mature adults and reaching early-aging at 18 months of age (Stone et al., 2000; Sengupta, 2013). Rats were born from lines of rats bred in our animal facility and following a previously described selection according to corticosterone secretion adaptation to stress during juvenile period (Walker et al., 2017; Huzard et al., Chapters 2 and 3). These lines were selected for low, intermediate or high glucocorticoid responsiveness to repeated stress at one month of age (called 'Low-', 'Inter-' and 'High-line' rats respectively). The CAST selection process was applied between postnatal days 28 and 30 (P28-30), during pre-pubertal juvenile period, to rats from the 8th generation of lines. At 5 months of age, males and females from same lines were housed together, for one week, in order to breed the following generation (F9) of CAST-lines. After mating, rats were left undisturbed until 11-12 months of age considered mid-life period (Arbel et al., 1994). Rats were then submitted to different behavioral, cognitive and stress challenged until sacrifice at 19 months of age. Anxiety-like behaviors were assessed in an open-field/novel-object test at 11 months of age, and in an elevated plus maze at 15 months of age. At 16 months of age, three consecutive days of stress were applied with a reaction to novelty stress (bucket test), a restraint stress and exposure to an elevated platform (EP). Learning and memory were assessed in a Morris water-maze test (17 months), followed by a forced swim test before sacrifice (19 months of age). As indicated in the timeline of Figure 1, the aging of the rats can be divided in several periods from Juvenile (pre-pubertal) time at one month of age, to young-adult time, between 3-5 months, as typically targeted in previous experiments with the lines (Walker et al., 2017; Walker and Sandi, 2018). Then rats reach mid-life at around 12 months old and we consider 15-18 months of age as "early-aging" (Sandi and Touyarot, 2006; Sengupta, 2013; Morterá and Herculano-Houzel, 2012).

iii. Open-field/novel-object test (OF/NO)

The open-field/novel-object test was performed to evaluate emotional and non-social exploratory behaviors in middle-aged animals (11 months of age) from the CAST lines. The open field test (OF) was conducted in a circular open arena (40 cm high, Ø = 1 m) by placing the animal facing the wall and let it explore freely the apparatus for 10 min. The OF was virtually divided in three parts for analysis: a central disk (Ø = 25 cm), an intermediate zone (annulus with Ø = 25-75 cm) and the remaining wall zone (annulus with Ø = 75 cm - 1 m). The total distance traveled and the time in the different zones were analyzed during the test using Ethovision software (Noldus, The Netherlands). Immediately after the OF testing, an object (green plastic bottle) was placed at the center of the arena and animals

were left 5 additional minutes to explore the arena (with the novel object). In addition to the localization of the rats, the time of active sniffing of the object was scored by an experimenter blind to the groups. The arena was cleaned with a 5% ethanol solution between animals.

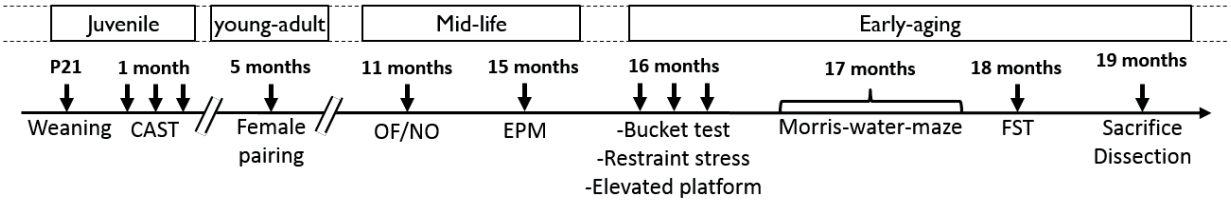

Figure 1: Experimental timeline of the different behavioral tests performed during aging from 12 to 19 months of age. Rats used in this experiment were bred from the 8th generation of CAST-line selection. Rats were weaned at P21 and pair-housed in same-line cages and submitted to the CAST protocol at one month of age. At young-adulthood (5 months of age), rats were used to breed the 9th generation of CAST-line rats (pair-housed with females during 1 week). At 11 months of age, rats were tested on an open-field/novel-object test and at 15 months old, anxiety-like behaviors were assessed with an elevated-plus-maze (EPM). At 16 months of age, three consecutive days of stress were performed and tail-blood was sampled after stress. At 17 months of age, the Morris-water-maze was executed. A blood sample was extracted after a FST at 18 months of age. Organ collection were performed at 19 months of age. See 'Material and methods" section for detailed protocols of behavioral tests. The red drop image indicates blood sampling.

iv. Elevated plus maze (EPM)

To assess the anxiety-like behaviors, the elevated plus maze was used at 15 months of age. The apparatus consisted of a plus-shaped elevated platform (50 cm above the floor) with two opposite open-arms (50 × 10 cm), two opposite closed-arms (50 × 10 × 38 cm) and a square central hub (10 × 10 cm) where all four arms meet, made of black PVC. Light intensity was set to 15–16 lux at the apex of the open-arms and 3-6 Lux in the closed-arms. The rats were placed individually in the maze facing a closed-arm and were allowed to explore the apparatus for 5 minutes. The maze was cleaned with 5% Ethanol between animals. Videos, recorded form above the maze were analyzed with Ethovision (Noldus, The Netherlands) and the distance as well as the time spent in the difference arms was extraction. The percentage of time spent in the open-arms, is classically used as a marker of anxiety-like behaviors (Pellow et al., 1985).

v. Repeated stress at early-aging (16 months of age)

At 16 months old, rats were challenged with three consecutive days of stress exposure. In order to avoid habituation to homotypic stress, three different stressors were used. First, rats were placed for 30 min in an unescapable bucket, previously presented as a novelty stress (Huzard et al., Chapter 2). On the second day, rats were submitted to 30 min of restraint stress. Finally, rats were individually placed on elevated-platforms in a bright room (350 lx) for 30 min.

In order to study CORT and ACTH in reaction to stress, different blood samples were collected across the three days of stress. Immediately after each stressor, tail-blood was sampled for the analysis of peak corticosterone. In addition, during restraint stress, blood was sampled at onset of stress to assess CORT and ACTH basal levels and after 15 min of restraint, another blood was sampled in EDTA capillary tubes for peak ACTH analysis (Herman et al., 2016).

vi. Morris water-maze (MWM)

The protocol for the Morris-water-maze test was adapted from (Vorhees and Williams, 2006; Inostroza et al., 2011). The maze consisted on a black circular pool (Ø = 200 cm, 45 cm high) filled with 30 cm of water at 23 ± 1 °C and virtually divided into four equivalent quadrants: northeast (NE), north-west (NW), southeast (SE) and south-west (SW). A circular rescue platform (Ø = 12 cm; distance between platform center point and pool wall: 30 cm) was

submerged 1–2 cm below the water surface and the testing room was illuminated with indirect lighting (50 ± 10 lx) to avoid reflections. To monitor animals during trials, a camera was mounted to the ceiling centrally above the pool. The water maze was surrounded by extra-maze cues of different shape, size and color on the walls of the room.

Spatial acquisition phase

The platform location (NE) remained the same for all trials, whereas the starting location varied between trials in a semi-random design (East, South or West) as reported in supplementary figure 1. Rats were tested successively in blocks of four animals in order to have an inter-trial interval of 5-10 min. Before starting the first training trial on the first day, the rat was placed for 30 s on the platform. A trial began by placing the rat into the water facing the wall of the pool. The training trials were divided into 5 consecutive days (D1-5), with 5 trials on D1-2 and 4 trials on D3-5. The rat was guided to the platform if it failed to escape within 120 s. After reaching the platform rat was left undisturbed for 15 s before returning to his homecage before next.

Retention trial (probe trial)

On D5, 2 h after training trials, we conducted a retention trial during which the escape platform was removed from the pool and the rat was allowed to swim for 60 sec (D'Hooge and De Deyn, 2001; Vorhees and Williams, 2006). For the probe trial, there was a new starting position. The rat was placed in the SW quadrant, opposite to the platform location (NE). In order to analyze memory for the location of the platform, the latency to reach the platform's previous location and the distance to the platform's previous location were analyzed.

Long-term memory assessment and reversal learning

On D17, we conducted a second probe trial, similar to the one of D5. It was followed by three additional training trials with similar platform location (NE). On D18, a reversal learning protocol was performed: the platform was moved to the opposite quadrant (SW) and rats were trained on 4 trials (starting positions: N, S, E and W respectively). Before the first reversal trial rat was placed for 30 s on the platform at the new location.

Analysis of MWM

For the spatial acquisition trials, the mean latency to reach the platform was recorded. During every trial, movements of the animals were video-recorded and tracked by Ethovision software (Noldus, The Netherlands). The latency to escape, the time spent in the difference zones of the maze (Thigmotaxis, center and proximity to the platform), the velocity and the distance travelled before escaping were analyzed. Additionally, more advanced analysis was performed on the exported trials using the RODA software (Gehring et al., 2015; Vouros et al., 2017) which produced a detailed classification of the swimming paths into multiple strategies (See supplementary *methods*). This approach divided the trajectories into segments. These segments, and not the full trajectories, were classified into different classes of behavior. Results from this analysis led to a detailed categorization of swimming paths and allowed the detection of mixed strategies within a single trial (Gehring et al., 2015). The different trajectories detected were: *thigmotaxis*, when a rat was swimming close to the walls. *Incursion,* when an animal started to move on the inward locations of the arena. The *scanning* strategy was defined by animal randomly searching in the whole arena (Graziano et al., 2003). The *focused search* strategy was attributed when an animal actively explored a particular region of the arena. The *chaining response* behavior was determined as an animal having memorized the distance of the platform from the arena wall (Wolfer and Lipp, 2000). During *Self-orienting*, an animal performed a loop and oriented itself inside the arena (Graziano et al., 2003). *Scanning surroundings*, was defined as the animal crossing the platform or a region very close to the platform (Gehring et al., 2015). An animal was *Scanning Target* when it was actively searching for the platform by *focus searching* the location where the platform is located.

vii. Forced swim test (FST)

The CORT response of rats was assessed immediately after 15 min of forced swimming on 18 months of age. Animals were individually placed in an unescapable plastic bucket (Ø = 25 cm, 45 cm deep) containing 30 cm of water (23 ± 1 °C). Blood was sampled from the tail immediately after and blood plasma was subsequently extracted and analyzed to determine CORT concentration.

viii. Dissection Procedures

Rats were sacrificed, at 19 months of age, by decapitation. Trunk blood was sampled in EDTA tubes for telomere analysis. Brains were removed from the skull, snap-frozen in isopentane on dry ice and stored at -80 °C until further processing. The pituitary glands, the adrenals, and the left ventricle from the heart were dissected and snap-frozen in liquid nitrogen and stored at -80 °C.

ix. Quantification of Plasma Corticosterone Levels

Plasma samples (diluted 20 times in assay buffer) were assayed using an ELISA immunoassay (Corticosterone EIA Kit, Enzo Life sciences, Switzerland) following manufacturer's guidelines in order to determine corticosterone concentration.

In order to determine stress adaptation between CORT responses between P28 and P30, we calculated the percentage of CORT adaptation with the formula: CORT adaptation = ([CORT(P30)] - [CORT(P28)]) / [CORT(P28)] * 100. As previously described (Ulrich-Lai and Engeland, 2002; Myers et al., 2016), the adrenal responsivity was calculated with the following formula: Adrenal responsivity = [CORT] / LOG_{10}([ACTH]).

x. Quantification of ACTH

Plasma ACTH levels were determined using ELISA kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, biotinylated and enzyme labeled antibody were added to standards or 200 µL plasma distributed in coated wells and incubated for 4 h. After incubation with 3,3',5,5'-tetramethylbenzidine substrate for an additional 30 min, the 'Stop' solution was added and absorbance at 450 nm was measured in a plate reader.

xi. Statistics

Data are presented as mean ± SEM (standard error of the mean). Prior statistical analysis, normality testing was performed and in order to guarantee proper use of ANOVA analysis, ROUT method for outlier exclusion was performed (GraphPad Prism, Version 7.02) and potential outliers were excluded from statistical testing. When applicable, outlier exclusion is reported in the figure caption. Overall, one rat from the High-line was excluded because of health issues before EPM testing. Data from the three lines were analyzed using one-way ANOVAs with the lines as main effect followed by a Fischer's LSD comparisons as posthoc statistics. When suitable, repeated-measures 2-way ANOVAs, with time as repeated effect, were performed.

Results

i. Anxiety-like and coping-style differences throughout aging

During the OF test at 11 months of age, there were no differences between the lines in the amount of time spent in the center ($F_{2,27}$ = 0.36, p = 0.69), intermediate ($F_{2,27}$ = 0.77, p = 0.47) or external ($F_{2,27}$ = 0.82, p = 0.45) zones of the field (Figure 2A). There was no difference in the total distance traveled during the OF test (Supplementary figure 2A). However, when an object was placed at the center of the maze, explorative behaviors of the lines were differently

affected and there was a line effect ($F_{2,27}$ = 3.63, p = 0.040) on the time spent in the outer zone of the maze (Figure 2B). Inter-line rats spent less time than Low- (p = 0.038) or High-line animals (p = 0.021) close to the walls (thigmotaxis). There was also a line effect on the amount of time rats spent In the intermediary ($F_{2,25}$ = 12.35, p < 0.001) and central zones ($F_{2,24}$ = 6.29, p = 0.006) of the OF. Inter-line rats spent more time than Low-line animals in the intermediate (p = 0.001) and center zones (p = 0.005). And Inter-line rats spent more time in the intermediate zone than High-line rats (p = 0.002). Interestingly, there were differences between the lines ($F_{2,26}$ = 11.16, p < 0.001) on the time spent sniffing the novel-object (Figure 2C). Low-line animals spent less time sniffing the object than the Inter-line animals (p = 0.0002) with only 3 rats (out of 9) sniffing the object. High-line rats spent less time sniffing the object than Inter-line animals (p = 0.020). Note that statistical analysis by removing the three Low-line rat that did not enter the central part or using non-parametric ANOVA (Kruskal-Wallis) gave similar statistical results.

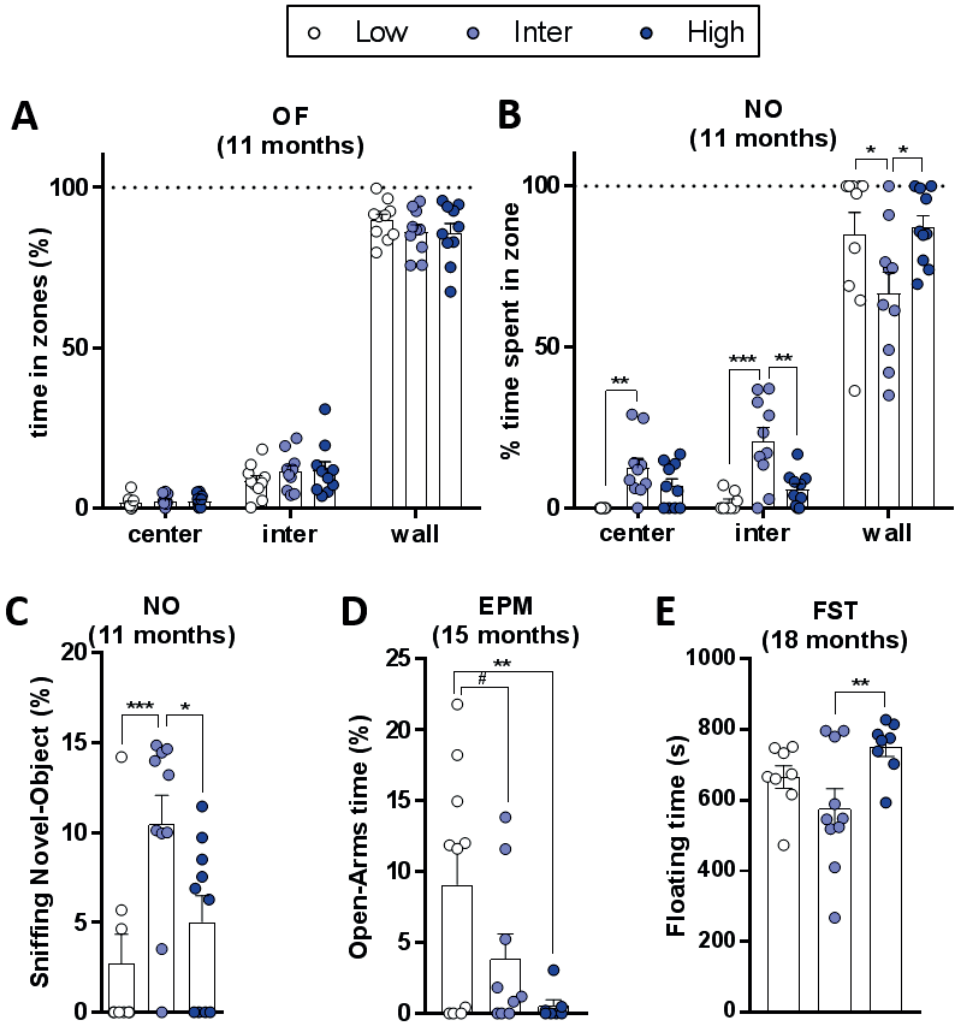

Figure 2: Difference in behavioral responses to stress between male rats of CAST lines. A, at 11 months of age, during OF there was no difference in time spent in the different zones (p > 0.1). **B,** After the OF, when an object was placed at the center of the maze Inter-line animals spent less time close to the wall than both Low- (p < 0.05) and High-line rats (p < 0.05). Only 3 rats entered in the center during NO. **C,** Inter-line rats spent more time sniffing this unanimated object than Low- (p < 0.001) and High-line rats (p < 0.05). **D,** During EPM test at 15 months of age, Low-line rats spent more time that Inter- (p < 0.1) and High-line rats (p < 0.05). **E,** At 18 months of age, during a FST, high-line rats floated more than inter-line rats (p < 0.05).

During the 5 min in the EPM, at 15 months old, there was a line effect ($F_{2,23}$ = 4.27, p = 0.026) on the time spent in the open-arms (Figure 2D). Low-line rats spent more time in the open-arms than Inter- (t_{23} = 1.87, p = 0.074) and High-line animals (t_{23} = 2.83, p = 0.009). When rats were exposed to 15 min of FST, at 18 months of age, there was a difference between the lines ($F_{2,23}$ = 4.21, p = 0.028) in floating time (Figure 2E). High-line rats spent more time floating than Inter-line animals (t_{23} = 2.89, p = 0.008) but there was no difference with Low-line rats (t_{23} = 1.36, p = 0.188). There was no difference in the total distance moving on the EPM (Supplementary figure 2B).

Altogether, these data show that low-line rats displayed lower anxiety-like behaviors than High- and Inter-line rats when measured at mid-life adulthood in the EPM. However Low- and High-lines had neophobic reaction to a novel-object in the OF. Finally, High-line rats displayed more passive coping style in a FST at early-aging compared to Inter-line animals.

ii. Difference in physiological markers between the lines throughout aging

The body weight was measured during pre-puberty, at weaning and juvenile period (Figure 3A) and demonstrated a significant line effect (p = 0.016). Although there was no significant difference in body weight during sexing (P20) between the lines (p > 0.199), at one month of age (P28 and P30) there was a difference with Inter-line animals being heavier than Low- (p = 0.035) and High-lines (p = 0.001). Throughout aging there was a line effect on the body weight of the animals (Figure 3B, $F_{2,26}$ = 3.62, p = 0.041) with Inter-line rats being heavier than Low- (p < 0.05) and High-line rats (p < 0.05). In later aging, after 13 months of age, Inter-line rats were significantly heavier than Low-line rats (p < 0.01) but not than High-line animals (p > 0.08).

In reaction to stress at P28, all rats showed high secretion of CORT (Figure 3B), but there were differences in the amplitude of stress response. There was a line effect on the stress response ($F_{2,24}$ = 40.2, p < 0.001) with Low-line animals exhibiting lower CORT concentration in blood plasma than Inter- (p = 0.01) and High-lines (p = 0.013). At P30, Low-line rats also secreted significantly lower CORT than Inter- (p = 0.005) and High-line animals (p < 0.001). Inter-line animals had a lower CORT concentration than High-line rats (p < 0.001). Differences in CORT production at P28 and P30 showed an important difference in the way the lines adapted to stress (Figure 3C). There was a highly significant line effect on CORT adaptation ($F_{2,23}$ = 128.4, p < 0.001) and Low-line animals habituated more to stress than Inter- (p = 0.009) and High-line animals (p < 0.001). Inter-line had higher CORT adaptation than high-line rats (p < 0.001). The High-line exhibited a sensitization to stress illustrated by a positive CORT adaptation (t-test (vs. 0): p = 0.002) with higher CORT response at P30 than at P28.

Fourteen months after CAST exposure, at 16 months of age, rats were exposed to three successive days of stress (d1-d3) in order to analyze stress responsiveness after aging. The concentration of CORT in blood plasma (Figure 3D) was different between the lines ($F_{2,26}$ = 6.58, p = 0.005) and across the days ($F_{2,52}$ = 24.8, p < 0.001). There was no significant interaction between days and lines ($F_{4,52}$ = 1.86, p = 0.131). Low-line animals had lower CORT than Inter- (t_{26} = 0.024) and High-line rats (t_{26} = 0.002). There was no difference between Inter- and High-line animals (t_{26} = 0.307). When CORT results were analyzed separately for the three stressors, it appeared that Low-line rats had lower CORT than Inter-line rats after exposure to restraint (d2, p = 0.016) and EP (d3, p = 0.006) but not after exposure to a bucket (d1, p = 0.57). Low-line rats had lower CORT than high-line rats after exposure to the three stressors (d1, p = 0.024; d2, p = 0.015; d3, p < 0.001). Inter-line animals had a tendency for lower CORT response than High-line rats after bucket-test only (p = 0.098). When data from the different days were compared within the different lines, it appeared that there was a higher CORT response after the bucket test (d1) compared to restraint (d2, Low: p = 0.083; Inter: p < 0.001; High: p = 0.047) and EP (d3, Low: p = 0.027; Inter: p < 0.001; High: p < 0.001). However, between restraint and EP there was an increase in CORT secretion for High-line rats (0 = 0.022) but not for Low- (p = 0.61) or Inter-line animals (p = 0.35).

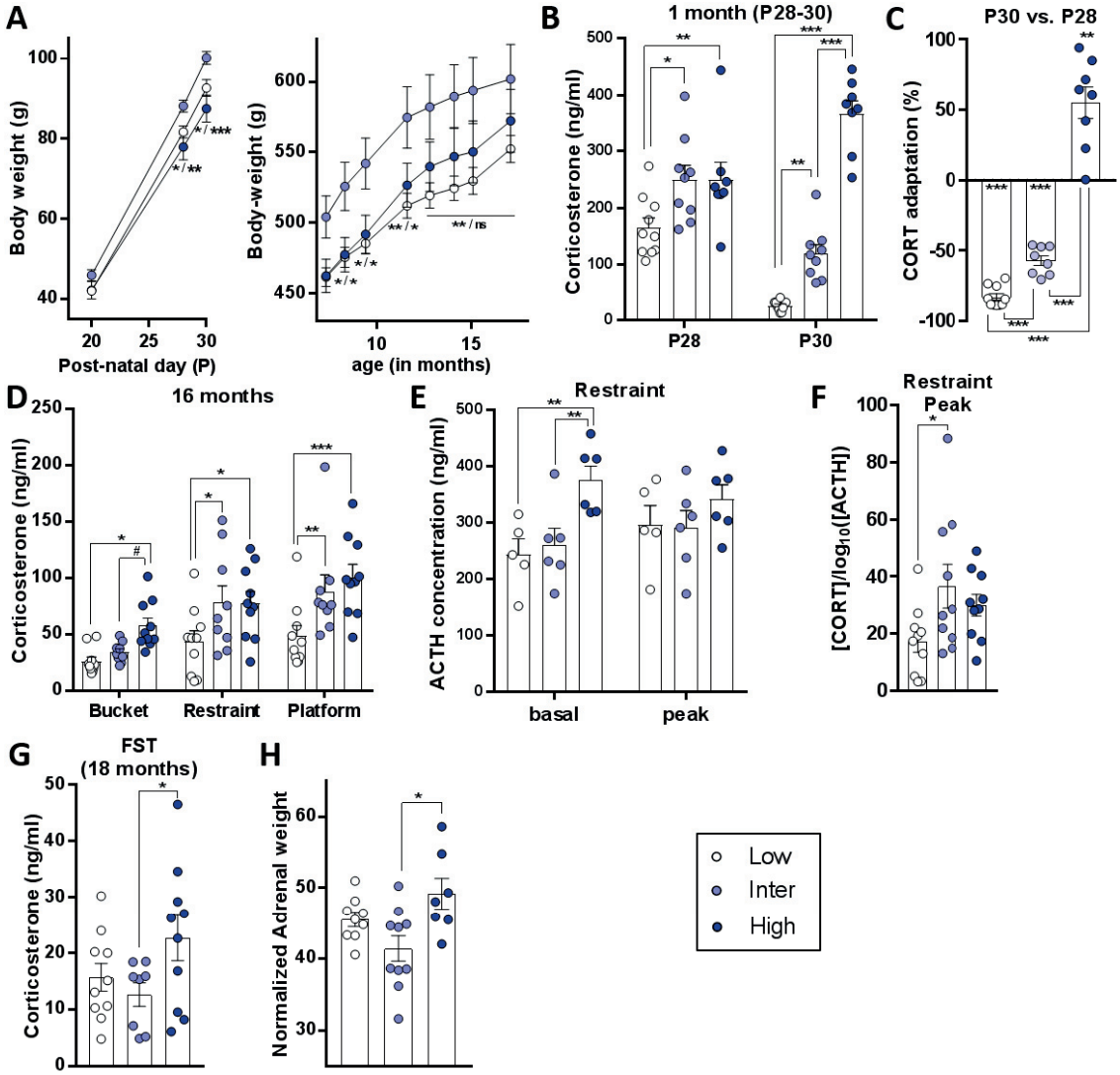

Figure 3: Difference in stress physiology between the lines throughout aging. A, During CAST protocol (**A, left panel**) Inter-line rats started having a higher body weight than Low- ($p < 0.05$) and High-line rats ($p < 0.01$). This difference remained till young-adulthood (**A, right panel**) and early-aging (with lower differences after 13 months of age). **B,** Plasma CORT during CAST showed differences at P28, with Low-line animals having lower CORT secretion than Inter- ($p < 0.05$) and High-line rats ($p < 0.01$). At P30, there were differences between the three line with Low-line rats having lower CORT than both Inter- ($p < 0.01$) and High-line rats ($p < 0.001$). High-line rats had higher CORT response than Inter-line rats ($p < 0.001$) at P30 than at P28 ($p < 0.01$). **C,** The adaptation to stress has been calculated by calculating the percentage of CORT response at P30 in comparison to P28. A positive adaptation illustrates a sensitization and a negative adaptation shows a habituation to stress. Low-line rats habituated more than Inter-line rats ($p < 0.001$) while High-line rats sensitized ($p < 0.01$). **D,** When challenged for 3 days in a row at 16 months of age, Low-line animals had consistently lower CORT Response than inter- ($p < 0.06$) and High-line rats ($p < 0.01$). One Inter-line rat was excluded (no blood sample after EP). **E,** During restraint stress, blood was sampled to analyze ACTH concentration and it appeared that High-line rats had higher basal ACTH concentration than Low- ($p < 0.01$) and Inter-line animals ($p < 0.01$). 2 Low-, 1 Inter- and 1 High-line rats were excluded by outlier analysis. **F,** Adrenal responsivity was calculated for peak response to stress by dividing CORT concentration by the logarithm of ACTH. In response to restraint stress, the adrenal responsivity differed between Low- and Inter- line animals ($p < 0.05$). **G,** After the FST, High-line rats had higher CORT concentration than Inter-line rats ($p < 0.05$). **H,** When normalized to BW, High-rats had bigger adrenals than Inter-line rats ($p < 0.05$). 1 low- and 1 High-line animals were excluded from adrenal analysis since only 1 adrenal gland was extracted.

During restraint stress, blood samples were screened for ACTH concentration at basal condition and at peak response to stress (Figure 3E). There was a line effect ($F_{2,14}$ = 4.73, p = 0.027) on ACTH concentration. High-line animals had higher ACTH values in basal condition than Low- (p = 0.004) and Inter-lines (p = 0.007). There was no difference in ACTH concentration after 15 min of restraint stress (p > 0.202), and there was no difference in ACTH between basal and peak levels for the three lines (p > 0.16). The ratio of CORT concentration divided by the logarithm of ACTH concentration was used to estimate the adrenal responsivity of the rats (Myers et al., 2016). There was a tendency for a line effect ($F_{2,27}$ = 3.26, p = 0.054) on adrenal responsivity to stress (Figure 3F). Low-line rats had lower adrenal responsivity than Inter-line rats (p = 0.018) but no difference with High-line animals (p = 0.111).

At 18 months of age, after 15 min of forced-swimming, tail blood was analyzed to establish CORT response (Figure 3G). There was a statistical tendency for a line effect ($F_{2,25}$ = 2.71, p = 0.086). High-line rats had higher CORT response to FST than the Inter-line animals (p = 0.035) but there was no difference with Low-line rats (p = 0.112).

At 19 months of age, adrenals glands were extracted and weighed in order to establish long-term effects of stress on physiology (Blanchard et al., 1995). In order to control for body size differences, adrenal weight was normalized by the body weight of the rats (Figure 3H, right graph). There was a line effect on normalized adrenals weight ($F_{2,23}$ = 5.2, p = 0.014), and High-line rats had heavier adrenals than Inter-line rats (p = 0.018).

Taken together, physiological information obtained during aging shower that the differential CORT response of the animals from the three lines was relatively stable throughout aging, with Low- and High-lines having opposite and extreme CORT responsiveness and Inter-line animals responding in an intermediary way. In appeared that Low-line rats may have reduced adrenal responsivity to stress, while similar adrenal responsivity under basal conditions. Finally, after 19 months of aging in laboratory conditions with exposure to various stressful experiences (CAST, breeding, stressors and behavioral tests) High-line rats had bigger adrenal glands, illustrating an accumulation of stress throughout their life.

iii. During MWM training Inter-line had lower level swimming strategy

In order to establish learning deficits during aging, rats were tested in a Morris water-maze and data were averaged for the two first days (D1&2) and two last days (D4&5) of training. The latency to escape the maze by reaching the submerged platform was used as an index of learning (Figure 4A). During the training there was no line effect on the latency to reach the escape platform ($F_{1,26}$ = 0.69, p = 0.511). There was an important effect of the training days ($F_{1,26}$ = 53.4, p < 0.001) illustrated by a decrease in latency to find the platform between D1&2 and D4&5 of training (Figure 4A). Accordingly the distance moved before finding the platform (Figure 4B) decreased between D1&2 and D4&5 ($F_{1,26}$ = 125, p < 0.001). The swimming distance during training significantly differed between the lines ($F_{2,26}$ = 3.98, p = 0.031). Overall, Inter-line rats had a higher swimming distance than Low- (t_{26} = 2.34, p = 0.027) and High-line animals (t_{26} = 2.51, p = 0.018). Differences between the Inter-line and the two other lines were significant during D1&2 sessions (p < 0.02) but not during D4&5 training (p > 0.121). The velocity of swimming (data not shown) also differed between the lines ($F_{2,26}$ = 5.5, p = 0.01) and Inter-line rats had higher velocity than Low- (t_{26} = 2.56, p = 0.012) and High-lines (t_{26} = 3.09, p = 0.005).

The different strategies obtained from the swimming paths during D1&2 and D4&5 of training are represented in Figures 4C-F. There was a line effect ($F_{2,25}$ = 4.62, p = 0.02) on the *thigmotaxis* strategy (Figure 4C) and Inter-line rats did more thigmotaxis strategies than Low- (t_{25} = 2.04, p = 0.052) and High-lines (t_{25} = 2.98, p = 0.006). There was an effect of training on the thigmotaxis strategy with a net decrease between D1&2 and D4&5 ($F_{1,25}$ = 69, p < 0.001). Differences in thigmotaxis were significant only during D1&2 between Inter- and High-lines (p = 0.004), it was a

statistical tendency between Inter- and Low-line (p = 0.063) and it was not significant during D4&5 (Low- vs. Inter-line, p = 0.32; Inter- vs. High-line, p = 0.213).

There was a line effect ($F_{2,25}$ = 3.71, p = 0.038) on the *incursion* strategy (Figure 4D) with Inter-line rats doing more incursions than Low-line rats (t_{25} = 2.72, p = 0.011) but no differences between High- and the two other lines (p = 0.19). There was a training effect of training on the number of incursions with a decrease between D1&2 and D4&5 ($F_{1,26}$ = 91, p < 0.001). There was no interaction between the lines and training ($F_{2,26}$ = 1.72, p = 0.2). During D1&2 Low-line rats did less incursions than Inter- (p = 0.011) and High-lines (p = 0.031) and there were no differences during D4&5 (p > 0.11).The *chain response* (Figure 4E) and *target scanning* (Figure 4F) swimming strategies showed similar patterns: both strategies did not differ between the three lines (p > 0.58) but displayed an increase during training (p < 0.021). There were no line effects (p > 0.137) on the four other swimming strategies (data not shown).

Linear regression analyses (data not shown) showed that the distance traveled during training sessions was positively correlated with thigmotaxis (D1&2: r = 0.734, p < 0.001; D4&5: r = 0.679, p < 0.001) and incursion (D1&2: r = 0.66, p < 0.001; D4&5: r = 0.87, p < 0.001) strategies.

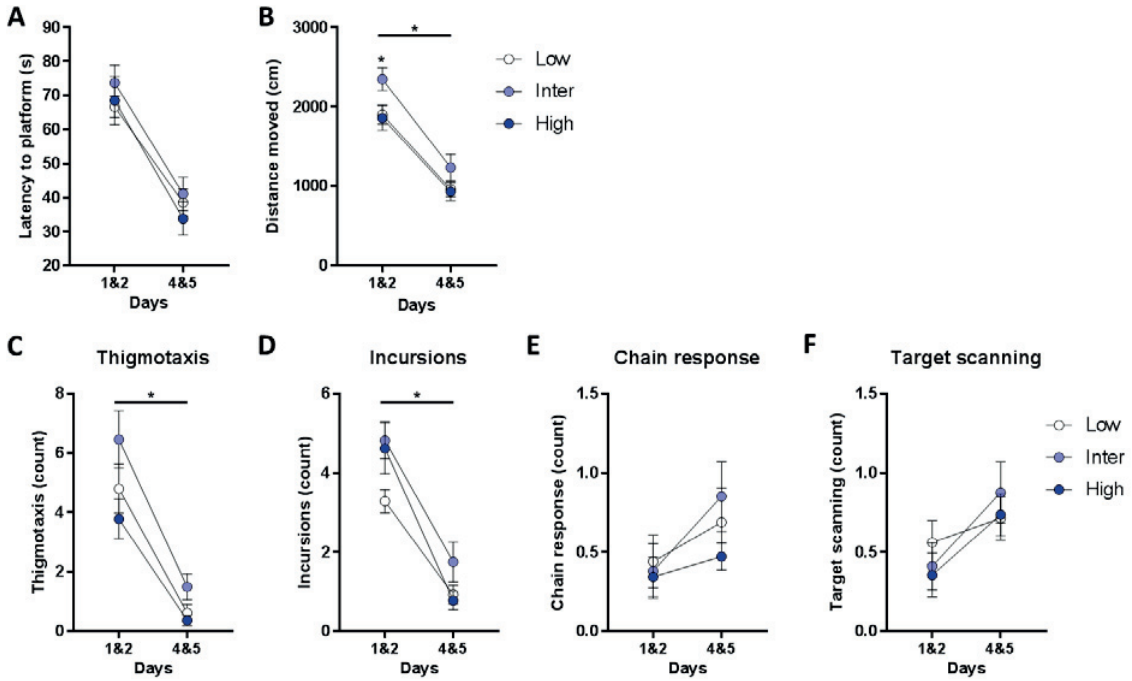

Figure 4: Inter-line animals used lower level strategies to escape during Morris-water-maze training at 17 months of age. A, All rats showed similar learning with a decrease of the latency to escape the pool across between D1&2 and D4&5 (p < 0.001). **B,** Inter-line rats traveled more distance (p = 0.031) than the Low- (p = 0.027) and High-line rats (p = 0.018) during training. Symbol (*) at D1&2 illustrates posthoc comparison between Inter-line and both Low- and High-lines (p < 0.05). **C,D,E,F,** The different swimming strategies evaluated with RODA software (Vouros et al., 2017), showed that Inter-line rats did more 'low-level' strategies by spending more time in the thigmotaxis (**C**) than Low- (p = 0.039) and High-line animals (p = 0.009). Inter-line rats also did more incursions (**D**) than Low- (p = 0.017) and High-line rats (p = 0.04). There were no differences in the amount of chain response (**E**) or target scanning (**F**) strategies.

After training, the learning of the exact localization of the platform was tested with a probe trial. There was no difference in the latency to reach the virtual extended-platform (p = 0.61).

Learning of the exact platform location was tested with probe trials (Figure 5) on D5 and two weeks later, on D17, during which the platform was removed and rats were allowed to swim for 60 s in the pool. There were no differences in the latency to reach the previous platform location at D5 ($F_{2,26}$ = 0.4873, p = 0.9) but there was a significant difference at D17 (Figure 5A) with High-line rats reaching faster the platform location than the two other lines ($F_{2,25}$ = 10.3, p < 0.001). High-line rats had a lower latency to reach the platform previous location than Low- (t_{25} = 4.13, p = 0.001) and Inter-line animals (t_{25} = 3.84, p = 0.002). High-line animals had a tendency to swim closer to the previous location of the platform than Low-line rats (supplementary Figure 3).

The distance moved during probe trials (Figure 5B) did not show any statistical difference between the three lines ($F_{2,26}$ = 2.011, p = 0.154). There was no line effect on the time spent in the platform quadrant (Figure 5C) but posthoc analysis at D17 revealed that Low-line rats spent less time in the platform quadrant compared to High-line rats (t_{52} = 2.12, p = 0.039).

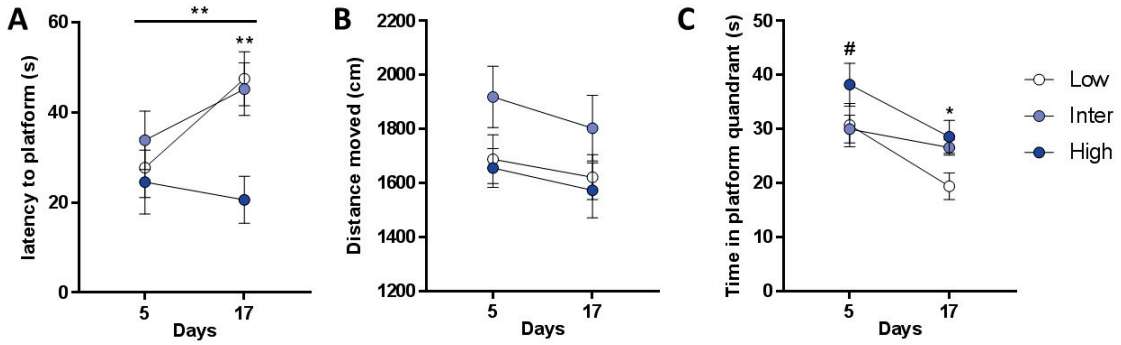

Figure 5: High-line rats retained more the platform location after 2 weeks without training. A, During the first probe trial at D5, all rats reached to platform location with the same latency (p = 0.9). During the second probe trial, High-line rats reached the platform location with a lower latency than the two other lines (p < 0.008). **B,** There was no statistical differences in the distance moved during probe trials. **C,** During D5, the High-line rats had a tendency to spend more time in the platform quadrant than Low-line rats (p < 0.1). During D17, Low-line rats spent less time in the platform quadrant than the High-line (p < 0.05).

iv. Low-line animals had long-term memory deficits

After the probe trial at D17, rats were re-trained to reach the platform in the NE quadrant with three additional training trials. There was a tendency for a line effect (p = 0.081) on the time to escape the maze (Figure 6A). Low-line rats had a longer escape latency than Inter- (p = 0.037) and High-lines (p = 0.086). Rats were then tested, on D18, for 4 reversal learning trials during which the platform was placed in the opposite quadrant (See supplementary methods). There was a line effect on the latency to reach the platform (Figure 6A) at the new location (p = 0.017). Low-line rats had a longer latency to escape the MWM than Inter- (p = 0.029) and High-line animals (p = 0.007). The distance traveled during re-learning and reversal learning (Figure 6B) showed a tendency for a line effect (p = 0.081), with no difference at D17, but Low-line rats swam more during D18 (p < 0.05). On both, D17 and D18, there was a significant line effect ($F_{2,26}$ = 3.65, p = 0.040) on the swimming velocity (Figure 6C) and inter-line rats swam faster than Low- (t_{26} = 2.15, p = 0.041) and High-line animals (t_{26} = 2.48, p = 0.02).

During reversal learning, swimming trajectories were analyzed and averaged over the 4 trials (Figure 6D-G). There was a line effect on the thigmotaxis behavior (Figure 6D; $F_{2,24}$ = 3.27, p = 0.055). Low-line rats did more thigmotaxis

trajectories than Inter- (t_{24} = 2.28, p = 0.032) and High-lines (t_{24} = 2.07, p = 0.049). Concerning the *chaining response* trajectories (Figure 6E), corresponding to a strategy in which the rat uses the distance to the wall in order to find the platform, there was significant line effect ($F_{2,26}$ = 5.08, p = 0.014). Inter-line rats used more *chaining response* trajectories than Low- (t_{26} = 2.72, p = 0.012) and High-line rats (t_{26} = 2.78, p = 0.010). During swimming, the *self-orienting* strategy (Figure 6F) was defined by loops performed by a rat to orient itself. There was a significant line effect on the self-orienting behavior ($F_{2,26}$ = 4.29, p = 0.024). Low-line rats self-oriented themselves more than Inter- (t_{26} = 2.44, p = 0.022) and High-line rats (t_{26} = 2.07, p = 0.049). Finally, the *focus searching* strategy (Figure 6G), corresponding to active searching for the platform, differed between rats from the three lines ($F_{2,26}$ = 3.16, p = 0.059). Inter-line rats did less *focus-searching* than High-line animals (t_{26} = 2.48, p = 0.02). There was no difference between Inter- and Low-line rats (t_{26} = 1.56, p = 0.132).

Taken together, during training in the MWM at 17 months of age, the three lines of rats successfully learnt a strategy to escape as shown by similar escape latencies. However, data suggests that Inter-line animals used lower level strategy to escape the MWM by spending more time in thigmotaxis and swimming more. Moreover, after 2 weeks, High-line animals retained more the previous location of the platform and Low-line rats had memory deficits since they needed more time to escape the maze during re-training. Finally, Low-line rats exhibited a deficit in reversal learning with longer latency to escape and by expressing lower level strategies. Furthermore, Inter-line rats exhibited also lower level strategies to escape the maze during reversal learning in comparison to High-line rats.

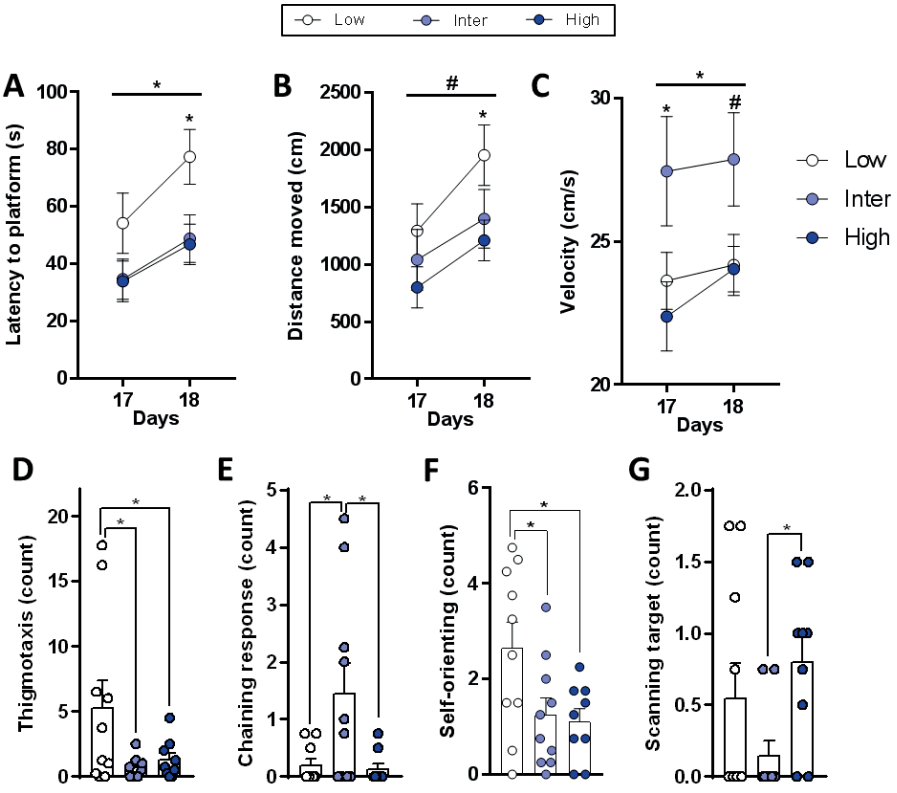

Figure 6: Low-line rats had reversal learning deficits. A, The latency to reach the platform at D17 and D18 was higher in the Low-line with higher deficit during reversal learning (p < 0.05). **B,** The distance traveled during re-training was the same between the three lines but Low-line rats swam more during the reversal learning sessions. **C,** Inter-line swam faster than the two other lines during D17 and D18 (p = 0.04). **D,** During reversal learning at D18, Low-line rats did more 'thigmotaxis' strategy (p = 0.056). **E,** Inter-line animals did more 'chaining response' strategy (p = 0.0137) than Low- and High-line rats (p < 0.05). **F,** Low-line animals did more 'self-orienting' during reversal learning (p = 0.024). **G,** High-line rats did more 'scanning target' strategy than inter-line animals (p = 0.02).

Discussion

Here, we characterized the behavioral, physiological and cognitive profile during aging of lines of rats genetically selected for their differential responsiveness to stress during the juvenile period. We validated and extended previous observations obtained at juvenile period (1 month of age) and young-adulthood (3-4 months of age) by illustrating that the three lines exhibited similar differences at mid-life (11-15 months of age) and at early-aging (16-19 months of age). Specifically, we showed that anxiety-like behaviors were stable between young-adulthood and mid-life and we found that stress responsiveness and coping strategies were consistent between young-adulthood and early-aging. Then, we assessed early-aging influences on both HPA axis parameters (CORT, ACTH, adrenal responsivity and adrenals post-mortem) at 16 months of age and a number of behaviors, with a main focus on the study of cognition (evaluated in the Morris water-maze) at 17 months of age. Our results indicated that the HPA axis indexes at early-aging differed in basal ACTH regulation, adrenal responsivity to stress and adrenal weight. Finally, during Morris water-maze experiments we illustrated that at early-aging: i) rats from the three lines had similar escape latency during training, ii) High-line rats had a higher memory retention of the platform's location, iii) Low-line rats had an diminished memory retention and an impairment in reversal learning and, iv) Inter-line rats exhibited lower-level swimming strategies.

Plasma CORT concentration in response to stress was assessed during juvenile period and early-aging in order to assess stability of the physiological responses to stress throughout life, and to compare the data to differences previously observed at 4 months of age (Walker et al., 2017). First, after the CAST protocol we selected rats from the three lines according to their, low, intermediate and high plasma CORT concentrations following the breeding procedure from Walker and colleagues (2017). Subsequently, after 16 and 18 months of age, we observed that the CORT responses to stress of the three lines were consistent with results obtained with young-adults from the lines (Walker et al., 2017). Taken together, those results showed that in response to stress, the pattern of CORT responsiveness of the three lines was stable from the juvenile period (Walker et al., 2017; Annex 1), to young-adulthood (Walker et al., 2017; Huzard et al., chapter 1) and to early-aging. Therefore, we validated here that the selection criteria of the three lines (i.e. differences in CORT response after 3 days of stress in juvenile period) induced stable CORT secretion differences in response to stressors throughout aging.

Subsequently, with the aim of comparing aging effects on anxiety-like behaviors and coping styles, we tested the lines in an OF/NO test (11 months old), an EPM (15 months of age) and a FST (18 months of age). While no differences were observed during OF exposure, results from EPM were in line with previous findings at young-adulthood (Walker et al., 2017; Walker and Sandi, 2018), with Low-line rats being less anxious than High- and Inter-lines. Previous studies also reported a lack of correlation between OF and EPM anxiety-like measures (Sudakov et al., 2013). Interestingly, insertion of a novel-object in the OF induced neophobia in Low- and High-lines, illustrated by their reduced time spent in center area and their low exploration of the object. During FST, in line with previous reports (Walker et al., 2017; Walker and Sandi, 2018), High-line rats spent more time passively floating than Inter-line animals. Taken together, those results illustrated that anxiety and coping styles of the three lines were stable during aging.

Dysfunctions of the HPA axis may contribute to aging-related diseases (Gupta and Morley, 2014) and the production of CORT from the HPA axis is controlled by ACTH (released from the pituitary gland). Thus, in addition to CORT levels, we assessed ACTH concentration at basal level and at peak response to restraint stress, at 16 months of age. High-line animals had higher basal ACTH than Low- and Inter-lines but there was no difference in peak ACTH concentration between the lines. Moreover, ACTH and CORT levels allowed an estimation of adrenal responsivity (Myers et al., 2016). We showed that, in response to restraint stress, Low-line rats had lower adrenal responsivity than Inter-line animals. Taken together those results show that High-line rats had higher basal ACTH and Low-line had lower adrenal

responsivity. ACTH is released from the pituitary gland in response to CRH and arginine vasopressin (AVP) from the PVN (Goncharova, 2013). Binding of CRH enhances transcription of POMC gene, which promotes ACTH release. Vasopressin works through AVP1B receptors to activate ACTH release but is not sufficient to drive significant ACTH at physiological levels (Herman et al., 2016). Interestingly, previous findings from the lines (Walker et al., 2017) showed higher *Avpr1b* expression in the pituitary gland of the High-line compared to the other lines and a higher *Pomc* expression in High-line compared to Inter-line. This may illustrate an increased sensitivity of the pituitary to AVP and a higher availability of POMC to generate more ACTH in the High-line. Higher basal ACTH in the High-line rats might be dependent on AVP signaling and might be eclipsed by CRH signaling during the stress response. It was shown in dogs that, in response to stress, the changes in adrenal sensitivity were not completely accounted for by changes in ACTH (Engeland et al., 1981). Indeed, it was shown that splanchnic innervation of the adrenal gland represented a control mechanism on stress-induced adrenal cortical responses *in vivo* (Ulrich-Lai and Engeland, 2002). Altogether, these data suggest that the reduced adrenal responsivity in the Low-line might be due to changes in autonomic innervation of the adrenal gland.

Chronic stress induces increased adrenal weight (Ulrich-Lai et al., 2006; Blanchard et al., 1995) but in a previous report, no differences in adrenal weight between lines were found at 4 months of age (Walker et al., 2017). Thus, we analyzed whether the three lines would differ in their adrenal weight at 18 months of age, particularly as they were exposed to the cumulative influence of behavioral challenges (that can be considered stressors) throughout life. High-line rats exhibited heavier adrenal glands than the Inter-line animals, but there were no differences between Low-line and the two other groups. Surprisingly, the Low-line rats did not have smaller adrenals, implying that their adrenal glands were not necessarily hypo-responsive. Gene expression from the adrenal glands in a previous rat lines generation (Walker et al., 2017) indicated increased expression of *Mrap* in the Low-line compared to the High-line. In the adrenal gland, MRAP is an essential accessory factor for the functional expression of the MC2R/ACTH receptor (Novoselova et al., 2013). Those results suggested a potential compensatory mechanism in the Low-line in order to respond to ACTH and secrete CORT. Altogether, these data from the Low-line illustrate a potential decrease in adrenal sensitivity to ACTH due to autonomic dysregulation, and a compensatory, but insufficient, mechanism through *Mrap*.

Rats were trained in the MWM at early-aging, in order to assess differences in learning and memory. The three lines successfully learnt an escape strategy as shown by the similar decrease in latency to reach the platform between the first and last training sessions. However, data from detailed swimming path analysis suggested that Inter-line animals used lower level strategies to escape the MWM during the initial phase of training. Indeed, Inter-line rats were swimming more distance, closer to the walls (*thigmotaxis*) and moving toward the center (*incursions*) to reach the platform. It was considered to be a “low level” strategy, since, instead of using spatial learning with surrounding visual cues, Inter-line rats learnt to escape the maze by targeting the platform from its distance to the wall. At the end of the training phase, all rats had similar strategies to reach the platform. Interestingly, we observed that low-level strategies decreased during the training phase and that “high level” swimming strategies (*chain response* and *target scanning*) increased. It also appeared that there was a positive correlation between swimming distance and *thigmotaxis* and *incursions* and further analysis would be required to determine whether the low-level strategies of Inter-line was due to differences in learning or due to a global increase in activity.

Long-term memory retention of the platform’s location was tested after 2 weeks, with a second probe trial. High-line animals retained better the exact location of the platform since they reached the platform's previous location faster than the other lines and swam closer to the platform. Interestingly, it was previously reported that rats exposed to higher stress during MWM training had a better spatial learning (Akirav et al., 2001), greater retention of the platform location (Sandi et al., 1997), and it was suggested that higher circulating CORT during MWM training increased the strength of memory (Akirav et al., 2004). However, re-learning following a reversal procedure was impaired in animals

exposed to higher stress (Kogan and Richter-Levin, 2008) suggesting that the quality of the memory formed is dependent on alterations in the limbic system following stress. Accordingly, since High-line rats secreted more CORT to behavioral challenges, it is reasonable to hypothesize that elevated CORT during MWM training improved long-term retention of the spatial location in those animals. Further investigation could establish activation patterns in a number of limbic regions and assess re-learning of the lines. It is important to mention that, 12 days before the retention probe trial, rats were tested on a first probe trial during which there was no difference in the latency to reach the platform location. It cannot be excluded that rats from the Low- and Inter-lines retained the lack of platform during the second exposure, explaining the longer latency to visit it on the second exposure, and describing a better extinction learning in comparison to the High-line. Further investigation would be required in order to test whether High-line rats had a better long-term memory.

Finally, Low-line rats had impaired reversal learning compared to Inter- and High-animals, illustrated by a longer latency to find the new platform and lower-level swimming strategies. Similarly as MWM training, Inter-line rats had similar latency to find platform but lower-level strategies than the High-line. It was shown that mice with mPFC damage had an impaired behavioral flexibility during reversal learning in a MWM but not during regular training (Latif-Hernandez et al., 2016). Moreover, alterations of neural encoding were observed in the in orbitofrontal cortex (OFC) of "reversal-impaired" aged rats (Schoenbaum et al., 2006). Whether the Low-line animals had deficits in mPFC of OFC after aging is unknown but it was recently reported that Low-line rats had lower basal ventral OFC activity than High-line rats at 4 months of age (Walker and Sandi, 2018).

It is interesting to note that, despite our hypotheses on deleterious effects of increased stress responsiveness during aging on cognitive function, our data illustrated the opposite relationship. Indeed, High-line animals outperformed the two other lines at early-aging. One might hypothesize that the aging period studied here was too early in order to observe the deleterious effects of aging, even though previous studies observed aging effects at early and similar aging states (Weiss and Thompson, 1991; Kadish et al., 2009). This is why we favor an alternative hypothesis relating the negative feedback efficacy and deleterious effects of glucocorticoids on the brain. Indeed, long-lasting and negative effects of stress have been related to impaired negative feedback (Sapolsky et al., 1983, 1985a, 1985b, 1986; Segar et al., 2009) and increased exposure to excitotoxins (Bishop et al., 2010). However, the three lines previously showed efficient negative feedback loops (Walker et al., 2017) and if anything, the Low-line rats appeared to have a less efficient feedback inhibition of the HPA axis (See Annex). In the future, it will be important to study aging processes in the context of lifelong exposure to stress. That would allow us to explore potential differences in negative feedback mechanisms between the lines, and their respective vulnerability to repeated stress exposure.

We observed that Inter-line rats were heavier than the two other lines starting at P28 and until early-aging. All lines had comparable inter-individual variability in body weight during juvenile period but Inter-line rats had a higher variability at mid-aging (after 10 months of age) compared to High- and Low-lines. When approaching early-aging, around 15 months of age, the variability of the body weight of High-line rats increased while it remained stable in Low-line rats. It has been shown that biological processes are highly affected by aging and that variability in health parameters, including body weight of rats, increases with age (Phillips et al., 2010). They demonstrated that the signs of deterioration due to aging appeared around 18 months of age and that body weight increased until 24 months and then remained stable, in Brown Norway rats (Phillips et al., 2010). Whether differences in body weight variability between the three lines were early signs of aging remain to be determined. Moreover, in term of physiological markers, CORT response to stress and adrenal gland weight, there were more inter-individual variabilities within the High-line. There were no clear differences in variability during behavioral analysis, except the wide range of responses during the elevated plus maze in the Low-line. Further examination of the results using a *behavioral profiling (*Ardi et al., 2016) and taking into account physiological and behavioral results could establish whether aging increased inter-

individual variability in the lines or if some lines were more susceptible to aging. indeed, during the Morris water-maze, the Inter-line showed the higher variability in swimming strategies and it might be related to the inter-individual differences reflected in their history and potentially related to aging processes.

Furthermore, we should mention that the Morris water-maze results presented here validated the use of a computational modelling of swimming paths with the RODA software (Gehring et al., 2015; Vouros et al., 2017). Indeed, it would not have been possible to differentiate learning strategies by analyzing only the latency to escape or other parameters obtained from the Ethovision software. Others also reported the importance to use tracking algorithms during MWM analysis (Hval et al., 2001; Wolfer and Lipp, 1992), because motor and other non-cognitive impairments might bias the behavior of the animals and might not be visible with standard MWM measures. This is important since previous research did not systematically use advanced analyses to assess swimming strategies and the current results show that such analysis allowed us to distinguish major differences in the way animals managed to escape. We suggest that a more systematic analysis of the swimming strategies should be used during MWM experiments and that further research might allow highlighting neuronal pathways involved in the different swimming strategies.

Few limitations of the study might be discussed. During ACTH measurements, while ACTH peak is supposed to be reached 15 min after onset of stress (Herman et al., 2016), there was no increase in ACTH between basal and peak measures of the lines. One potential explanation would be that we did not measure clean basal levels since ACTH secretion might have few minutes before during moving of the animals to the experimental room. Moreover, we cannot assess the effects of aging on the MWM results since the same test was not performed in younger animals. It would be interesting to test young adults in order to assess whether the differences observed here due to aging or to constitutive learning and memory differences between the lines.

Conclusion and future perspectives

In summary, we report that constitutive stress responsiveness is stable throughout early-aging of rats and is associated with stable anxiety-like and coping-style profiles. Rats with high glucocorticoid response to repeated stressors had enhanced expression of anxious- and passive coping behaviors. Additionally, the lines differed in learning, long-term retention and reversal flexibility during a spatial learning task given after early-aging. We showed that the increased exposure to glucocorticoids throughout life did not lead to detrimental effects on cognition. We hypothesize that this is due to efficient negative feedback mechanisms in the three lines despite their observed differences in peak responses. Furthermore, the biobehavioral findings reported here implicate these genetically lines as a useful model with which to further explore mechanisms related to stress responsiveness, coping strategies and cognition.

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Contributions

DH, designed and performed behavioral experiments, analyzed the results and wrote the manuscript. AV an EV, provided the RODA software knowledge and expert analysis of swimming strategies. CS, designed the experiment and helped finalizing the manuscript.

Disclosure of interests

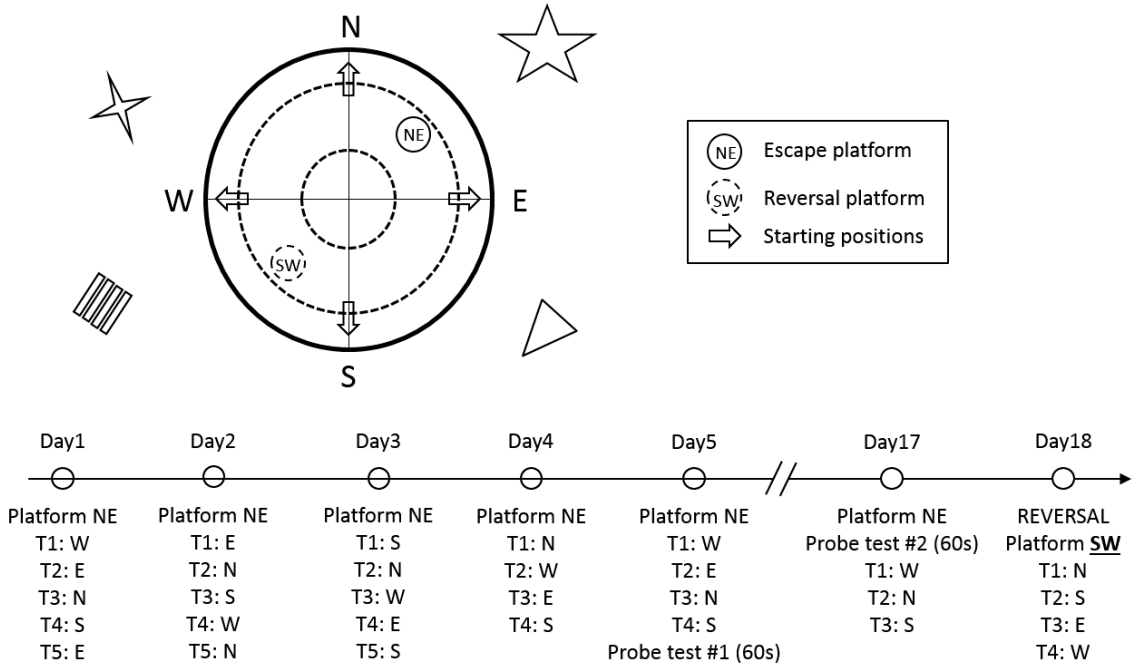
The authors report no conflicts of interest.

Supplementary Material

i. Supplementary methods

Morris-water-maze planning

The starting position for the MWM training followed a semi-random design in order to avoid prediction of the starting position and in order to have one training with each starting position per day (Supplementary figure 1).



Supplementary Figure 1: Morris-water-maze organization. The circular pool was surrounded of different visual cues (different shapes, colors and distances from the maze). The platform was placed in the NE quarter during the training procedure and in the SW quarter for the reversal training on D18. The starting positions were semi-randomly allocated as illustrated on the timeline.

MWM swimming paths analysis:

The RODA Software: The behavioral analysis of this study was performed by using the RODA software version 4.0.2 which implements an updated classification procedure based on (Gehring et al., 2015).

Trajectories Segmentation: Based on our previous studies (Gehring et al., 2015), the swimming paths of the animals were split into segments of length equal to 2.3 times the arena radius and 70% overlap percentage. The segments overlap is important since there is no prior knowledge of the length of each animal behavior thus information of importance which would be lost due to an unfavorable segmentation is now secured. The classification of the reversal trials was performed manually because a small amount of segments was available (216 total trials -including both males and females, which yield 3995 segments).

Classification Procedure: According to the classification procedure of Gehring et al. (2015) a small amount of labels needs to be provided on the segments. The classification algorithm will then use a set of eight trajectory features to create clusters of data (group segments based on their similarity) and then the labels to merge the clusters into 8

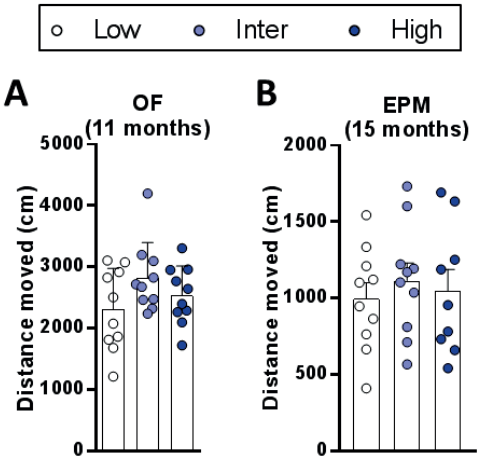
classes of behavior (see classes of behavior section below). Based on the indication of Gehring et al. (2015) and RODA. Labels are provided to 11% of the data. In addition, because of the clustering procedure, a number of clusters (known as K) needs to be selected which will indicate the number of clusters that will be created by the algorithm. Identifying an optimal K is a difficult machine-learning problem thus the default boosted classification procedure of RODA was used. This procedure tests different classification tunings (different Ks) and generate different classifiers. The classifiers are assessed based on their 10 fold cross validation error and the ones with error lower than 25% are selected to form an ensemble. The ensemble in the merged classification result of all the classifiers based on majority voting; for each data-point the classifiers vote in which class it falls into and finally it is assigned to the class with the most votes.

Classes of Behaviors: The following classes of behavior were used for the behavioral analysis within the Morris-Water-Maze:

- Thigmotaxis: The animal moves on the periphery of the arena, close to the walls.
- Incursion: The animal starts to move on the inward locations of the arena.
- Scanning: The animal randomly searches the whole arena and turns away from walls if it touches them (Graziano et al., 2003).
- Focused Search: searching a particular region of the arena, which is not where the platform is located.
- Chaining Response: The animal has memorized the distance of the platform from the arena wall and swims circularly in order to find it (Wolfer and Lipp, 2000).
- Self-Orienting: The animal performs a loop and orients itself inside the arena (Graziano et al., 2003).
- Scanning Surroundings: The animal crosses the platform or a region very close to the platform (Gehring et al., 2015).
- Scanning Target: Actively searching for the platform by focus searching the location where it is located.
- Direct Finding: Small unsegmented trajectories are automatically assigned to this class indicating that the animal swam straight away towards the platform.
- Transitions: Strategy transitions is the amount of times that each animal switches between different strategies.

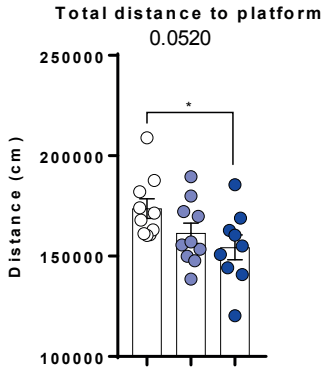
ii. Results.

During the OF, at 11 months of age (supplementary figure 2A), and EPM, at 15 months of age (supplementary figure 2B), there were no differences in locomotor activity between the lines ($p > 0.16$).



Supplementary Figure 2: Distance traveled in anxiety-like behavioral testing during mid-life (11 months) and early-aging (15 months). A, at 11 months of age, during 10 min in an OF, there was no line effect on the total distance traveled (One-way ANOVA: $F_{2,27} = 1.99$, $p = 0.156$). **B,** At 15 months on age during 5 min on an EPM, there was no line effect on the total distance traveled (One-way ANOVA: $F_{2,26} = 0.255$, $p = 0.78$).

During the second probe trial done in the MWM at D17, we measured the distance of the rats to the center of the previous location of the platform (supplementary figure 3). There was a tendency ($p = 0.052$) for a difference in the total distance from the center of the previous platform's location during the second probe trial (at D17). Low-line rats swam further from the platform than High-line rats ($p < 0.05$).



Supplementary Figure 3: Morris water-maze result. There was a tendency ($p = 0.052$) for a difference in the total distance from the center of the previous platform's location during the second probe trial (at D17). Low-line rats swam further from the platform than High-line rats ($p < 0.05$).

General Discussion

General discussion and future perspectives

This part summarizes our experimental findings and presents a general overview of how our studies can be integrated within the larger context of existing knowledge of the central regulation of stress and its interactions with autonomic, metabolic and aging mechanisms. In this thesis, a model of rats genetically selected for differential responsiveness to stress was used to study these links. Here we used rats with constitutive differences in glucocorticoid responses to repeated stress during their juvenile period. These rats display different biobehavioral phenotypes and can be classified into three separate lines namely "High", "intermediate", and "low" as shown previoulsy (Walker et al., 2017; Walker and Sandi, 2018).

The HPA axis is the central neuroendocrine component of the stress response, allowing proper energy distribution in the organism to allow behavioral, physiological and metabolic coping with environmental challenges. The HPA axis is highly connected to the autonomic nervous system and the mediators of metabolism. Therefore, studies in the dissertation were designed to test the novel hypothesis that selection for constitutive differences in HPA axis responses leads to differences in cardiac modulation between the lines (chapter 1) and general and central metabolism (chapter 2). Moreover, throughout life, stress interacts with aging processes in numerous levels and, we investigated the evolution of the biobehavioral phenotypes of the lines during aging (chapter 3).

i. Validation and extension of the biobehavioral phenotypes of the lines

First, we summarize in this section the different results reported in the thesis illustrating a stability of the biobehavioral phenotypes of the lines across generations and aging. We reported (see Annex 1) an experiment performed in order to validate and deepen our knowledge concerning the stress protocol (CAST) used to select the rats for the breeding of different lines. We applied a new CAST protocol (TMT-CAST), including the same stressor (TMT), at the same circadian time (morning), for the same duration (30 min) and we showed that Low-line rats, had a constitutive blunted HPA axis response (already on first day of stress) and a potentially less efficient negative feedback. On the other hand, High-line rats had higher CORT response to stress and a lack of habituation but an efficient negative feedback loop of the HPA axis. Importantly, my studies show that the lines were not habituated to homotypic stressor, thus we conclude that the three lines constitute a model of general differential response regardless of whether the stressor were homotypic or heterotypic in nature. Note that both stressors used were different (predator scent and elevated platform) but from the same 'environmental fear-inducing' type and that testing the lines with stressors from different natures (e.g. social stress) might allow use to extend for broader heterotypic habituation.

In chapters 1, 2 and 3, we showed that rats from the different lines had stable differences in their biobehavioral phenotypes as described previously in earlier generations (Walker et al., 2017; Walker and Sandi, 2018). We found that at adulthood, rats from the High-line exhibited: higher CORT secretion in reaction to stress (Chapter 1, 2 and 3; Annex 1), higher anxiety-like behaviors in the EPM (Chapter 1, 2 and 3) and in the LD-box (Chapter 1), higher aggressive behaviors (Chapter 2), more passive coping strategies in FST (Chapter 3) and in shock-probe burying test (Chapter 2), compared to Low-line rats. These results were obtained at different stages of adult life, from young-adulthood, between 3-5 months of aging (Walker et al., 2017; Walker and Sandi, 2018; Chapter 1 and 2), mid-life, around 11-15 months of age (Chapter 3) and early-aging, between 16-19 months of age (Chapter 3). All the findings for the biobehavioral phenotypes of the lines obtained are summarized in Table 1 (including the previous findings and the results presented in this thesis).

Previous studies in rats stressed during puberty indicated that stressed animals displayed increased emotionality, aggressive behaviors and decreased sociability (Tzanoulinou et al., 2014). Moreover, it was suggested that the High-

line may be anti-social in nature (Walker and Sandi, 2018) and consistently, we observed that the High-line rats displayed lower sociability than Low-line animals at mid-life when tested in a three-chambered test (data not shown). Taken together, differences in anxiety, emotionality, aggressiveness, coping and sociability parameters are gathered in the "biobehavioral phenotype" of the lines. This biobehavioral phenotype notion might be comparable to the "temperament trait" describing the idea that "individual behavioral differences are repeatable over time and across situations, covering numerous traits, such as aggressiveness, avoidance of novelty, willingness to take risks, exploration, and sociality" (Réale et al., 2007). The biobehavioral phenotype concept will be used for this general discussion since it includes all the differences observed between the lines.

ii. Cardiac autonomic regulation imbalance in Low- and High- lines

In my thesis, the first chapter includes characterization of the cardiac autonomic regulation in rats from the three lines. We demonstrate a potential link between the HPA axis control and the ANS modulatory systems, which are two key mediators of the stress response with some shared mechanisms (Cohen et al., 2015a; Coughlin, 2011), and being key risk factors for cardiovascular and psychopathological disorders (Beauchaine and Marsh, 2015; Beauchaine et al., 2007). Investigations of the autonomic correlates of rats differing in stress-responsiveness was performed with HRV analysis and evaluation of the vagal tone, which are indicators of cardiac health, representing behavioral and physiological flexibility of an organism and stress adaptation abilities (Porges, 1995). The results indicate that rats from the three lines differed in HR and HRV with the Low- and High-lines exhibiting higher resting HR and lower HRV compared to the Inter-line animals. Moreover, we show that with sympathetic blockade by atenolol injection, the three lines did not differ in sympathetic modulation of heart rate. Furthermore, the studies done in chapter 1 show that the selection for low or high CORT levels, in response to stress during juvenile period, is associated with a basal cardiac autonomic imbalance (low vagal tone) compared to intermediate stress reactivity. However, the High- and Low-lines showed no differences in the decrease in vagal tone induced by stress between the three lines, suggesting that the cardiac responsiveness and HR sensitivity to stress (Goldberger, 1991a; Carnevali et al., 2014) were not impaired in the high and low lines. Collectively, our findings indicate that differences in glucocorticoid levels during stress did not affect cardiac regulation. This is in accordance with previous studies showing that CORT treatment had no significant effect on arterial pressure and HR, while moderate elevation in CORT for several days induced pressure-independent modulation of baroreflex control of HR (Scheuer and Bechtold, 2002). The HPA axis and the ANS are extremely complementary (Ulrich-Lai and Herman, 2009) with autonomic control of HR from brain regions involved in the HPA-axis regulation and glucocorticoids acting on those regions as well as the adrenal gland being involved in CORT and norepinephrine production. Therefore, we suggest here that the inherent differences in HPA axis functioning between the lines had restricted outcome on HR responsiveness to acute stressors.

Constitutive differences in glucocorticoid responsiveness to stressors in the three lines were shown to be involved in the expression of psychopathology-like behaviors at adulthood (Walker et al., 2017). Previous studies with humans and rodents suggest a link between affective disorders including anxiety, depression- aggressive behaviors and psychopathologies with autonomic imbalance (Beauchaine et al., 2007; Carnevali and Sgoifo, 2014, Sévoz-Couche et al., 2013; Carnevali et al., 2014, Grippo et al., 2006, 2004; Wood et al., 2012). Furthermore, PTSD has been associated with a lower vagal tone (Sahar et al., 2001; Zoladz and Diamond, 2013; Shah et al., 2013), a blunted basal HPA-axis (Cohen et al., 2006; Danan et al., 2018), and an enhanced negative feedback inhibition of the HPA axis, with an exaggerated CORT sensitivity (Goenjian et al., 1996; Grossman et al., 2003; Yehuda et al., 2004). Thus, the links between ANS imbalance, HPA axis dysregulation, cardiovascular and psychopathological disorders emphasizes our lines as potent models for the investigation of the development of psychopathologies in humans. Specifically, our studies suggest that the High-line could be used as a model for depressive disorders while the Low-line could be studied as a model of PTSD susceptibility.

Behavioral Test	Measurement	Line	Effect	Reference	
Elevated-plus-maze (EPM)	Anxiety-like behaviors (time in open-arms)	Low	↓	Walker et al., 2017	Behavioral profile
		Inter	↔	Walker et al., 2018	
		High	↑	Chapter 1, 2 & 3	
Resident-Intruder (RI)	Aggressive behaviors	Low	↓	Walker et al., 2017 Walker et al., 2018	
		Inter	↔		
		High	↑		
Resident-Intruder (RI) After peripubertal stress	Aggressive behaviors	Low	↗	Walker et al., 2018	
		Inter	Not reported		
		High	↔		
Territory competition test	**Aggressive behaviors**	**Low**	↓	**Chapter 2**	
		Inter	↔		
		High	↑		
Forced-swim-test (FST)	Passive floating time	Low	↓	Walker et al., 2017 Walker et al., 2018	
		Inter	↓		
		High	↑		
FST at early-aging	**Passive floating time**	**Low**	↔	**Chapter 3**	
		Inter	↓		
		High	↑		
Shock-probe burying test	**Active burying**	**Low**	↑	**Chapter 3**	
		Inter	↔		
		High	↓		
Morris water-maze	**Learning / retention / reversal / swimming strategy**	**Low**	↔ / ↓ / ↓ / ↔	**Chapter 3**	**Cognitive profile**
		Inter	↔ / ↓ / ↑ / ↓		
		High	↔ / ↑ / ↑ / ↑		
Restraint stress	[CORT] in blood plasma	Low	↓	Walker et al., 2017	Endocrine profile
		Inter	↔		
		High	↑		
Basal HPA axis (diurnal peak and trough)	[CORT] in blood plasma	Low	↔	Walker et al., 2017	
		Inter	↔		
		High	↔		
HPA axis response at 16 months of age	**[CORT] in blood plasma**	**Low**	↓	**Chapter 3**	
		Inter	↔		
		High	↑		
Adrenal glands (P100)	Weight (normalized by body-weight)	Low	↔	Walker et al., 2017	
		Inter	↔		
		High	↔		
Adrenal glands at early-aging (18 months of age)	**Weight (normalized by body-weight)**	**Low**	↔	**Chapter 3**	
		Inter	↓		
		High	↑		
Autonomic nervous system	**Basal HR / SNS / PNS**	**Low**	↑ / ↔ / ↓	**Chapter 1**	**Autonomic profile**
		Inter	↓ / ↔ / ↑		
		High	↑ / ↔ / ↓		

Table 1. Summary of the biobehavioral phenotypes of rats from the three lines. Results from previous research from the lab (as shown in Introduction) supplemented with results presented in this thesis (in bold).

iii. Differences in energy metabolism between the lines and effects of social interactions on metabolism

In the second chapter, my studies demonstrate that rats from the three lines, in same-line pairing conditions, had differences social behaviors and in central energy metabolism measured by indirect calorimetry. Low-line rats were shown to use more carbohydrates as energy fuel compared to Inter- and High-lines which appeared to be contradictory to previous literature linking low CORT levels and increased fatty-acid use (Schiffer and Wertheimer, 1947; Hamelink et al., 1994; Patterson et al., 2013). These data suggested that some metabolic mechanisms paired with glucocorticoid secretion might be involved and potentially altered in Low-line rats, for example the co-release of NPY and ghrelin or the neuronal function in the hypothalamus (e.g. ARC). Indirect calorimetry measurements showed that the Inter-line had a lower energy expenditure than the two other lines (although the difference between Inter- and Low-lines was a statistical tendency) and previous studies showed a link between increased energy expenditure and heart rate regulation (Kuperman et al., 2010; Keytel et al., 2005). Thus, the lower energy expenditure measured for the Inter-line (Chapter 2) might be due to their lower heart rate values compared to Low- and High-lines, which is due to a higher parasympathetic activity (chapter 1) and related to a healthier cardiac phenotype (Porges, 1995).

Additionally, in chapter 2, we show that after one month of mixed-line pairing with High-line animals, central and brain metabolism and peripheral physiology were affected in Low- and Inter-lines. After one month of housing in mixed-line dyads, Low- and High-line rats lost more BW in LvH pairs than in LvI and IvH pairs. Even though, the effects of stress during calorimetric recording on body weight is not clear from the literature, we suggest that it could be interpreted as a higher stress response. Taken together this implied an increased stress responsiveness in Low-line rats when paired with High-line for a month. Moreover, Low-line rats from LvH pairs used more fatty acids as fuel, which is consistent with an altered relationship between the HPA axis regulation and the metabolism system in the Low-line. On the other hand, Inter-line rats did not have differences in RER or EE when paired with High-line rats but displayed an increase in both parameters when paired with Low-line rats. Altogether, these results suggested that mixed-social pairing could induced a normalization of the metabolic phenotypes, with Low-line converging toward High-line phenotype and the Inter-line converging toward the Low-line phenotype, whereas the High-line exhibited a more stable phenotype. Low-line rats paired with High-line animals had lighter gonadal fat indicating less body fat (Rogers and Webb, 1980) and had a higher use of fatty acids as fuel. Taken together, the studies suggest that the higher use of fatty acid reduces the amount of body fat in Low-line animals paired with High-line rats, possibly due to social pairing with High-line rats. Moreover, fat storage capacity and long-term adiposity might also be affected in those animals (Patterson et al., 2013b) but echo-MRI experiments would be required to determine precise body-composition.

We additionally sought to investigate the interaction between social housing in mixed-line dyads and mitochondrial respirometry in the NAc and mPFC. We first showed that, by analyzing data from the same lines together, High-line rats had lower mitochondrial respiration in the NAc compared to Low- and Inter-lines. In line with studies linking high anxiety-like behaviors, subordination and lower mitochondrial function or energy metabolites in the NAc (Hollis et al., 2015; Larrieu et al., 2017), within IvH pairs, more Inter-line rats became dominant than High-line rats. Interestingly, an opposite pattern was observed with rats from the LvH pairs implying that the high respirometry measured in the Low-line was not related to an increased dominancy propensity. Instead, the increased mitochondrial respiration in the Low-line might come from an altered metabolism following mixed-line pairing. When, mitochondrial respirometry data were analyzed according to the mixed-line dyads, we observed that Low- and Inter-lines paired with High-line rats had higher mitochondrial respirometry in NAc and mPFC. These increases

might be related to the energy required, to cope with High-line rats, in behavioral, emotional or cognitive brain circuitry.

iv. HPA axis regulation at early-aging

The third chapter of my thesis characterizes early-aging influences on HPA axis parameters (CORT and ACTH levels, adrenal responsivity and post-mortem adrenal weight) at 16 months of age. As previously mentioned, we validated that the differences in CORT response after 3 days of stress in juvenile period was stable throughout aging. Moreover, High-line rats exhibited heavier adrenal glands than the Inter-line, illustrating that they were exposed to a higher cumulative influence of behavioral challenges (that can be considered stressors) throughout life (Ulrich-Lai et al., 2006; Blanchard et al., 1995). There were no differences between Low-line and the two other groups in adrenal weight inferring that their adrenal glands were not necessarily hypo-responsive. High-line animals had higher basal ACTH than Low- and Inter-lines, which can be interpreted as a hypersensitivity to initiate a HPA axis response in these animals. Indeed, the High-line had higher *Avpr1b* and *Pomc* expression in the pituitary gland compared to the Inter-line (Walker et al., 2017). This illustrates an increased AVP sensitivity of the pituitary and a higher POMC availability (Herman et al., 2016) might be responsible for the higher basal level of ACTH in the High-line rats, and which might be overridden by CRH signaling during the stress response.

The adrenal responsivity was calculated from ACTH and CORT values (Myers et al., 2016) and we noticed that, in response to restraint stress, the Low-line had lower adrenal responsivity than the Inter-line. Low-line had increased expression of *Mrap* in the adrenal gland (Walker et al., 2017) compared to the High-line, which indicates a potential compensatory mechanism in order to promote the response to ACTH and to secrete more CORT (Novoselova et al., 2013). Furthermore, changes in adrenal sensitivity are not only due to changes in ACTH and control mechanism by the splanchnic innervation of the adrenal gland can induce cortical responses of the adrenal (Engeland et al., 1981; Ulrich-Lai and Engeland, 2002). Altogether, these data suggest that the reduced adrenal responsivity in the Low-line might be due to changes in sympathetic innervation of the adrenal gland and that a compensatory, but insufficient, mechanism through increased *Mrap* at the adrenal level was established.

v. Aging, learning and memory protection by glucocorticoids

During Morris water-maze experiments with the three lines at early-aging we showed that: i) rats from the three lines had similar escape latency during training, ii) High-line rats had a better long-term memory of the platform's location, iii) Low-line rats had an impaired memory retention and reversal learning and, iv) Inter-line rats exhibited lower-level swimming strategies. Interestingly, during the training, the escape latency was the same between the three lines but Inter-line rats did not use the same strategy to escape. Instead of using a classic spatial learning, they used a strategy during which they were swimming faster in a circular way by assessing the distance to the wall of the pool. Further studies and more systematic determination of the swimming strategies are warranted in research using the Morris water-maze in order to gain knowledge on the neural basis of the different strategies and the implications of the different level of strategies. Low-line rats had impaired reversal learning and it was reported that the Low-line had lower basal ventral OFC activity than High-line rats at 4 months of age (Walker and Sandi, 2018). Moreover, the OFC has been implicated in aged animals with reversal impairment (Schoenbaum et al., 2006), thus it would be important to determine whether OFC was altered and involved in this reversal learning impairment of the Low-line.

Importantly, High-line rats had better long-term memory retention of the platform's location, which might be explained by a higher production of CORT during swimming (Sandi et al., 1997; Akirav et al., 2001; Akirav et al., 2004). These data are of great interest since they illustrate that, despite the high levels of CORT during stress experiences during life of the High-line, there are no noticeable long-term detrimental effects, and on the opposite, high CORT

secretion during behavior allowed the High-line to outperform the other lines. Previous studies noticed deleterious aging effects at similar early-aging state (Weiss and Thompson, 1991; Kadish et al., 2009) and long-lasting and negative effects of stress have been related to impaired negative feedback (Sapolsky et al., 1983, 1985a, 1985b, 1986; Segar et al., 2009) and to the subsequent brain exposure to excitotoxins (Bishop et al., 2010). Therefore, we hypothesize that the negative feedback is a central mechanism of deleterious effects of glucocorticoids on the brain during aging. However, the three lines previously showed efficient negative feedback loops (Walker et al., 2017; Annex 1) and if anything, the Low-line rats appeared to have a less efficient feedback inhibition of the HPA axis (Annex 1). In the future, it will be important to study aging processes in the context of lifelong exposure to stress, such as exposing the rats from the lines to chronic stress protocols. A recent study, submitting Low- and High-lines to a peripubertal stress protocol, showed higher susceptibility in Low-line rats to develop aggressiveness (Walker and Sandi, 2018). Moreover, as shown by increased adrenal weight in the High-line, these animals had a long-term effect of higher cumulative exposure to glucocorticoids throughout life. Thus, using a chronic stress protocol might trigger a sufficient increase in CORT exposure to show deleterious effect on the High-line rats.

vi. Biobehavioral phenotypic differences and coping strategies of the lines

In chapter 2, we demonstrate differences in social interactions between rats from the three lines and we show that High-line rats exhibited more aggressive behaviors than the other lines. Additionally, pairs including High-line rats displayed more aggressive behaviors and more self-grooming illustrating an increased arousal state potentially inhibiting social behaviors. Interestingly Low-line rats were more susceptible and showed more behavioral differences between pairing with Inter- or High-lines. Low-line rats paired with High-line animals had lighter testes and were more subordinate, which is in line with reports showing a reduction in plasma testosterone in subordinate males (Tamashiro et al., 2004; Hardy et al., 2002).

Taken together, results provided from the three chapters of my thesis might be combined in order to discuss the implications of the differences in biobehavioral phenotypes as well as to suggest potential future investigations. The results from the different coping strategy experiments do not allow a clear categorization for the coping style of the lines. According to the literature, individuals having higher aggressive behaviors also exhibit higher burying strategies, and both strategies are considered “active” (Boer and Koolhaas, 2003; de Boer et al., 2017). However, High-line animals were more active (aggressive) in a resident-intruder test but were more passive in a FST (floating) and in a shock-probe burying test (less burying). As mentioned in the introduction, coping style is a multi-dimensional concept, thus, we suggest that High-line rats behave actively in social contexts, whereas they used passive strategies against non-social threats. Whether this difference is due to differential subjective threat valences for social or non-social situations is unknown, but further investigation on this will greatly enlighten the current literature on coping style. Interestingly, It has recently been shown that rats displaying higher aggressive behaviors did not necessarily access dominant status (Buwalda et al., 2017) and active copers in burying test, in case of subordination, exhibited more pronounced allostatic load (Boersma et al., 2017). In chapter 2, we show that High-line rats did not exhibit higher social dominance when paired with Inter-line rats than that in LvH pairs, High-line rats were more dominant and Low-line rats showed more behavioral and metabolic changes. Additionally, the link between active aggression and active burying has not always been consistently described in the literature (Veenema and Neumann, 2007).

Other studies related to coping strategies, HPA axis, autonomic and metabolic responses. For example, in fish, higher energy expenditure was found in passive individuals, with longer latency to escape in a confinement stress (Martins et al., 2011), and active copers exhibited typical neuroendocrine responses such as lower HPA-axis activity (Silva et al., 2010) and reactivity (Overli et al., 2005). Additionally, active fish were characterized by higher SNS reactivity and lower PNS (Verbeek et al., 2008) compared to passive copers. Moreover, passive copers may habituate to stress while

active copers might show sensitization to stress (Martins et al., 2011). It was suggested that active copers react more based on “internally organized” responses, while passive copers are more influenced by environmental stimuli and should be interpreted as a highly adaptive strategy for conserving energy and does not specifically reflect depression (Molendijk and de Kloet, 2015; de Boer et al., 2017). However, the lower sensitivity to environmental stimuli in active (aggressive) copers is challenged by the fact that active (burying) individuals becoming subordinate are more susceptible to social stress (Boersma et al., 2017) and we emphasize the importance of distinguishing the social and non-social aspects of coping style.

Another point that may explain several findings reported in the thesis is the fact that behavioral flexibility (changing behavioral response) is an important element of one’s fitness since it requires energy. Indeed, changing phenotype is energetically demanding (Stamps and Groothuis, 2010), thus saving energy by maintaining one’s phenotype in a challenging environment might increase fitness (Réale et al., 2007). Moreover, social contagion phenomena of negative emotional state of an animal, can affect the emotional, behavioral, cardiac state and HPA axis modulation of social partners (Carnevali et al., 2017). Overall, pairing rats with different biobehavioral phenotypes together, might lead to a situation in which, either both individuals maintain their phenotype, saving energy, but inducing social instability, or one individual might invest energy to adapt his phenotype, leading to less social conflicts. As illustrated in Chapter 2, Low- and Inter-lines were probably more susceptible to change their phenotypes when paired with High-line animals and increased brain energy capacity, illustrated by increased mitochondrial respirometry. On the other hand, High-line rats might represent an extreme non-flexible biobehavioral phenotype, saving energy but potentially related to negative outcomes. However, further experiments are required to test whether social contagion, also normalized other aspects of the phenotype (e.g. anxiety and CORT response).

vii. The arcuate nucleus of the hypothalamus and ghrelin: two potential mediators for biobehavioral phenotypic differences between the lines

Among, the different hormones regulating homeostasis and food intake, ghrelin appeared to be involved in all the previously mentioned system. Indeed, ghrelin acts on the ARC, through a vagal afferent pathway and NTS neurons, and enhances appetite by activating NPY/AgRP neurons and suppressing POMC neurons, it also indirectly activates CRH neurons in the PVN (Cabral et al., 2012) and directly acts on the anterior pituitary gland and facilitates ACTH release. Moreover, ghrelin was implicated in the regulation of anxiety-like behaviors (Spencer et al., 2015). The arcuate nucleus of the hypothalamus appears to be a potential central region for differences between the lines. It has been showed to control glucocorticoid secretion, through PVN projection (Leon-Mercado et al., 2017), to mediate sympathetic modulations controlling appetite and energy expenditure, involving POMC and MC4R pathways (Lu et al., 2003; Cansell et al., 2012), and to regulate heart rate depending on arterial pressure (Rahmouni, 2016). Taken together, we hypothesize that rats from the Low- and High- line show similar cardiac autonomic regulation but might display central difference in ARC, leading to differences in metabolism and in arterial pressure control. Further studies should focus on assessing arterial pressure of the lines and its control in response to stress, with radiotelemetric techniques. However, no data from the lines are available yet, but future studies should focus on the analysis of gene expressions and protein level assessment in order to evaluate key regulators of cardiac, HPA axis and metabolic systems that might be differently regulated in the lines. Potential targets may subsequently be specifically targeted (inhibited or over-activated) in order to establish causality between gene expression and in the observed phenotype.

General Conclusion and future perspectives

In this thesis, I have demonstrated that our model of rats genetically selected for differential glucocorticoid response to stress can be used as a fundamental tool in the investigations of the link between HPA axis regulation,

cardiovascular, metabolic and aging processes. Moreover, we suggest that it could be applied in the study of immunology, gut-microbiota and a numerous of systems that have been showed to interact with the stress system.

Finally, an important observation from my thesis is that the two extreme lines, hypo- and hyper-responsive to stress need to be further studied in order to deepen literature knowledge relating coping style, stress response, stress habituation, as well as stress susceptibility depending on social contexts. In Figure 1, a simplistic representation of the differences between Low- and High-lines in the social and non-social contexts and potential aspects requiring further investigation (illustrated by "?") has been schematically represented.

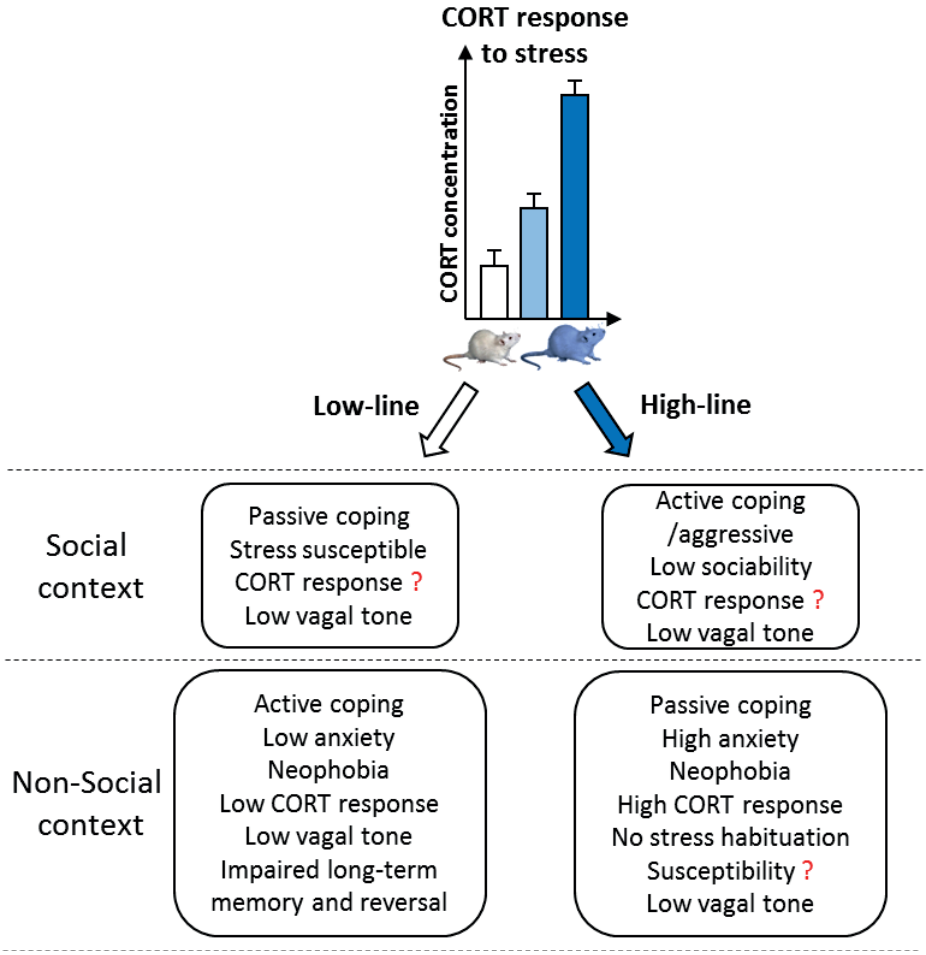


Figure 1. Graphical summary of the biobehavioral phenotypic differences of the Low- and High-lines according to the social context of behavioral experiments. Rats from the Low- (white bar and rat) and High- (dark blue) lines may show different coping strategies when facing social or non-social threats.

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Annex 1

Selecting lines of rats for differential stress responsiveness and adaptation

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Introduction

This annex focuses on the analysis of the corticosterone (CORT) response to the 'corticosterone-adaptation-stress-test' (CAST) selection process applied to the rats used as a model of differential stress responsiveness and glucocorticoid adaptation during juvenile period.

The CAST protocol is used in our laboratory to produce rats from three lines showing differential stress responsiveness and adaptation to stress and has been performed for 12 generations so far. The protocol used during juvenile period and before puberty, between post-natal days 28 and 30 (P28-30), is a combination of exposures to fear-induction procedures. It is a truncated version of the peripubertal stress protocol developed in our laboratory (Toledo-Rodriguez and Sandi, 2011; Márquez et al., 2013) which, though clearly stressful, has been shown to be insufficient in inducing the behavioral alterations associated with the full protocol at adulthood (Toledo-Rodriguez and Sandi, 2011; Tzanoulinou et al., 2014). Tail blood samples are taken at two timepoints on two separate days of the protocol (P28 and P30). Three breeding lines were established according to the outcome of the CAST. Rats with extremely low (<100ng/ml) or extremely high (>200ng/ml) secretion of CORT on the final day of the CAST (i.e. animals showing habituation or not respectively) were selected as the 'Low-' and 'High-' breeding lines. A third breeding line, 'Inter-line', was consisted of animals with intermediate CORT values in the CAST (between 100ng/ml and 200ng/ml).

The CAST protocol allowed a successful selection of rats according to their CORT secretion at P30 (Walker et al., 2017; Walker and Sandi, 2018), several questions remained to be elucidated concerning the CORT secretion values, the adaptation to different stressors and the circadian influences (Atkinson and Waddell, 1997). Indeed, as represented in Figure 1A several parameters of the protocol used can be questioned. First, the stress sessions were not performed at the same time of the day and a circadian fluctuation of CORT has been showed with low CORT values at the beginning of the inactive light-phase and increasing CORT levels during the second part of the light-phase reaching a maxima at the onset of the dark-phase (Bertani et al., 2010). Secondly, the duration of the stressors were not the same on P28 (30 min) and P30 (50 min). Therefore, differences in stress response between the 2 days cannot accurately be compared since alterations of the HPA axis negative feedback might change the CORT values evolution with stressors of different durations. In addition, different stressors were used, elevated platforms (EP) and Trimethylthiazoline (TMT = predator scent), so it is impossible to exclude the existence of a specific sensitivity against one of the stressors in a line which could affect the interpretation of the adaptation results. Finally, the recovery from stress was measured after 30 min in a neutral cage and in an unfamiliar environment, which can be seen as a novelty stress, thus CORT measurements obtained cannot be defined as "recovery from stress".

In order to test whether those parameters influenced the CORT response of the rats from the different lines, we applied a TMT-CAST protocol, as illustrated in Figure 1B. The TMT-CAST protocol was a controlled version of the classic CAST protocol. Rats were exposed for 3 consecutive mornings (P28-30) to the same stressor (TMT) for 30 min. Recovery from stress was measured on P28 and P30 after 45 min in their homecage. Finally, a different stressor was applied at P31 (EP), in order to address for stress specificity of the previous responses measured.

Materials and Methods

i. Animals and CAST procedures

Male Wistar-Han rats were bred in our animal facility and used as subjected during the CAST protocol.

Classic CAST

Classic-CAST procedure is represented in Figure 1A. Following exposure to 5 min in an open field (50 x 50 x 30 cm) on P28, the stress protocol consisted of the presentation of two different stressors, each one lasting 25 min. These were

either; exposure to the synthetic fox odor trimethylthiazoline (TMT) or to an elevated platform (EP). TMT exposure was administered in a plastic box (38 x 27.5 x 31 cm) via a scent-charged paper tissue. The box was placed under a bright light (> 200 lx). The elevated platform (12 x 12 cm, elevated 95 cm from the ground) was also under direct bright light (> 400 lx). Following each stress session, animals were returned to neutral cages for 15 minutes. A transparent Plexiglas wall perforated with holes separated pairs of cagemates during this time. Following the holding period, animals were returned to their home cage. The stressors were applied during juvenility, on three consecutive days across p28–p30, during the light phase and following an unpredictable schedule. Tail blood samples were taken on p28 and p30, once at the offset of stress and again 30 minutes later.

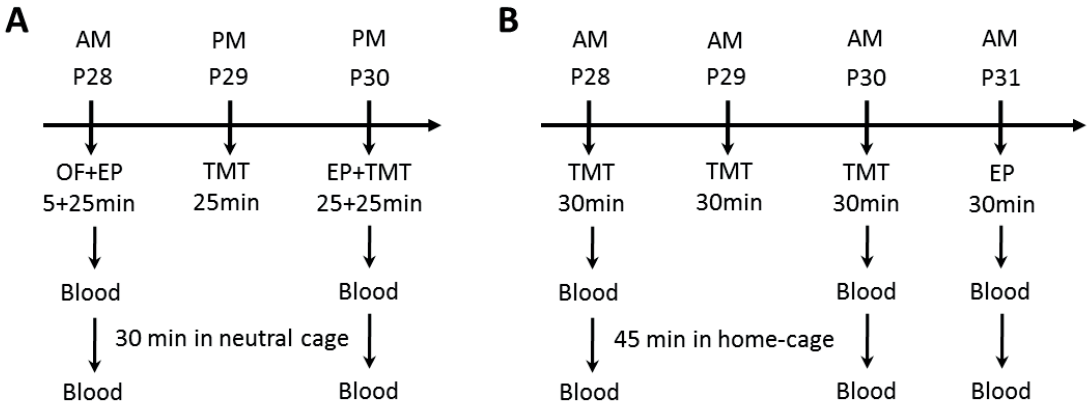

Figure 1: Experimental timelines of classic-CAST (A) and TMT-CAST (B) protocols. A, The classic-CAST is a combination of different stressors applied at different time points of the day in order to create unpredictable stress between P28-P30. After stress exposure, tail-blood was sampled immediately after stress exposure and following 30 minutes in a neutral cage. **B,** The TMT-CAST protocol was designed to control for stress, duration and time of application. During P28-30, rats were exposed to 30 minutes of TMT CAST during the morning. An additional stress exposure was performed at P31 with the elevated platforms. Tail-blood was sampled at P28, P30 and P31, immediately after stress and after 45min in homecage. OF = Open-field, EP = Elevated-Platform, TMT = trimethylthiazoline (predator/fox scent).

TMT-CAST

The TMT-CAST protocol is illustrated in Figure 1B. During P28-30, rats were submitted to the 30 min TMT scent in a bright (> 200 lx) and unescapable environment between 9-12 am. On P30, rats were placed in another bright testing room (> 400 lx) on elevated-platforms for 30 min. Following stressors at P28, P30 and P31 tail-blood was sampled and rats were placed in their homecage with separators in order to avoid social physical contact with their cagemates and left undisturbed for 45 min before a 2nd blood sample.

ii. Corticosterone analysis

All blood samples were collected into ice-cold heparin-coated capillary tubes (Sarsted, Switzerland) and kept on ice until centrifugation (10000 rpm; 4 °C; 4 min). Blood plasma was stored at -20 ^{o}C until analysis. Free corticosterone was measured in the plasma samples (dilution 1/40) using an enzymatic immunoassay kit, performed according to manufacturer's instructions (Enzo Life Sciences, Switzerland). Levels were calculated using a standard curve method.

CORT adaptation between P28 and P30 was calculated with the formula: ([CORT(P30)] - [CORT(P28)]) / [CORT(P28)] * 100.

CORT recovery between peak CORT response and recovery, 45 min later, was calculated with the formula: ([CORT(45min)] - [CORT(peak)]) / [CORT(peak)] * 100.

iii. Statistics

Results of rats from 3 different generations (F7, F8 and F11) submitted to the classic-CAST were averaged and analyzed as reported in Figure 2 and supplementary figure 1. One-way ANOVA with the lines as main effect were performed. Rats from generation F12 (n = 9 per line) were used in the TMT-CAST experiment and reported in Figure 3 and supplementary figure 3. Repeated-measures two-way ANOVA statistics were performed with the lines as between-subject factor and days as within-subject factor. Posthoc analyses were performed using Fischer's LSD comparisons. Prior statistical analysis, data distribution were checked for normality and outlier were Excluded with the ROUT method. Excluded animals are reported in the description of the figures. Data are reported as mean ± SEM. Statistical significance was set at $p < 0.05$. Statistics and graphs were performed with GraphPad (Prism, Version 7).

Results

i. Classic-CAST

During the classic-CAST protocol, Low-line animals produced less CORT than Inter- ($p < 0.001$) and High-line animals ($p < 0.05$) at P28 (Figure 2A). There was no difference in CORT response at P28 between Inter- and High-line animals ($p > 0.1$). At P30, there was a line-effect ($p < 0.001$) on the CORT response to stress (Figure 2B). High-lines rats secreted more CORT than Inter- ($p < 0.001$) and Low-lines rats ($p < 0.001$). Low-line animals had lower CORT values than Inter-line rats at P30 ($p < 0.001$). Stress response adaptation was measured by the ratio of the difference in CORT between P30 and P28 over the CORT value of P28. A negative value illustrated a decrease in CORT (habituation) and a positive ratio showed an increase in CORT between P28 and P30 (sensitization). There was a line effect ($p < 0.001$) on the percentage of CORT adaptation (Figure 2C). Low- and Inter-line rats had lower adaptation than High-line animals ($p < 0.001$) and exhibited a significant habituation (one sample t-test (vs. 0%): $p < 0.001$ and $p < 0.01$ respectively). On the other hand, High-line rats had a significant sensitization to stress (one sample t-test (vs. 0%): $p = 0.05$). Low-line rats had lower adaptation (higher habituation) than Inter-line rats ($p < 0.001$).

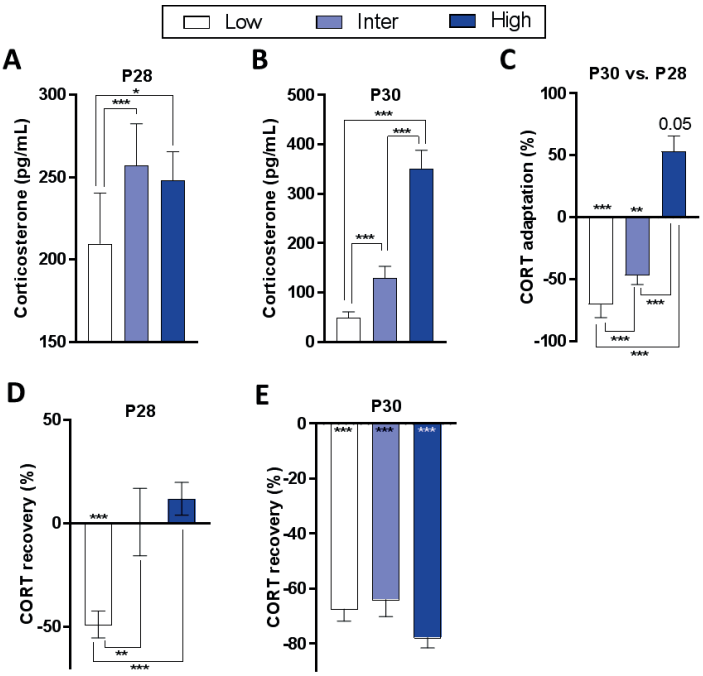

Figure 2: Corticosterone responses and adaptation to the classic-CAST protocol differ between rats from the three lines. A, During P28, rats from the Low-line produced less CORT than Inter- and High-rats. **B,** At P30, there were important differences in stress responses. High-rats produced more CORT than Inter and Low-animals ($p < 0.001$) and Low rats produced less CORT than Inter-rats ($p < 0.001$). **C,** The CORT adaptation was calculated as the percentage of CORT produced at P30 in comparison to P38. Low-rats habituated more than Inter-rats ($p < 0.001$) and high rats showed a sensitization response to stress ($p = 0,05$). **D,** Rats from the Low-line displayed higher recovery to stress at P28 in comparison to Inter- ($p < 0.01$) and High-animals ($p < 0.001$) which did not show significant recovery after 30 minutes in a neutral cage post-stress. **E,** On P30, all lines showed important and similar recovery from stress ($P = 0.11$). Statistical comparisons (Fischer's LSD) are indicated as follow: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # $p < 0.1$

Following, 30 min in a neutral cage and in an unfamiliar environment, plasma corticosterone was analyzed to assess recovery from stress. Recovery was computed as the CORT adaptation, with negative values showing efficient recovery and positive values illustrating lack of recovery (increase in stress response). On P28 (Figure 2D), there was a significant line effect ($p < 0.001$) in the percentage of CORT recovery. Low-line rats had lower recovery percentage than Inter- ($p < 0.01$) and High-line rats ($p < 0.001$). There was no difference between Inter- and High-line animals ($p > 0.1$). Efficient recovery was observed for Low-line rats only (one sample t-test (vs. 0%): $p < 0.001$) since Inter- and High-line rats did not differ from 0% (illustrating no recovery after 30 min). During P30 (Figure 2E), there was no difference between the lines ($p = 0.11$) in the recovery from stress and the three lines exhibited significant recovery after 30 min (one sample t-test (vs. 0%): $p < 0.001$).

ii. TMT-CAST

Following the TMT-CAST protocol, blood samples were analyzed for different blood samples. Corticosterone concentration following 30 min of TMT (P28-30) or EP (P31) are represented in Figure 1A. There was a significant line effect ($p < 0.001$) across the three days as well as a significant effect of the day ($p = 0.004$), but no interaction between lines and day ($p = 0.508$). Low-line animals had a lower CORT secretion during the different days than Inter- ($p = 0.003$) and High-line rats ($p < 0.001$). Inter-line rats had lower CORT than High-line animals ($p < 0.001$). Those differences were stable across the 3 days and Low-line rats had lower CORT than the Inter- and High-lines already at P28 ($p < 0.1$ and $p < 0.001$ respectively).

The CORT adaptation, as measured with classic-CAST data, between P28 and P30 showed a significant line-effect ($p = 0.02$). Low- and Inter-line rats had a lower CORT adaptation than High-line rats ($p < 0.01$ and $p < 0.05$ respectively). There was no difference in adaptation between Low- and Inter-lines rats ($p > 0.1$). When tested against 0%, it appeared that Low- and Inter-line rats had a significant habituation ($p < 0.001$ and $p < 0.01$ respectively) while there was no difference for High-line rats ($p > 0.1$).

The values of corticosterone concentration after 45 min of recovery in homecages are illustrated in Figure 3C. Globally, there was a main difference between the lines ($p < 0.001$), a main effect of the testing day ($p < 0.001$) and an interaction between lines and day ($p < 0.001$). When Days where analyzed separately, it appeared that High-line animals had higher CORT than low- ($p < 0.01$) and Inter-line rats ($p < 0.001$) after recovery at P28. There were no differences in CORT values after recovery at P30 and P31.

The percentage of CORT recovery was determined for the three days (Figure 3D). On P28, there was a significant line-effect ($p = 0.04$) on the percentage of recovery. Low-line animals had a higher recovery value (less decrease in CORT) than Inter- ($p < 0.1$) and High-line rats ($p < 0.05$). Stress recovery percentage tested against 0% with one-sample t-test showed significant recovery for Inter- ($p < 0.001$) and High-line rats ($p < 0.001$) but not for the Low-line. During P30 and P31, similar results were observed, with significant recovery for the three lines ($p < 0.001$) but lower recovery percentage for Low-lines rats in comparison to Inter- ($p < 0.01$) and High-lines ($p < 0.001$).

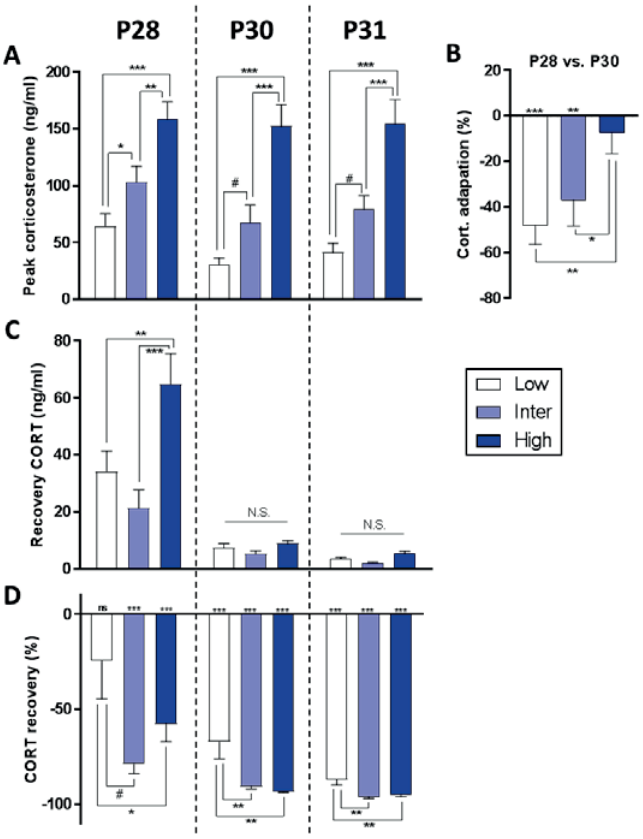

Figure 3: Corticosterone responses, adaptation and recovery to the TMT-CAST protocol differed between the three lines. A, At P28, low animals had had a lower CORT response than Inter- ($p < 0.1$) and High-rats ($p < 0.001$) and Inter produced less CORT than high-animals ($p < 0.01$). Same response were obtained at P30 and P31. After 30 minutes of recovery, Low and Inter-rats had lower CORT than high rats ($p < 0.001$). One High-line rat excluded by outlier analysis from P28. **B,** The stress adaptation between P28 and P30 was the same for low and Inter-animals that habituated more than High-rats ($p < 0.05$). High-rats did not show habituation to stress. **C,** After 45 minutes in homecage, recovery CORT was assayed and Low animals had lower CORT than High-rats at P28 ($p < 0.05$). There was no difference in recovery CORT at P30 and P31. Two Inter- and one high-line rats were excluded as outliers from P28 and P30 respectively. **D,** The percentage of CORT recovery was assessed and low animals showed no recovery at P28, in comparison to inter- ($p < 0.1$) and High-animals ($p < 0.05$). Low rats had less recovery at P30 and P31 ($p < 0.01$). Two Inter- and one high-line rats were excluded as outliers from P28 and P30 respectively. Statistical comparisons (Fischer's LSD) are indicated as follow: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # $p < 0.1$

Discussion

A previous report from the lab (Walker et al., 2017) showed that rats from a previous generation of the lines (F7) did not differ in CORT response at P28. We showed here, with the TMT-CAST protocol, that the following generations (from F7 to F12) elicited a different CORT secretion to stress at P28 and Low-line rats secreted less CORT than both the Inter- and the High-lines at P28. Note that the difference between Low- and Inter-lines was a statistical tendency in the TMT-CAST protocol but data from the classic-CAST illustrated a significant difference between Low- and Inter-line rats. Taken together we suggest that the genetic selection applied, led to a constitutive difference in CORT responsiveness between the three lines, already noticeable at the first stress exposure during juvenile period.

In the report from Walker and colleagues (2017) the CORT response at P30 relative to P28, for the High-line, showed a sensitization (P30 CORT relative to P28 = 134.1 ± 5.1 %) and a one-sample t-test (tested against 100%) was significant ($t_9 = 6.69$, $p < 0.001$). Moreover, in line with these findings, with the classic-CAST, rats from the Low- and Inter-line rats significantly habituated to stress whereas high-line animals showed sensitization. However, with the TMT-CAST protocol, the High-line did not exhibit sensitization. Thus, we suggest that the sensitization to stress obtained from the classic-CAST protocol was an artifact due to the fact that the stress applied at P30 was longer than at P28, and that the High CORT response from the High-line was due to longer CORT secretion. Altogether, we show that rats from the lines differ in stress habituation but the High-line do not have sensitization to homotypic stress.

With the classic-CAST protocol, Low-line rats showed higher recovery after 30 min in a neutral cages on P28 but all rats had similar recovery at P30. We suggest that the exposure to a neutral cage in an unfamiliar environment was considered as a "novelty" stressor on P28 and not on P30. It is difficult to assess whether rats from the lines had different recovery from stress on P28 or whether the 30 min period was perceived as more stressful for Inter- and

High-line rats compared to the Low-line. With the TMT-CAST protocol, at P28, High-line rats had higher CORT after 45 min of recovery in homecage but, the relative recovery (in comparison to peak CORT) was higher in High-line animals than Low-line rats. Moreover, on P30 and P31, Low-line rats had less recovery than Inter- and High-line animals. However, the magnitude of the differences in small and the differences observed are potentially due to peak CORT differences. In the TMT-CAST protocol, the exposure to a supplementary day with a novel stress showed that rats exhibited the same CORT pattern response.

Altogether, those results showed that Low-line rats, have a lower CORT response to stress and a less efficient recovery, which might suggest a blunted HPA axis response and a less effective negative feedback compared to Inter- and High-line. On the opposite, High-line rats have extreme CORT secretion in reaction to stress and a lack of habituation but an efficient negative feedback loop. The stress responses measured are not stress specific and the lines constitute a model of general differential habituation to stress.

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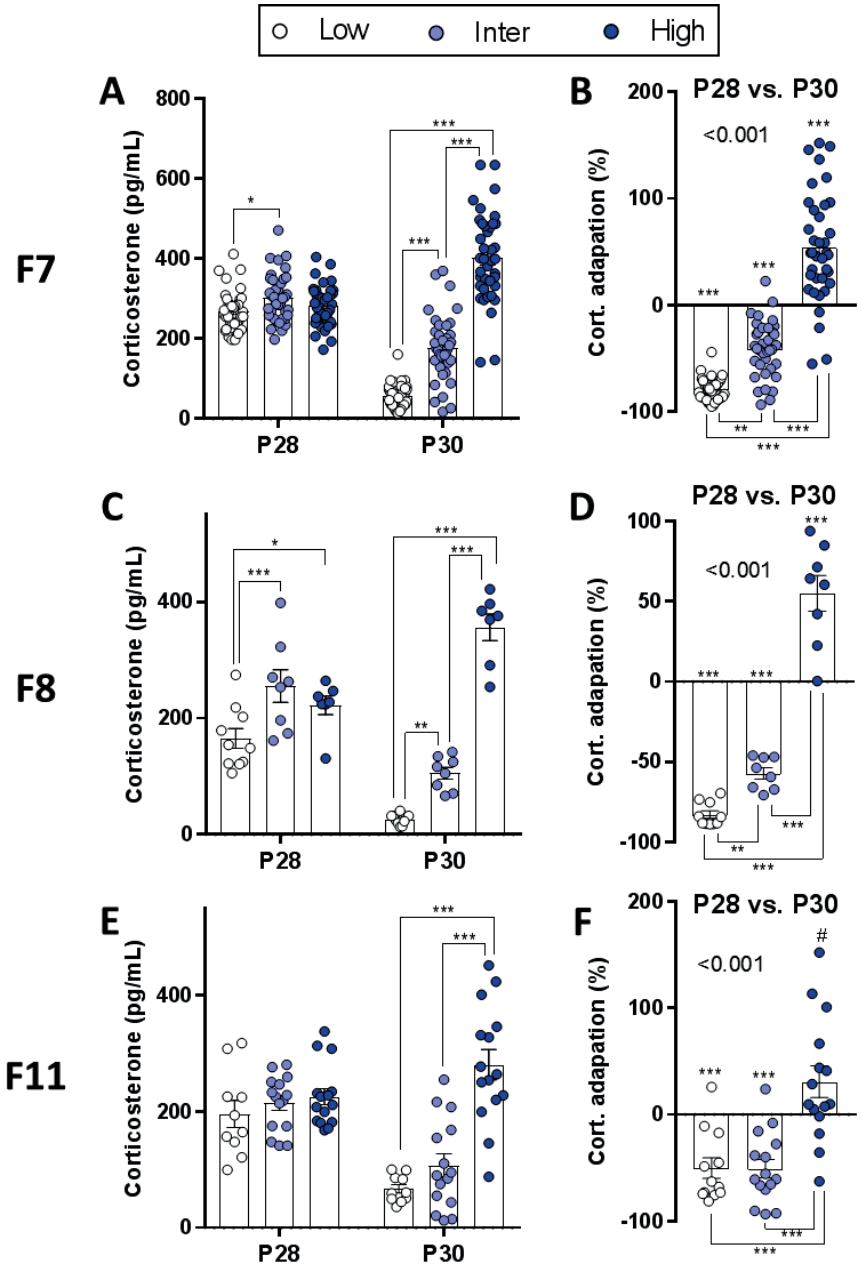
Supplementary Material



Supplementary Figure1. Corticosterone responses and adaptation to the classic-CAST from generations F7, F8 and F11 represented in Figure 2A-C. A, With F7, there was a higher CORT production in Inter than Low-rats at P28 ($p < 0.05$) and significant differences between the three lines at P30 ($p < 0.001$). **B,** Low and Inter rats showed important habituation to stress while High-rats showed sensitization ($p < 0.001$). **C,** During CAST of F8, Low rats had lower CORT response at P28 and P30 in comparison to Inter and High-rats ($p < 0.05$). Inter and high-rats differed in CORT response only at P30 ($p < 0.001$). **D,** Inter and Low rats habituated a lot whereas High-rats sensitized in response to stress ($p < 0.001$). **E,** During F11, there was no difference in CORT response at P28 and Low and Inter rats had low CORT at P30 in comparison to High-animals ($p < 0.001$) but no difference between them. **F,** There was a difference in habitation between high- and both Low- and inter-lines ($p < 0.001$) but no difference between Low and Inter. High-rats had a tendency for a higher stress response at P30 than at P28 ($p < 0.1$). Statistical comparisons (Fischer's LSD) are indicated as follow: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # $p < 0.1$

Annex 2

The link between aberrant hypothalamic-pituitary-adrenal axis activity during development and the emergence of aggression – Animal studies

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Abstract

Aggressive behavior is not uniform, including proactive and reactive forms of aggression. Aberrant functioning of the hypothalamic-pituitary-adrenal (HPA) axis is frequently associated with abnormal aggression. Here, we review the rodent literature in order to assess whether developmental abnormalities in the HPA axis can be causally linked with the emergence of abnormal aggression. We examine studies that involve genetic models and life challenges (e.g., early life stress, drug exposure) that course with developmental alterations in the HPA axis. Although the lack of systematic studies hinders development of an integrated model, existing evidence supports a U-shaped function regarding differences in HPA axis functioning during development and the emergence of aggressive phenotypes. Thus, developmentally low or high HPA axis reactivity are typically found to be aligned with the emergence of aggressive phenotypes; however, existing information is insufficient to causally link divergent HPA axis aberration with specific types of aggression. Progress in this field is needed to support interventions in children aimed at ameliorating social dysfunctions associated with aberrations in HPA axis function.

Keywords

Abnormal aggression; Aggression; Animal models; Behavior; Corticosterone; Drugs; Early life stress; HPA axis; Inbred strains; Mouse; Rat; Selective breeding;

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Disclaimer

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Introduction

Aggression is a behavioral adaptation ubiquitously expressed throughout the animal kingdom. However, aggression is not uniformly expressed and may manifest in several forms. A general and widely accepted distinction discriminates between reactive, normally considered emotional-impulsive, and proactive, cold, gain-oriented, aggression (Haller, 2014a). Although the neurobiological mechanisms leading to the expression of these different types of aggression are still unclear, progress in this field is currently blooming (Blair, 2016; Waltes et al., 2015; Yang & Raine, 2009).

Altered functioning of the hypothalamus-pituitary-adrenal (HPA) axis has been frequently found to be associated to pathological forms of aggression. Along with the sympathetic nervous system (SNS), the activated HPA axis coordinates metabolic, behavioral and physiological responses to stressful challenges. Although findings from the human literature are not always consistent, probably due to the difficulties in systematizing its collection (timing, circadian characteristics, basal vs. reactive, etc.), substantial evidence indicates that individuals characterized by elevated levels of reactive aggression show heightened activation of the stress systems (Lopez-Duran et al., 2009). Conversely, one of the most consistently reported findings is that individuals with elevated affective psychopathic traits display blunted activation of the physiological stress systems (including blunted cortisol) to stressful situations (O'Leary et al., 2007; O'Leary et al., 2010; but see Johnson et al., 2015 for evidence in incarcerated male offenders showing that some psychopathic individuals show normal cortisol stress responses). Remarkably, substantial evidence indicates that similar alterations in the HPA axis are already observable during childhood (Fairchild et al., 2008; Hawes et al., 2009). Thus, HPA axis hypo-activity is frequently reported for children and adolescents with callous-unemotional traits (a large part of those diagnosed with conduct disorders, and those with a higher probability to show criminal behaviors at adulthood) (Loney et al., 2005; McBurnett et al., 2000; van Goozen et al., 2000 but see Gordis et al., 2006). On the other hand, HPA axis hyper-activity is observed in cases of child and adolescent antisocial behavior in those with low levels of callous-unemotional traits (Lopez-Duran et al., 2009).

An important and unresolved issue is whether such alterations in the stress systems, and particularly in the functioning of the HPA axis, are a mere correlate of the different types of aggressive behavior or, instead, play a causal role in the emergence of the respective aggressive phenotypes. Studies aimed at distinguishing the causal role of glucocorticoids – the final products of the activated HPA axis – in the regulation of aggressive behaviors are scarce. Most of the existing evidence that arrogates a key role of glucocorticoids in aggression has been obtained by manipulating circulating levels of these hormones at adulthood (Kim & Haller, 2007; Haller, 2014b). Whether or not a similar picture would be observed when HPA axis alterations occur during development is a question that has not been systematically addressed. One study that applied injections of the HPA axis hormone, corticosterone, during the peripubertal period in rats reported increases in play fighting during adolescence and increased aggression at adulthood (Veenit et al., 2013), suggesting a causal role for enhanced corticosterone levels during development in the emergence of aggression. However, conclusions extracted from a single study are insufficient.

The purpose of this review is to analyze the relevant data from the animal literature that shed light on the potential link between deviation in normative HPA axis activity during development and the emergence of aggressive behaviors. We place a particular focus on rodent studies and, as most data has been gathered in males, we primarily review data obtained from male rodents. We first introduce the HPA axis and its developmental characteristics from a translational perspective in rodents and humans. Following on from previous reviews (Neumann et al., 2010; Veenema & Neumann, 2007), we focus on evidence obtained via genetic approaches, using lines of rodents selected either for HPA axis function or aggressiveness that deviate from normative levels throughout the individuals' life. We then explore the literature in which developmental variation in HPA axis function and aggression phenotypes are induced by manipulations occurring early in life, including stress and exposure to a diversity of drugs. Finally, we

evaluate the knowledge extracted from the reviewed evidence regarding a potential link between developmental variation in HPA axis function and the emergence of aggressive phenotypes, and propose an integrative model that implies specific predictions that can be tested in future studies in the field.

i. The hypothalamus-pituitary-adrenal axis and its development

The HPA axis is a key physiological stress system. its activation involves a cascade of responses that starts with the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) by the paraventricular nucleus (PVN) of the hypothalamus. In the pituitary, CRH and AVP stimulate the production and release of the adrenocorticotropic hormone (ACTH) into the bloodstream. When ACTH reaches the adrenal cortex, it stimulates the secretion and production of glucocorticoids (primarily cortisol in humans; corticosterone in a variety of rodents, including mice and rats). The HPA axis is inhibited by glucocorticoids, which exert negative feedback through actions on the hippocampus, the PVN and the pituitary (Ulrich-Lai and Herman, 2009).

Glucocorticoids act through two receptors systems, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The GR is widely distributed in the brain and exhibits lower affinity for glucocorticoids compared to the MR (de Kloet et al., 2008). Upon glucocorticoid binding, corticosteroid receptors translocate to the nucleus, where they act as transcription factors. Through association with GR responsive elements, or through interactions with other transcription factors, these activated receptors induce or repress expression of genes critical for the modulation of many different processes, including inflammation, metabolism, behavior and cognition (Biddie et al., 2012; de Kloet, 2013). In addition to these genomic actions, membrane-bound MR and GR can also exert rapid, non-genomic, membrane-mediated effects (Groeneweg et al., 2011). Non-genomic glucocorticoid effects are thought to help encoding stress-related information as well as facilitating behaviors such as locomotion, aggression and other stress-related adaptive behaviors (de Kloet et al., 2008; Groeneweg et al., 2011; Makara & Haller, 2001; Sandi et al., 1996).

When translating developmental research studies between humans and rodents, it is important to note that there are important differences in the timing of the HPA axis development between these species (Lupien et al., 2009). For example, in humans, the HPA axis is highly responsive at birth whereas it is still under development during the first week of a rodent's life (Lupien et al., 2009). In rodents, the two weeks following birth are characterized by a "stress hypo-responsive period" (Schapiro, 1968), during which stress glucocorticoid responses have been reported to be largely blunted (Levine et al., 1994; Meaney et al., 1985). A comparable period of HPA axis hypo-responsivity may also exist in humans during childhood (Gunnar and Cheatham, 2003) and around puberty (Gunnar and Quevedo, 2007). It has been hypothesized that maternal care, social contact and parental buffering might be responsible for the maintenance of a hypo-responsive state both in rodents (Lupien et al., 2009) and humans (Gunnar and Cheatham, 2003). On the contrary, in rodents, during adolescence and early adulthood, the HPA axis is hyper-responsive due to an as yet underdeveloped negative feedback system (Klein and Romeo, 2013; McCormick and Mathews, 2010).

ii. Genetic models of variation in HPA axis development in rodents: Consequences for aggression

Genetic animal models can help address the key question discussed in this review. More precisely, they allow the comparison of differences in the functioning of the HPA axis due to genetic factors with corresponding social behavior and aggression phenotypes. So far, existing data have been generated through two main approaches: selective breeding of rodents to generate lines differing in the functioning of the HPA axis, and, comparison of inbred lines that were generated according to other traits but eventually differing in HPA axis function.

A. Rodents selectively bred for extremes in HPA axis activity

The selective breeding strategy starts from an outbred population. Animals displaying extremes in the 'target' phenotype are bred together for several generations after which the resulting lines ought to display stable differences in the phenotype of interest.

Mouse lines selected for extremes in HPA axis responsiveness to stress have been generated recently (Touma et al., 2008). Specifically, C57Bl/6 mice were selected and bred according to their plasma corticosterone response to 15 minutes of restraint stress, producing high-reactive (HR), low-reactive (LR) and intermediate-reactive (IR) lines (Touma et al., 2008). Once the lines were established, although they did not show differences in corticosterone levels at circadian nadir, HR mice had significantly higher diurnal corticosterone than the IR and LR lines (Touma et al., 2008). Following exposure to a stressor, and as compared to LR mice, HR animals were more reactive and showed higher activation of the paraventricular hypothalamic nucleus. Moreover, HR mice exhibited higher corticosterone responses to an ACTH injection and impaired negative feedback inhibition following a combined dexamethasone/CRH test (Heinzmann et al., 2014; Touma et al., 2008). The IR line displayed intermediate responses in these measurements. In one study, mice from these lines were tested for their aggressive behavior in the resident-intruder test. In this test, an unfamiliar mouse ('intruder') is introduced into the homecage of the experimental animal ('resident'). In this study, analyses were focused on the time the resident mouse took to attack the intruder – i.e., latency to attack, used as a proxy of aggressiveness – following the placement of the latter in the resident's cage. LR mice were the fastest to attack and 92% of them performed an attack within 300s vs only 42% of HR mice. The IR line behaved at an intermediate level, with 70% performing an attack within 300s (Touma et al., 2008). Therefore, low HPA axis responsiveness was linked to enhanced reactivity to attack an intruder conspecific and, hence, aggressiveness, in this study, while high HPA axis responsiveness had a negative link with aggression.

In addition to these mouse lines, there are several lines of rats that, although originally bred for extremes in behavioral traits relating to exploration or anxiety, show additional differences in HPA axis function and for which information about their aggressiveness has been gathered. These lines include: (i) the Roman high/low avoidance (RHA/RLA) lines, whose selection criterion was based on their ability to acquire a two-way active avoidance task (Bignami, 1965); (ii) high/low anxiety-related behavior (HAB/LAB) lines, selected based on their behavior in the elevated plus maze and, then, crossbred in an early generation with lines selected for high and low active avoidance (Liebsch et al., 1998); and (iii) high/low responder lines (bHR/bLR), selected according to their locomotor behavior in a novel context (Stead et al., 2006). In each case, the line that shows enhanced HPA axis function, both in terms of diurnal corticosterone levels and in response to stressors, displayed higher levels of aggression than the counterpart line, or, in the case of HAB/LAB lines, in comparison to non-selected controls (Clinton et al., 2008; Kerman et al., 2011; Steimer et al., 1997; Steimer & Driscoll, 2003; Coppens et al., 2012; Coppens et al., 2013; Díaz-Morán et al., 2012 ; Landgraf et al., 1999; Neumann et al., 2005; Neumann et al., 2010b; Veenema et al., 2007 ; Beiderbeck et al., 2012). Although this is in contrast with the findings from mouse lines selected for divergent HPA axis responses described above, it is important to note that these studies did not always analyze the same parameters in the aggression test, nor was information routinely given about qualitative differences in aggressive behaviors, which potentially indicate presence of pathological reactions. For example, no information was provided as to whether attacks were delivered to vulnerable body parts or at a time when the intruder showed a submissive posture and, therefore, differences in aggression between the lines discussed here should be considered quantitative in nature.

B. Inbred rat strains

The second approach that we have chosen to discuss in this section is the comparison of phenotypes presented by inbred rat strains, which are generated by mating siblings across many consecutive generations. This process results in a strain in which only one version of each gene is present, and all animals are therefore genetically identical,

somewhat akin to twins. Specifically, we discuss here strains of rats that present differences in the functioning of their HPA axis and that have been tested for their aggressive responses.

Such a comparison can be established, for example, between Fischer 344 (F344) and Lewis inbred rat strains, which were both derived from the Sprague Dawley strain. As noted by several studies, although these lines do not differ in basal corticosterone levels at diurnal nadir (Jongen-Rêlo et al., 2002), following exposure to stressors, such as restraint or tail shock, F344 rats had higher ACTH and corticosterone levels than Lewis rats (Gómez et al., 1998; Jongen-Rêlo et al., 2002). In agreement with this finding, F344 rats were found to have lower hippocampal GR expression, suggestive of less effective negative feedback regulation of HPA axis (Jongen-Rêlo et al., 2002). When these lines were compared for juvenile play behavior paired with counterparts from either their same strain or Sprague Dawley, the F344 line showed less play fighting than Lewis juveniles (Siviy et al., 2003). These differences were not altered by cross-fostering, which indicates a strong genetic basis for these differential behaviors (Siviy et al., 2003). In line with these findings at juvenility, analysis of social behaviors at adulthood showed similar differences. Specifically, when exposed to a same strain partner in a neutral environment following two weeks of social isolation, F344 animals engaged in significantly fewer bouts of pinning and fighting with their opponent and launched fewer biting attacks than Lewis rats (Berton et al., 1997). In a subsequent resident-intruder test, although both F344 and Lewis rats were relatively unaggressive, F344 again were the ones that showed less aggressiveness, as they initiated fewer fights and spent a greater amount of time engaged in defensive behavior (Berton et al., 1997). Therefore, the strain with lower HPA axis responsiveness in this case showed enhanced aggression.

Another comparison can be drawn between normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rat inbred strains, both derived from Wistar rats. These lines do not differ in their HPA axis hormone levels under basal conditions, but had divergent responses to stress – such as handling or restraint – with SHR rats showing higher plasma ACTH and corticosterone levels than WKY rats (Dickey et al., 2012; Roman et al., 2004). In this instance, the more HPA axis-reactive SHR rats are the ones reported to be more aggressive, when compared to WKY, across several experimental situations (Berton et al., 1997). Specifically, SHR were more aggressive: (i) in a colony-housing model, where they performed more attacks on novel intruders, and subordinates in the colony had significantly higher number of scars (Toot et al., 2004); (ii) in muricidal tests; (iii) when challenged with shock-induced fighting (Potegal and Myers, 1989).

These two examples of inbred rat strains indicate a mixed relationship between differential HPA axis function and the associated level of aggressive behavior that seems to depend on the background strain of the particular line. Specifically, in the strains derived from Sprague Dawley rats (i.e. Fischer 344 and Lewis strains), higher HPA axis reactivity is linked with decreased sociability and decreased aggressiveness. Conversely, in the strains derived from Wistar rats (i.e. WKY and SHR), higher HPA axis reactivity is associated with increased aggressiveness. Interestingly, in direct comparisons of Sprague Dawley-derived and Wistar-derived inbred strains, Wistar-derived rats have been shown to have higher HPA axis response to acute stress, less vulnerability to the effects of chronic social stress on bodyweight gain and higher overall aggressiveness (Berton et al., 1997). Although without the direct analyses of these different rats within a specific study, it is difficult to cross-compare findings; it is tempting to speculate the existence of a U-shape effect for the results described above. Specifically, high aggression levels seem to be displayed by the Lewis and SHR strains showing, respectively, the lowest and highest HPA axis reactivity, while low aggression levels correspond to the strains (i.e., F344 and WKY) showing intermediate HPA axis responses.

iii. Genetic models of variation in aggressiveness in rodents: Consequences for HPA axis function

A further approach to collect information about a potential link between developmental differences in HPA axis function and aggression is taking the converse strategy with regard to line selection to the ones described above. Here, we discuss data obtained from rodent lines selectively bred for extremes in aggressiveness and scrutinize whether they present significantly different HPA axis function. We review data from three mouse selection lines and one from rats.

One of the oldest documented lines selected for extremes on aggressiveness are the Turku aggressive (TA) and non-aggressive (TNA) mice, which were derived from an original cohort of Swiss albino outbred mice in 1959 (Sandnabba, 1985). As compared to TNA, TA mice have proven to be more aggressive in several parameters and testing situations. Thus, in a resident-intruder test, they perform more attacks, more threats and are less social than TNA mice (Caramaschi et al., 2008a). They also display reduced latency to attack a conspecific whether they are the resident, the intruder, or whether the social interaction takes place in a neutral cage (Nyberg et al., 2004). Importantly, TA mice are more likely to attack females in the homecage (Caramaschi et al., 2008a) or in a resident-intruder test (Nyberg et al., 2004), indicating presence of an abnormal aggressive phenotype in these mice. Although little is known about HPA axis function in these mice, some evidence indicates that TA mice had blunted diurnal peak corticosterone in comparison to TNA mice (Caramaschi et al., 2008b).

Other relevant lines include the low- (NC100) and high-aggressive (NC900) mice established from two sets of outbred ICR (Institute for Cancer Research) stock (Petitto et al., 1993). NC900 mice displayed significantly shorter attack latency, emitted more attacks, more sustained attack bouts, more threats, and were less social than NC100 (Caramaschi et al., 2008a). The aggressive phenotype of NC900 mice was not ameliorated by cross-fostering (Granger et al., 2001), indicating an intractability to environmental influences. Although there is limited information regarding HPA axis function in these mice, evidence shows that, relative to NC100 mice, NC900 have a lower basal (Petitto et al., 1993) and diurnal peak corticosterone levels (Granger et al., 1996). Curiously, this was found in conjunction with higher hypothalamic CRH content in the same animals (Granger et al., 1996). This may suggest of blunted sensitivity of the pituitary to CRH tone in NC900 aggressive mice.

One of the best studied mouse lines in this context are the ones originally selected from wild house mice according to their short (SAL) or long (LAL) latency to attack a conspecific mouse (van Oortmerssen and Bakker, 1981). SAL mice displayed higher number of attacks and higher duration of aggressive behavior than LAL mice (Caramaschi et al., 2008a). Importantly, SAL mice have been described as abnormally aggressive as they attack females and anesthetized intruders, and ignore submissive postures of their opponents (Caramaschi et al., 2008a). Analysis of their HPA axis function indicates abnormal reactivity in SAL mice. Thus, although no differences between SAL and LAL mice were described under basal conditions (Veenema et al., 2003), SAL mice showed a flatter circadian corticosterone rhythmicity; the typical upshift of corticosterone during the dark phase being blunted in comparison to LAL mice (Korte et al., 1996). Furthermore, following exposure to novelty, administration of ACTH or forced swim stress, SAL mice displayed blunted corticosterone response relative to LAL mice (van Riel et al., 2002; Veenema et al., 2003), and mild psychosocial stress-induced corticosterone increases were short, as opposed to longer-lasting responses observed in LAL mice (Veenema et al., 2003).

Lines of rats derived from wild-caught Norway rats were selected according to their low ('domesticating') or high (maintenance of 'wild') aggressiveness toward a glove (Naumenko et al., 1989). Domesticated rats showed no aggressiveness toward humans by the 10th generation of selection (Plyusnina and Oskina, 1997). In terms of social

behavior, wild rats emitted considerably more fighting bouts in shock-induced fighting tests than tame rats, but, at the 19th generation of selection did not display more inter-male aggression when not provoked by shock, nor were they more frequently muricidal (Naumenko et al., 1989). Later generations of the lines showed relatively higher inter-male aggressive behavior and lower social interaction in wild rats relative to domesticated rats (Gulevich et al., 2015). Regarding their HPA axis, wild line rats display higher basal corticosterone levels than tame rats (Gulevich et al., 2015; Naumenko et al., 1989). This finding was sustained when studying fecal matter obtained in the absence of any human interaction, which would presumably constitute a stressor, particularly to the wild line (Albert et al., 2008). Additionally, wild line rats showed higher corticosterone responses to novelty than domesticated rats, and had higher adrenal weight, indicative of both situational and general hyperactivity of the HPA axis (Naumenko et al., 1989; Plyusnina and Oskina, 1997).

The view depicted by the models discussed above suggests a species-dependent relation between aggressiveness and the HPA axis. The global message from mouse models is that selection for aggressive behaviors (that in in the case of TA, NC900 and SAL lines has co-segregated with pathological forms of aggression) were related with a blunted HPA axis activity and/or reactivity. However, the opposite pattern is observed in the rat lines, as the more aggressive line had higher HPA axis reactivity. However, an important caveat is that direct comparison of these models with other selection models is not possible since the definition of aggressive behaviors is relatively different between studies.

iv. Developmental stress leading to variation in HPA axis function: Consequences for aggression

In addition to genetic selection, early life experiences can also have profound consequences on the development of the HPA axis. In particular, exposure to stressful experiences during different stages of development are known to have long-term consequences on HPA axis function and behavior. Early life stress can result in different psychopathologies, such as depression, anxiety, and alterations in social behaviors including changes in sociability and aggressiveness (Haller et al., 2014; Sandi & Haller, 2015; Veenema, 2009). The brain undergoes important changes during prenatal, postnatal and pubertal periods, which renders it highly vulnerable to stress (Lupien et al., 2009). Importantly, adverse experiences during early life and adolescence can also divert the development of the HPA axis which, in turn, can affect social behaviors (Sandi & Haller, 2015). We review here the relevant literature involving stress application at different early developmental periods in which an association between divergent HPA axis function and aggressiveness has been established.

A. Prenatal stress

Acute prenatal stress – administered on gestation days 10 and 19 – in an inbred strain of male rats (DA/Han) was found to result in increased stress-induced HPA axis reactivity (Patin et al., 2002) as well as reduced aggressiveness and increased submissiveness (Patin et al., 2005). Using a protocol of chronic prenatal stress, from gestation day 11 until delivery, in male Sprague-Dawley rats increased reactivity of the HPA axis following restraint stress was also observed. This was accompanied by decreased social play behavior (Morley-Fletcher et al., 2003). Conversely, chronic prenatal stress during the last week of pregnancy resulted in an increase of aggressive behaviors during a social interaction test, without effect on social play frequency, in juvenile male Wistar rats. Levels of corticosterone were not found to be different under basal conditions but were enhanced at diurnal peak and following exposure to forced-swim stress (Koehl et al., 1999; Schroeder et al., 2013). In voles, different types of prenatal stress (including exposing pregnant females to either confrontation, immobilization or crowding on days 13, 14 and 15 of gestation) led to prolonged stress-induced activation of the HPA axis and increases in aggressiveness in male offspring (Marchlewska-Koj et al., 2003). Therefore, the opposite association between HPA axis reactivity resulting from

prenatal stress exposure and aggression levels were found between rats and voles. Although it is not possible to conclude about species differences given the many additional differences in the studies discussed here (e.g., different nature, duration and timing of gestational stressors), higher HPA axis reactivity was found associated with lower aggression in rats, while it was related with higher aggression in voles.

B. Early postnatal stress

Separation of the young from the mother is one of the most used and best-studied models of early life adversity, aiming to mimic deficits observed in socially neglected children. We discuss here studies that have examined the consequence of this manipulation for HPA axis function and aggressive behaviors in rodents. Additionally, we mention relevant studies addressing the same question and evaluating similar parameters in monkeys.

In Wistar rats, maternal separation during the first two weeks of life led to a pattern of changes in endocrine and behavioral responses differential according to developmental stage (Veenema et al., 2006). Maternally-separated juvenile male rats showed an increase in HPA axis activity at basal level in the early dark phase, but no difference with regards to controls following social interaction. These juveniles exhibited increased play fighting and reduced submissive behaviors (Veenema and Neumann, 2009). However, when assessed at adulthood, HPA axis responsiveness was similar between stressed and control rats, both at baseline and after acute stressor. Maternally separated adult rats showed a faster increase in corticosterone levels after stress. In common with juvenile rats, adult animals were more aggressive during a resident-intruder test (Veenema et al., 2006).

In C57Bl/6 mice, however, maternal separation during the first two weeks of life is known to lead to increased reactivity of the HPA axis in response to stress (Parfitt et al., 2004), reduced play fighting in juvenility (Tsuda et al., 2011) and reduced intermale aggression at adulthood (Veenema et al., 2007). However, increased aggressiveness has been reported when a shorter maternal separation protocol was applied in Balb/C mice (Hohmann et al., 2013). To our knowledge, the HPA axis reactivity of these mice has not been assessed, though behavioral similarities with C57Bl/6 mice led the authors to hypothesize HPA axis hyperactivity in this strain following stress (Hohmann et al., 2013).

In monkeys, juveniles reared in isolation were found to display elevated baseline cortisol levels, though acute stress-induced cortisol levels was not different to controls at adulthood (Meyer & Bowman, 1972; Sackett et al., 1973). Young monkeys, that were maternally-separated at birth, hand-reared for the first month and subsequently raised with same-age peers for the next 5 months, displayed higher levels of impulsive aggressive behaviors during play-fighting (Higley et al., 1996). Monkeys with this early life history were toward the bottom of the social hierarchy when housed with mother-reared peers (Suomi, 1997) and when challenged by a period of social separation, peer-reared monkeys exhibited extreme behaviors and higher HPA axis responses (Higley et al., 1991; Higley & Suomi, 1989). Furthermore, studies on monkeys maltreated by the mother during infancy have reported increased plasma cortisol levels in infant monkeys and exaggerated aggressive behaviors during adolescence (Howell et al., 2013). Conversely, other studies of peer-reared monkeys found low basal cortisol and low HPA axis response to stress as well as no differences in basal and stress-induced levels of cortisol (Clarke, 1993; Winslow et al., 2003; Champoux et al., 1989; Feng et al., 2011). Thus, no clear picture of the effects of peer-rearing stress on the HPA axis is evident. Recent studies have focused on explaining some of this variability, determining genetic factors and emphasizing the importance of gene-environment interactions linking stress, HPA axis and aggression (Novak et al., 2013). (Novak & Suomi, 2008) applied a rearing model in which monkeys were raised with an inanimate surrogate mother and provided daily exposure to playmates. Surrogate/peer-reared monkeys were more aggressive and displayed abnormal aggressive behaviors, as they did not respond to submissive postures of their opponents (Novak & Suomi, 2008). Furthermore,

monkeys exhibited lower levels of circulating cortisol and showed blunted HPA axis response to a period of social separation (Capitanio et al., 2005; Davenport et al., 2003; Shannon et al., 2005; Shannon et al., 1998).

Overall, the picture arising from early stress protocols in different species emphasizes, once more, a complex relationship between variation in developmental HPA axis function and the emergence of aggression. Higher stress-induced HPA axis in rats was related to increased aggression, as previously described in several other models using this species. However, in monkeys, the two opposing patterns have been described, one that fits with the findings in rats and another one that links low HPA axis reactivity with higher aggression. Globally, all the findings summarized so far may be illustrated by a U-shaped relation between HPA axis regulation and the development of aggressive behaviors (Figure 1).

C. Peripubertal and adolescent stress

In humans, social neglect and bullying are two stressful experiences occurring in adolescence that are known to lead to hormonal alterations and behavioral deficits later in life (Tzanoulinou & Sandi, 2016). Corresponding rodent models, post-weaning social isolation and social subjugation, attempt to model alterations observed in humans (Haller et al., 2014). Exposure to fearful situations during peripuberty has been modeled with a peripubertal stress model of psychopathology (Márquez et al., 2013).

Studies employing post-weaning social isolation in male Wistar rats have reported that isolation from the point of weaning, over seven weeks, led to exaggerated corticosterone levels after aggressive encounters or social stress while not altering basal levels (Toth et al., 2011; Tulogdi et al., 2014). Isolated males also exhibited a pattern of abnormal or pathological aggression, including increased propensity to target their counterparts vulnerable body parts, such as throat, belly or head (Toth et al., 2011) and propelling unsignaled attacks toward their opponents (Toth et al., 2011). Moreover, socially deprived male rats showed increased defensive behaviors and initiated most of their attacks from defensive postures, suggesting aggression ambiguity. The aggressive behaviors of isolated rats were fragmented, with rapid switching from one behavior to another during resident-intruder encounters (Toth et al., 2011). A period of resocialization following isolation failed to ameliorate abnormal behaviors exhibited by socially deprived animals (Tulogdi et al., 2014). Interestingly, a study showed that the exposures to post-weaning social isolation shorter than seven weeks are sufficient to lead to alterations in social behaviors (Wall et al., 2012). When tested in late adolescence, following just four weeks of isolation, socially deprived Sprague Dawley rats showed enhanced play-fighting behavior and higher social interaction (Wall et al., 2012). This effect was found in both male and female rats. Chronicity of isolation appears to be a mediating factor, however. In mice, five days of peripubertal isolation did not lead to enhanced aggressive behavior, nor changes in HPA axis function, later in life (Pietropaolo et al., 2004). In summary, increased HPA axis reactivity was found to be associated with enhanced and pathological aggression in rats.

Bullying, or social abuse, is modelled in rodents via means of repeated social subjugation. Social subjugation of juvenile rats, by daily exposure to an aggressive adult, was shown to lead to enhanced basal corticosterone levels as well as exaggerated aggressive behaviors after both physical and social provocation, including towards larger opponents (Cunningham and McGinnis, 2008). In hamsters, juveniles (P26-38) exposed for 20 minutes daily in the homecage to an aggressive adult male (Delville et al., 1998), while not showing alterations in basal corticosterone levels, had increased stress-induced corticosterone responses (Wommack and Delville, 2003). Subjugated hamsters attacked less intruders of similar size, but exhibited increased aggressive behavior (specifically, more biting) towards smaller opponents (Delville et al., 1998; Wommack & Delville, 2003; Wommack et al., 2003). Subjugated animals also showed premature transition from play-fighting behavior to adult-like patterns of attack, and displayed high levels

of aggression at adulthood (Wommack et al., 2003). Other studies reported that hamsters subjugated during puberty (P26-38) showed high levels of aggression toward intruders and blunted release of cortisol (Ferris et al., 2005).

The peripubertal stress model of psychopathology developed originally in rats comprises a variable sequence of psychogenic, fear-inducing stressors, including exposure to elevated platform and predator odor, on seven scattered days across the peripubertal period (Márquez et al., 2013; Toledo-Rodriguez & Sandi, 2011). Although no difference in basal corticosterone was observed, peripubertal stress-exposed males and females had a blunted corticosterone response to stress and exhibited exaggerated aggression (Cordero et al., 2013; Márquez et al., 2013). In addition to several behavioral disturbances, male rats exposed to peripubertal stress showed evidence of pathological aggression at adulthood, as they showed increased intermale aggression, even towards juveniles and animals showing subordinate postures, and increased aggression towards a cohabitating female partners (Cordero et al., 2012; Márquez et al., 2013; Tzanoulinou et al., 2014). Although the corticosterone response induced by the resident-intruder test did not differ, the testosterone to corticosterone ratio was higher in peripubertal stress animals, which has been shown to be a marker of aggressive-impulsive behaviors in humans (Terburg et al., 2009).

Given all the findings reported above, we can argue that the relationship between stress and the development of alterations in HPA axis functions and aggressive behaviors that emerges from this data is complex. Again, rats stressed at peripuberty and/or adolescence tend to develop higher HPA axis reactivity and increased aggression. An exception seems to be for the peripubertal stress model in which lower HPA axis reactivity was linked to increased aggression. In this particular case, the discrepancy may be explained by considering the novelty of the stress stimulus used to assess HPA axis reactivity relative to the nature of the stress experienced earlier in life. Rats submitted to peripuberty stress, a stress consisting of repeated exposure to unpredictable and fearful situations, show blunted HPA axis response to a novel environment in adulthood (Márquez et al., 2013). By contrast, rats exposed to post-weaning social isolation showed HPA axis hyper-responsiveness to a social encounter, a wholly novel experience (Toth et al., 2011). This represents a more general problem in comparing across studies, and highlights the undue influence that single-point analyses of HPA axis function may have on interpretation of trends. Critically, the effects seem to be highly dependent on the developmental period when stress is given, but also depend on the protocol and species used. Given the limited number of studies, further research is needed to disentangle the impact of different types of stress over time and at varying intervals of brain development in relation to aggressive behavior.

v. Developmental exposure to drugs: Effects on HPA axis function and aggression

In addition to genetic factors and early life stress, the HPA axis can be affected during developmental periods by exposure to a range of substances. We have a special focus here on drugs of abuse and antidepressants. The rationale to review the literature on drugs of abuse rests on the well-known, close and bidirectional interaction of the HPA axis and the mesolimbic dopamine system, the latter being a major site of action for these drugs (Koob & Kreek, 2007; Ungless et al., 2010). Moreover, mesolimbic dopamine plays a critical role in motivation towards both social and non-social stimuli (Salamone and Correa, 2012). Antidepressants are included in this section as there is documented evidence that they can affect neurodevelopmental trajectories of individuals.

A. Cocaine

Evidence indicates that prenatal cocaine exposure blunts HPA axis reactivity to novel and stress inducing stimuli in rats (Johns & Noonan, 1995; Johns et al., 1994), whilst also leading to enhanced aggressiveness (Johns & Noonan, 1995; Johns et al., 1994; Wood & Spear, 1998). Conversely, chronic cocaine exposure during adolescence appeared to give rise to a hyperactivity of the HPA axis in response to stress exposure (Alves et al., 2014) as well as leading to

enhanced aggressiveness in both rats (Alves et al., 2014) and hamsters (Harrison et al., 2000; Jackson et al., 2005; Knyshevski et al., 2005).

B. Alcohol

Prenatal exposure to ethanol, via a variety of administration routes, gives rise to a hyperactive HPA axis responsiveness to a range of stressors (rats: Gabriel et al., 2000; Gangisetty et al., 2014; Kim et al., 1999 ; mice: Wieczorek et al., 2015). No differences in basal HPA axis tone, nor diurnal rhythmicity is evident however (rats: Glavas et al., 2007; mice: Wieczorek et al., 2015). Prenatally exposed rats demonstrated higher levels of play fighting and adult aggression relative to controls (Hamilton et al., 2010, 2014; Royalty, 1990).

There is little research exploring the effects of adolescent exposure to ethanol on either HPA axis function, aggression or both. The sole paper published thus far indicates that, in rats, there is dissociation in the effects of ethanol exposure between the early and late adolescent period (Varlinskaya et al., 2014). Specifically, early adolescent ethanol led to a decrease in social motivation, without concomitant alteration in HPA axis function, whereas late adolescent ethanol enhanced both fighting behavior and corticosterone response to this social challenge (Varlinskaya et al., 2014).

D. Cannabinoids

Perinatal administration of Δ9-THC or synthetic cannabinoid receptor type 1 (CB1R) agonists led to decreased HPA axis activity in adult male rats (del Arco et al., 2000; Rubio et al., 1995). Rats exposed to similar regimens of perinatal Δ9-THC displayed a reduction in play fighting at adolescence and in aggression at adulthood relative to vehicle-treated controls (Newsom & Kelly, 2008; Trezza et al., 2008). Exposure to a CB1R agonist at the postnatal time point only also led to reduced social interaction duration, including fighting behavior, when measured in late adolescence (O'Shea et al., 2006).

Conversely, pubertal exposure to CB1R agonists was associated with hyperactivity of the HPA axis in response to restraint stress in adult rats (Lee et al., 2014). Animals treated with a similar drug during adolescence showed alterations in social behavior in adulthood. Specifically, CB1R agonist exposed rats were more likely to behave defensively when attacked, as well as emitting more attacks and more pins themselves (Schneider and Koch, 2005).

E. Antidepressants

Research into the effect of antidepressant exposure during development on HPA axis and social function is limited. The existing literature indicates that pre and perinatal exposure to the selective serotonin re-uptake inhibitor (SSRI) class of antidepressant drugs gives rise to hyperactivity of the HPA axis in basal conditions as well as blunted corticosterone response to mild stress (Bourke et al., 2013). Mice treated prenatally with SSRIs displayed enhanced aggressive behaviors relative to vehicle-treated controls in a number of studies (Kiryanova & Dyck, 2014; Svirsky et al., 2015; Coleman et al., 1999). SSRIs have been shown to both decrease levels of circulating corticosterone and lead to impaired negative feedback regulation of corticosterone in rats (Gobinath et al., 2016; Pawluski et al., 2012). Route of administration, dose and time of testing influence the outcome. That noted, mice exposed to a similar treatment regimen to the one that impaired HPA axis negative feedback regulation (Gobinath et al., 2016), demonstrated reduction in aggressive behavior at adulthood relative to control (Yu et al., 2014).

Exposure to addictive substances and medicines *in utero* can lead to both hypo and hyperactivity of the HPA axis later in life. Whether alterations in HPA axis activity in response to challenge represent general hypo- or hyper-function of the axis remains unknown. Drug-induced alteration of HPA axis function is associated with both increase and decreases in aggressive behavior depending on the drug in question. Effects of drug exposure during adolescence,

on the other hand, render a more coherent picture. Across drug classes, evidence, though scant, indicates that adolescent exposure leads to enhanced HPA axis response to stressors, as well as enhanced aggression.

Discussion

We have reviewed the existing literature to assess the potential presence of a link between aberrations in the development of the HPA axis in a diversity of animal models and the emergence of aggression (summarized in Table 1.). The literature described is generated from genetically-selected and inbred strains of rodents, as well as on the effects of developmental exposure to stress or drugs of abuse. A major drawback in establishing any firm conclusion is the lack of systematic studies including equivalent manipulations (e.g., timing and duration of treatments) and common protocols for the measurement of the HPA axis and aggressive behaviors.

Thus, although a general unifying picture cannot be extracted from the reviewed data, there are certain commonalities that ought to be emphasized. We found several examples suggesting that aberrations towards abnormally low or abnormally high HPA axis functionality taking place during development are associated to increased aggression, frequently characterized by pathological features. Thus, the reviewed literature suggests the existence of a U-shape function between developmental HPA axis reactivity and the emergence of aggressive phenotypes (Figure 1). Note, however, that the U-shaped function presented here is naturally speculative, since the majority of models outlined have assessed HPA axis (re)activity and aggression in two groups only. It is thus important for future research to perform experiments taking into account an intermediate group. Of the studies that considered three groups, the HAB/LAB rat lines have come closest to supporting the hypothesized U-shape. In that case, both of the selected lines showed enhanced aggression relative to non-selected controls, and also demonstrated high and low HPA axis reactivity, respectively (Beiderbeck et al., 2012; Neumann et al., 2005, 2010). However, crucial information regarding the relative level of HPA axis reactivity in the control group has not been so far reported. Therefore, the currently available data can only partially support our hypothesis, and further experiments considering three experimental groups are warranted.

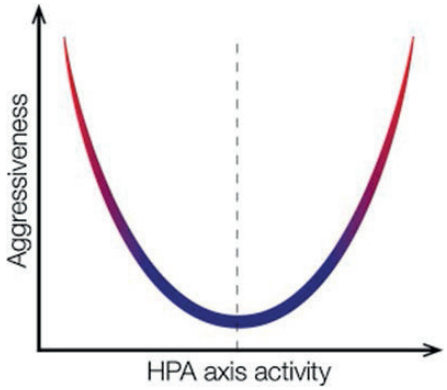

Figure 1. U-shaped relationship between HPA axis functioning and aggressive behavior.
HPA axis activity driven toward either hypo- or hyper-function is linked to exaggerated emission of aggression, often with abnormal features. Species, substrain and developmental stage also influence this relationship.

Other data from the animal literature, not reviewed here, show that the direct manipulation of glucocorticoids at adulthood, leading to both abnormally low (Haller et al., 2004; Haller et al., 2001) or high (Haller et al., 1997; Kruk et al., 2004) glucocorticoid levels can lead to pathological aggression. This is found alongside alterations in the activity of brain regions and circuits implicated in the control of aggression (Haller, 2014a, 2014b). However, a critical issue is whether aberrant HPA axis has a causal implication in the development of aggressive phenotypes.

Research linking both aspects from a developmental perspective is scarce and it is thus difficult to outline a comprehensive view that implies any particular link between extremes in HPA axis variation and features of

pathological aggression. Indeed, the HPA axis is not a single unit and various outcomes may arise from a unique modification in the system. For example, a decrease in corticosterone production may lead to differential behavioral outcomes whether it is associated with a hypersensitivity to a regulator of aggression or in a negative feedback of the systems. Discrepancies between studies and outcomes, in addition to the already mentioned differences in protocols and species, may arise from the inappropriately focal picture observable using single-point analyses of HPA axis function employed by many studies in the field. Traditionally, in line with the 'hypoarousal theory' of violence (Raine, 1996), blunted activation of the stress systems has been proposed to be particularly associated with symptoms of psychopathy. However, enhanced HPA axis reactivity was also related to pathological aggression in several rodent models (e.g., post-weaning social isolation; lines bred for high anxiety, and lines bred for maintenance of wildness), potentially mimicking emotional-impulsive types of aggression. Importantly, recent evidence in humans suggests that, even within individuals high in psychopathic traits, there might be subtypes presenting not only blunted, but also high HPA responses to stress (Johnson et al., 2015).

Various experimental results summarized in this review show some inconsistencies and it is important to note that translation of such results must be taken with great care. For example, the effects of stress highly depend on the developmental stage at the time of stress, and this may not be directly comparable between species (Matthews, 2000; Lupien et al., 2009). Some species give birth to immature young animals (e.g., rats and mice) while others deliver mature young ones (e.g., guinea pigs and primates). Immature young animals continue to display considerable higher rates of neuronal development after birth (Sapolsky and Meaney, 1986) and exhibit a higher glucocorticoid receptor sensitivity than the young of species with substantial rates of maturity at birth (Claman, 1972). In addition, it is important to note that the "stress hypo-responsive period" displayed by rats on the two weeks following birth has been interpreted from as an adaptation to enable proper brain development (Sapolsky & Meaney, 1986; Schmidt et al., 2005).

We should also note that critical differences in the ontogeny of aggression between the two rodent species reviewed here, namely rats and mice, may influence the conclusions drawn from the reviewed literature. For example, as summarized above, mice and rats exposed to maternal separation were found to display opposite patterns of aggression in the context of similar HPA axis reactivity (Boccia & Pedersen, 2001; Hohmann et al., 2013; Parfitt et al., 2004; Tsuda et al., 2011; Veenema et al., 2006; Veenema & Neumann, 2009). In particular, we know that these two species differ in their engagement in social play. Thus, rats engage heavily in play-fighting during the juvenile period (Pellis and McKenna, 1995) and, when deprived of such play experience they show aberrant social behaviors at adulthood, from hyper-defensiveness and failure to display appropriate submissive behavior, to exaggerated aggression (Potegal & Einon, 1989; Vanderschuren et al., 1997). These observations led to the hypothesis that engagement in social play was critical to the development of appropriate social behaviors (Vanderschuren et al. 1997, Pellis & Pellis 1998). However, as compared to rats, the social play repertoire of mice is impoverished (Pellis & Pasztor, 1999; Pellis & Pellis, 1998; Terranova et al., 1998). Accordingly, the social development, including aggressiveness, of these two species may not follow the same developmental pattern. Given that HPA axis activity relating to social play has been implicated in the effective learning of social interaction patterns (Achterberg et al., 2014), elevated corticosterone levels during early life might influence the way in which play and aggressive behaviors are embedded differentially in these two species.

Additionally, the nature and duration of stressors given in different studies could also be critical in determining differences in aggression. It has been shown that various stressors, and other environmental challenges, do not elicit the same glucocorticoid secretion in rodents (Koolhaas et al., 2011) and that the quality of the stressor might also shape (or prime) future stress responses (Branchi et al., 2013). Additionally, the results of stress may be altered by housing conditions early in life. For example, it was shown that socially-enriched environments, while being stressful

due to the inherent increase in social interactions, may actually be protective against stress and favor stronger cognitive abilities (D'Andrea et al., 2007; Huzard et al., 2015). It is thus important to take factors such as this into account in order to compare models but data remain too disparate to be able to draw clear conclusions from additional factors.

Furthermore, we should also note that one limitation with animal studies is the difficulty to clearly differentiate reactive and proactive types of aggression. Although various studies have reported abnormal forms of aggressive behaviors in rodents, it remains difficult to distinguish whether the motivation to aggress. As previously described (Haller, 2014a), "aggression is not a unitary phenomenon" and can be divided is various subtypes. It is often suggested that hypo-reactive (hypoaroused, "cold") individuals perform proactive aggression whereas hyper-reactive (hyperaroused, "hot") individuals display reactive aggression (Blair, 2001; Caramaschi, 2008a; Haller, 2014a; Provençal et al., 2015). Subdivision of aggression likely involves a complex brain network. One may suggest that distinct components are taking place in the emergence of aggressive behaviors: the onset of an aggressive bout, the intensity (low or highly aggressive), the nature of the outcome (normal or abnormal), and the type/context (reactive or proactive). The neural pathways responsible for such components might be distinct but interconnected, giving rise to the broad panel of aggressive responses reported in the literature. The different components of aggression could be altered independently by developmental changes. However, at present there is no systematic analysis of those different criteria allowing a precise description of the variations induced by stress at different developmental stages. Finally, the U-shape model may not only applicable to "aggressiveness" as a whole but to the different components of aggression, leading to a multi-dimensional model comprising multiple outcomes, as observed in the diversity of aggressive behaviors described in the literature.

A further limitation regards the difficulty in assessing whether the findings of studies using male animals generalize to females. The neurobiology underlying aggressive behavior, in terms of the downstream effects of signaling molecules, as well as in neuroanatomy, shows sexual dimorphism (Haller, 2014a). Additionally, the influence of the HPA axis on female aggression is little studied, an issue more deeply entrenched with regard to the developmental aspect under review here. From the available evidence, the relationship in females between altered HPA axis activity during development and later aggressiveness appears to align with the findings observed in males. For example, in rat lines originally bred for variation in anxiety-like behavior (HAB/LAB: Liebsch et al., 1998), female HAB rats showed enhanced aggressiveness. This was observed both in lactating and virgin females (Bosch & Neumann, 2010; de Jong et al., 2014). Though no differences in basal levels or in stress-induced levels of HPA axis activity were observed, the aggressive HAB line females showed blunted HPA axis responsiveness to an injection of CRH (Neumann et al., 1998). Additionally, in rats selectively-bred for aggressiveness toward humans ("wild" vs. "tame"), female wild rats were more aggressive than tame rats, and showed enhanced levels of corticosterone under basal conditions (Albert et al., 2008). This alignment also holds with regard to some developmental stress models. Indeed, peripubertally stressed female rats were found to show enhanced maternal aggression, as well as enhanced aggression prior to mating (Cordero et al., 2013). No differences in circulating corticosterone were found but increased basal vasopressin was observed (Cordero et al., 2013). In contrast, maternally-separated female rats showed reduced maternal aggression in the context of lower basal levels of corticosterone and enhanced responsiveness following stress (Aisa et al., 2008; Boccia & Pedersen, 2001; Koe et al., 2014; Slotten et al., 2006). Taken together these studies indicate that females showing alterations in HPA axis activity during development may display dysfunctional aggression later in life. Moreover, from the examples described above, it appears that female rats in which the HPA axis is under enhanced feedback-mediated inhibitory control may be more aggressive (Bosch & Neumann, 2010; de Jong et al., 2014; Neumann et al., 1998; Albert et al., 2008; Cordero et al., 2013; Boccia & Pedersen, 2001; Slotten et al., 2006; Koe et al., 2014; Aisa et al., 2008). These findings support a link between HPA axis hypo-responsiveness and enhanced

aggression in females, in line with observations reviewed above in males. However, owing to the paucity of studies in females, we cannot draw any conclusion regarding the potential existence of a U-shape for them.

Glucocorticoids are crucial for typical brain development and GRs and MRs are highly expressed in the developing brain (Kitraki et al., 1997; Seckl, 2004). Thus, abnormal levels of glucocorticoids during developmental stages have been shown to lead to detrimental effects on neuronal structures and processes (Matthews, 2000). Excess of glucocorticoids during development not only alter structures from the HPA axis but also brain regions closely regulating the stress system and involved in the control of aggression (e.g. amygdala, prefrontal cortex and hypothalamus). For example, the hippocampus, via negative feedback is one of the main limbic regulators of the HPA axis (Jacobson and Sapolsky, 1991) and can be altered by high concentration of glucocorticoids during development (Eiland and Romeo, 2013). This alteration has been shown to be induced by a decrease in GR and MR density in the hippocampus, leading to long-term decrease in HPA axis feedback sensitivity (Seckl, 2004). It has also been demonstrated that exposure to high prenatal corticosterone concentration can increase CRH in central amygdala at adulthood (Welberg et al., 2000). Interestingly, the amygdala is involved in both the regulation of aggressive behaviors (Haller, 2014a) and the stimulation of the HPA axis via CRH and serotonin signaling (Feldman and Weidenfeld, 1998). The role of the prefrontal cortex in the control of aggressive behaviors has been extensively reported but there are contradictory findings on the direction of such control (Biro et al., 2016). Overall, glucocorticoids have been closely related to the aberrant development of neural networks controlling aggression, thereby facilitating aggressiveness (Haller, 2014a).

In conclusion, the reviewed evidence highlights a complex, but potentially critical link between developmental HPA axis activity and the development of social disturbances. In order to capture the causal link between these two elements in a time- and dose-controlled manner, future animal experiments should aim toward specific manipulation of HPA axis function using a variety of experimental approaches. This research is much needed, given the suggestion that children with callous-unemotional traits might benefit from interventions capable of normalizing their blunted cortisol levels (van Goozen et al., 2007). Importantly, the data reviewed here indicate that genetic differences or other factors might critically affect neurodevelopmental trajectories influenced by aberrations –either high or low- in HPA axis function.

	Model or treatment	Developmental age	Species	HPA axis reactivity	Measurement time	Aggressiveness	References
Genetic models	Bred for extreme HPA axis reactivity	-	C57Bl/6 mice	LR:↓	Diurnal peak + stress	Resident-intruder: ↑ (LR)	Heinzmann (2014), Touma et al. (2008)
	Bred for extreme anxiety traits	-	Rats	↑	Diurnal peak + stress	Resident-intruder: ↑ (low anxious) Abnormal aggression: ↑ (low anxious)	Beiderbeck et al. (2011), Landgraf et al. (1999), Neumann et al. (2005, 2010), Steimer et al. (2003), Veenema et al. (2006)
	Bred according to exploration	-	Rats	↑	Diurnal peak + stress	Resident-intruder: ↑ (high explorer)	Clinton et al. (2008), Kerman et al. (2011, 2012), Stead et al. (2006)
	Bred according to active avoidance	-	Rats	↑	Diurnal peak + stress	Resident-intruder: ↑ (low avoidance)	Bignami et al. (1965), Coppens et al. (2012, 2013), Diaz et al. (2012)
	Inbred strain derived from Sprague-Dawley (F344 and Lewis) and from Wistar (WKY and SHR)	-	Rats	SHR > WKY F344 > Lewis Wistar > SD	Stress	Play-fighting behavior: SHR, Lewis > WKY, F344 Resident-intruder: SHR, Lewis > F344, WKY Both: Wistar > Sprague Dawley	Berton et al. (1997), Dickey et al. (2012), Gomez et al. (1998), Jongen-Relo et al. (2002), Potegal et al. (1989), Roman et al. (2004), Sviy et al. (2003), Toot et al. (2004)
	Short-latency to attack (SAL) lines	-	Mice	↓	Dark phase + stress	Resident-intruder: ↑ Abnormal aggression: ↑	Caramaschi et al. (2008a), Korte et al. (1996), van Oortmerssen & Bakker (1981), van Riel et al.(2002), Veenema et al. (2003)
	High-aggressive NC900 lines	-	Mice	↓	Basal + diurnal peak	Resident-intruder: ↑	Caramaschi et al. (2008a), Granger et al. (1996, 2001), Petitto et al. (1993),
	Turku aggressive lines	-	Mice	↓	Diurnal peak	Resident-intruder: ↑ Abnormal aggression: ↑	Caramaschi et al. (2008a), Nyberg et al. (2004), Sandnabba et al. (1985)
	Wild-caught Norway lines	-	Rats	↑	Basal + stress	Resident-intruder: ↑ Shock-induced fighting: ↑	Albert et al. (2008), Gulevich et al. (2015), Naumenko et al. (1989), Nikulina et al. (1992), Plyusnina & Oskina (1997)
Early life stress models	Maternal stress during pregnancy	Prenatal	Inbred rats	↑	Stress	Social interaction: ↓	Patin et al. (2002, 2005)
		Prenatal	Sprague-Dawley	↑	Stress	Social interaction: ↓	Morley-Fletcher et al. (2003)
		Prenatal	Wistar	↑	Stress	Social interaction: ↑	Schroeder et al. (2013)
		Prenatal	Bank vole	↑	Stress	Aggression test: ↑	Marchlewska-Koj et al. (2003)
	Maternal separation	Early life	Juvenile Wistar	↑	Basal	Play-fighting behavior: ↑	Veenema et al. (2006), Veenema & Neumann (2009)
		Early life	Adult Wistar	=	Basal + stress	Resident-intruder: ↑	Veenema et al. (2006)
		Early life	Long-Evans	↑	Stress	Social interaction: ↓	Boccia & Pedersen (2001)
		Early life	C57Bl/6 mice	↑	Stress	Maternal aggression: ↑ Play-fighting behavior: ↓ Resident-intruder: ↓	Parfitt et al. (2004), Tsuda et al. (2011), Veenema et al. (2007)
		Early life	Balb/C mice	↑	Stress	Resident-intruder: ↑	Hohmann et al. (2013)
	Isolated rearing	Early life	Juvenile monkeys	↑	Basal + stress	Play-fighting behavior:↑	Higley et al. (1991, 1996), Higley & Suomi (1989), Suomi (1997)
		Early life	Adult monkeys	=	Stress	Playroom test: ↑	Meyer & Bowman (1972), Sackett et al. (1973)
	Peer-rearing	Early life	Monkeys	↓ or =	Basal + stress	Aggression test: ↑	Champoux et al. (1989), Clarke (1993), Feng et al. (2011), Winslow et al. (2003)
	Maternal maltreatment during infancy	Early life	Monkeys	↑	Basal	Aggression test: ↑	Howell et al. (2013)
	Surrogate/peer-rearing	Early life	Monkeys	↓	Basal + stress	Aggression test: ↑ Abnormal aggression: ↑	Capitanio et al. (2005), Davenport et al. (2003), Novak et al. (2013), Novak & Suomi (2008), Shannon et al. (1998, 2005)
	Post-weaning social isolation	Adolescence	Wistar	↑	Stress	Resident-intruder: ↑ Abnormal aggression: ↑	Haller et al. (2014), Toth et al. (2008), Toth et al. (2011), Tulogdi et al. (2014)
		Adolescence	Sprague-Dawley	↑	Stress	Play-fighting behavior: ↑ Social interaction: ↑	Wall et al. (2012)
	Repeated social subjugation	Adolescence	Golden hamsters	↑	Stress	Resident-intruder: ↑ (toward smaller opponents)	Delville et al. (1998), Wommack & Delville (2003), Wommack et al. (2003)
		Adolescence	Golden hamsters	↓	Stress	Resident-intruder: ↑	Ferris et al. (2005)
		Adolescence	Long-Evans	↑	Basal	Resident-intruder: ↑	Cunningham & McGinnis (2008)
	Peripuberty stress	Childhood and adolescence	Wistar	↓	Stress	Resident-intruder: ↑; Abnormal aggression: ↑ Aggression toward cohabitating female: ↑	Cordero et al. (2012), Cordero et al. (2013), Marquez et al. (2013), Toledo-Rodriguez & Sandi (2011)
Developmental exposure to drugs	Cocaine	Prenatal	Sprague-Dawley	↓	Stress	Resident-intruder: ↑ Maternal aggression: ↑	Johns et al. (1994), Johns & Noonan (1995), Wood & Spear (1998)
		Adolescence	Wistar rats & hamsters	↑	Stress	Resident-intruder: ↑	Alves et al. (2014), Harrison et al. (2000), Jackson et al. (2005), Knyshevksi et al. (2005)
	Alcohol	Prenatal	Rats and mice	↑	Stress	Play-fighting behavior: ↑ Resident-intruder: ↑	Gabriel et al. (2000), Gangisetty et al. (2014), Glavas et al. (2007), Kim et al. (1999), Wieczorek et al. (2015), Hamilton et al. (2010, 2014), Royalty (1990)
		Adolescence	Sprague-Dawley	↑	Stress	Play-fighting behavior: ↑	Varlinskaya et al. (2014)
	Cannabinoids	Perinatal	Wistar & Long-Evans	↓	Stress	Play-fighting behavior: ↓ Social interaction: ↓	Del Arco et al. (2000), Newsom & Kelly (2008), O'Shea et al. (2006), Rubio et al. (1995), Trezza et al. (2008)
		Adolescence	Wistar & Sprague-Dawley	↑	Stress	Social interaction: ↑	Lee et al. (2014), Schneider & Koch (2005)
	Antidepressants	Prenatal	Mice	?	-	Resident-intruder: ↑ Social interaction : ↑or ↓	Bourke et al. (2013), Coleman et al. (1999), Kiryanova & Dyck (2014), Svirsky et al. (2015), Yu et al. (2014)
		Prenatal	Rats	↑ or ↓	Stress	?	Gobinath et al. (2016), Pawluski et al. (2012)

Table 1. Summary of the literature describing the link between HPA axis function and aggressive behavior. ↑: represents an increase; ↓: represents a decrease; = not different; ?: not known; SHR: Spontaneously Hypertensive Rat; WKY: Wistar-Kyoto rat; F344: Fisher 344 rat; SD: Sprague-Dawley rat.

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Addendum

Curriculum Vitae

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Work Experience

PhD Candidate — *May 2014 – Apr. 2018*
École polytechnique fédérale de Lausanne (EPFL)
BMI - Brain Mind Institute (Prof. Carmen Sandi)

The aim of the project is to determine the behavioral and cardiovascular differences between rats that are reacting differently to stress (in term of HPA-axis activation).

Research Internship — *Nov. 2013 – May 2014*
École polytechnique fédérale de Lausanne (EPFL)
BMI - Brain Mind Institute (Prof. Carmen Sandi)

Finalization and writing of an article on results obtained during my Master project performed in Montréal (Cf. Below & Publications).

Research Internship (Master's Thesis) — *Feb. 2013 – Oct. 2013*
Concordia university, Montréal
Center for Studies in Behavioral Neurobiology (Prof. Dave Mumby)

I studied the beneficial effects of semi-natural environment on laboratory rats submitted to stress on cognition and anxiety-like behaviors.

Education

Diploma in Neurosciences (~B.Sc & M.Sc.) — *Sept. 2008 – Oct. 2013*
École polytechnique fédérale de Lausanne (EPFL)
Department of Life Sciences (sciences et technologies du Vivant)

Selected Publications And Conferences

Selected Journal Articles:

- *Low empathy-like behaviour in male mice associates with impaired sociability, emotional memory, physiological stress reactivity and variations in neurobiological regulations.* G. Laviola, F. Zoratto, D. Ingiosi, V. Carito, **D. Huzard**, M. Fiore, S. Macrì. Plos One. 2017

- *The link between aberrant hypothalamic-pituitary-adrenal axis activity during development and the emergence of aggression-Animal studies*. *Walker SE, *Papilloud A, ***Huzard D**, Sandi C. Neurosci Biobehav Rev. 2016.

- *Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents*. Ryu D, Mouchiroud L, Andreux PA, Katsyuba E, Moullan N, Nicolet-Dit-Félix AA, Williams EG, Jha P, Lo Sasso G, **Huzard D**, Aebischer P, Sandi C, Rinsch C, Auwerx J. Nat Med. 2016.

- *The effects of extrinsic stress on somatic markers and behavior are dependent on animal housing conditions*. **Huzard D**, Mumby DG, Sandi C, Poirier GL, van der Kooij MA. Physiol Behav. 2015

Selected Conference Papers / Poster / Talks:

- *Developing lines of rats responding differently to stress during puberty.* Webinar for the Juniors of EU MATRICS FP7 consortium, Feb 2018

- *The impact of physiological stress systems on memory formation and extinction*. Talk at BMI retreat June 2017.

- Oral Presentation on yearly updates at General Assembly Meeting of EU FP7 MATRICS consortium (Apr. 2017)

- *Heart-rate variability of rats stressed during puberty or selected for stress reactivity*. **D. Huzard**, A. Papilloud, S.E. Walker, C. Sandi, Poster at FENS 2016

- Oral Presentation on yearly updates @ General Assembly Meeting of EU FP7 MATRICS consortium (Apr. 2015)

- *Physiological and Behavioral Effects of stress on Socially and Environmentally Enriched groups of Rats*. **D. Huzard**, G. Poirier, D.G. Mumby, C. Sandi. Poster at FENS 2014

Extra (non-scientific journals and talks):

- TV interview for Canal9 "*Anxiété, des soucis plein la tête*" about social status and trait anxiety of rats (Link 1 & 2).

- Webinar from Feb. 2018: Youtube link