Genetic risk factors and early-life stress interact to shape endophenotypes of affective disorders

Behavioral, neuroendocrine, and molecular consequences of a gene × environment interaction

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Nature is relentless and unchangeable, and it is indifferent as to whether its hidden reasons and actions are understandable to man or not.

Galileo Galilei

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CHAPTER 1:

GENERAL INTRODUCTION

1.1. Major Depressive Disorder

"I didn't want to wake up. I was having a much better time asleep. It was almost like a reverse nightmare, like when you wake up from a nightmare you're so relieved. I woke up into a nightmare." – **Ned Vizzini,** *It's Kind Of A Funny Story*

For a healthy individual it will always be difficult to fathom what suffering from major depressive disorder (MDD) really feels like and what it means to a person. The above quote, taken from a personal account of a depressed patient, is an attempt to express the subjective sensation of major depression. When asked, many patients describe a feeling of inescapable emotional hurt or pain, together with a perceived inability to move or to act (Abramson et al., 1989, Uleyn, 1976, Joiner, 2001). MDD is a highly debilitating mood disorder that negatively affects a person's social and work life, their sleeping and eating habits, and general health (Blais and Peterson, 2004, Kennedy et al., 2001, Kupferberg et al., 2016). It often shows a chronic diseases course, where relapse is common, and it is extremely difficult to treat (Mrazek et al., 2014, Leonard, 1991). Epidemiologists estimate that up to 17% percent of the worldwide population will suffer from MDD during their lifetime (Andrade et al., 2003, Tsuang et al., 2004). All these factors converge to make MDD one of the leading causes of disability worldwide (WHO, 2001), as well a heavy economic burden (Greenberg et al., 2015). In spite of intense research efforts, the causes and the neurobiological pathways leading to MDD are still not fully understood. This lack of understanding is exemplified by the fact that the available treatment options remain insufficient, as 30-50% of MDD patients do not respond to established antidepressant medications (Menard et al., 2016). Many currently used drugs for the treatment of MDD are based on serendipitous discoveries (Penn and Tracy, 2012), and their mechanisms of action, leading to the amelioration of the depressive symptoms in some patients but not in others, are the topic of ongoing research. Part of the difficulty in understanding and treating MDD is likely due to the heterogeneity of the disease (Goldberg, 2011). In the diagnostic and statistical manual of mental disorders (DSM), version V (APA, 1994), the diagnostic criteria for MDD include cognitive and emotion-related symptoms (low mood, loss of interest or enjoyment, difficulties in concentrating, feelings of guilt or self-blame, thoughts of death and suicide) and vegetative symptoms (fatigue, psychomotor changes, disturbances of sleep, changes in appetite and bodyweight). Further, at least two subtypes of MDD can be distinguished, based on specific symptoms during an episode or over the course of several

episodes. Patients with a melancholic/psychotic depression subtype experience insomnia with early morning awakening, variable mood (worse in the morning), appetite loss, weight loss, psychomotor disturbances (agitation), and neuroendocrine dysregulation of the stress hormone system with hyper-cortisolemia (Fink and Taylor, 2007, Gold and Chrousos, 1999, Antonijevic, 2006). Almost the inverse pattern is seen in patients with atypical depression, where symptoms include hypersomnia, reactive mood, increased appetite, weight gain, leaden paralysis, interpersonal rejection sensitivity, and neuroendocrine dysregulation with hypo-cortisolemia (Gold and Chrousos, 1999, Stewart et al., 2009, Thase, 2009). The existence of these divergent subtypes, together with the observation that the dysregulated stress hormone secretion often normalizes before remission of clinical symptoms (Baghai et al., 2008, Ebert, 1996, Hirschfeld, 1999, Holsboer, 1983), highlights the central role of the neuroendocrine stress response system in MDD pathology.

1.2. The stress response system

In our everyday life, the word "stress" is used ubiquitously as a term referring to a situation, a feeling or a state that is perceived as stressful. In the field of stress research, a more precise definition is necessary to avoid confusion and redundancy. Hence, "stress" has been defined as the nonspecific response of an organism in answer to a stressor (Selye, 1976). The stressor can be any perceived threat to homeostasis, be it a real-word physical danger or psychological phenomenon. Upon perception of the stressor, two endocrine systems become rapidly activated to enable the organism to cope:

First, the sympathetic nervous system (SNS) signals to the adrenal medulla to initiate the release of catecholamines (epinephrine and norepinephrine) into the blood stream. These hormones act in the periphery to increase cardiovascular tone and blood supply to vital organs and skeletal muscles, and to stimulate energy metabolism from lipids and glucose. At the same time catecholamine signaling reduces appetite, digestive functions, and sexual drive, thereby focusing the body on essential actions in order to survive (the so called "flight-or flight" response (Cannon, 1915)). In the brain, norepinephrine is released from cells in the *locus coeruleus* and binds to receptors in limbic structures, such as the amygdala and the hippocampus, where it can modulate cognitive function (Valentino and Van Bockstaele, 2008).

Second, the hypothalamic-pituitary-adrenal (HPA) axis is activated, illustrated in Figure 1. Parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN) release

corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal blood stream to the pituitary gland, where they trigger the release of adreno-corticotropic hormone (ACTH) into the blood circulation to stimulate the synthesis and release of glucocorticoid (GC) hormones from the adrenal cortex (De Kloet et al., 1998, Herman and Cullinan, 1997, Sapolsky et al., 2000). GCs (mainly cortisol in humans and corticosterone (CORT) in murine rodents) act in the periphery to mobilize glucose reserves, stimulate gluconeogenesis, and suppress inflammation, and, together with AVP, to increase vasorestriction and water retention, which is especially important when injuries occur (Sapolsky et al., 2000). The lipophilic GCs easily pass the blood brain barrier and interact with various neurotransmitter systems in the brain (e.g. the dopaminergic, the endocannabinoid, the neurotrophic, or the noradrenergic system) to affect cognitive function (Czyrak et al., 2003, Daskalakis et al., 2015, Hill et al., 2010, Krugers et al., 2012). For instance, in the prefrontal cortex (PFC) GC signaling can promote attention and vigilance (de Kloet et al., 2005, Henckens et al., 2012), in the amygdala the stress hormones modulate emotion processing (McIntyre and Roozendaal, 2007, Phelps and LeDoux, 2005), and in the hippocampus they can reinforce memory formation and retention (Oitzl and de Kloet, 1992, Schwabe et al., 2012, Schwabe et al., 2008). GCs exert their function by binding to two types of receptors, the glucocorticoid and the mineralocorticoid receptors (GR and MR), which have very distinct functional profiles (de Kloet et al., 2005, Reul and de Kloet, 1985): The intracellular MR has a high affinity to GCs (~0.5 nM) and is therefore mostly bound, even under basal conditions. The GR has an approximately 10-fold lower binding affinity and thus only becomes activated under high GC conditions (Joels and Baram, 2009). Both receptor types are co-expressed in many cortical and limbic brain areas, including the hippocampus and amygdala, and in the PVN, but the relative proportions differ between regions. Both MR and GR are ligand-activated transcription factors, i.e. when bound, they can translocate to the nucleus, where they regulate gene expression by binding to glucocorticoid response elements (GREs) in the DNA and thus initiate a biochemical cascade leading to the transcription or repression of genes (Dostert and Heinzel, 2004, Pearce, 1994). In this way the GR has been reported to regulate up to 10-20% of all genes in the human genome (Oakley and Cidlowski, 2013). In an acute stress response, the elevated level of circulating GCs promotes their binding to GRs, which, in the PVN, stimulates a negative feedback signal directly onto the CRH-releasing neurons. A negative feedback signal also comes from GR activation in the pituitary and in the hippocampus, transmitted via *gamma*-Aminobutyric acid (GABA)-ergic projections to the PVN, leading to a cessation of the CRH release and a return of the HPA axis to baseline (Joels and Baram, 2009).

The negative feedback loop of the HPA axis is essential to maintain a delicate balance between an activated stress response state, optimal for coping with potentially life-threatening challenges at the expense of self-sustaining physiological and reproductive functions, and a baseline state, which is necessary to recover, rest and reproduce (McEwen, 2005). Thus, a well-controlled stress response is highly adaptive and often essential for survival (de Kloet et al., 1999, Sapolsky, 2015). However, extended exposure to high levels of GCs can exert neurotoxic effects, affecting cell proliferation, synaptic plasticity, and dendritic arborization throughout the brain, ultimately impairing cognitive function and mental health (Cuesta and Singer, 2012, de Kloet et al., 2005, McEwen and Gianaros, 2010, Holsboer and Ising, 2010, Lupien et al., 2009). This "tearing and wearing" effect of the stress response on cells and systems of the brain is summarized in the term "allostatic load"(McEwen, 2004). Thus, as long as the balance of stress and recovery phases is maintained, the stress response is beneficial and highly adaptive, but when the balance is tipped this can lead to adverse consequences for health and well-being. A dysregulated stress response is associated with several forms of metabolic, immune and affective disorders (McEwen, 2005, Sapolsky, 2015, Schneiderman et al., 2005, Segerstrom and Miller, 2004) and is likely to play a central role in their etiology and pathophysiology.

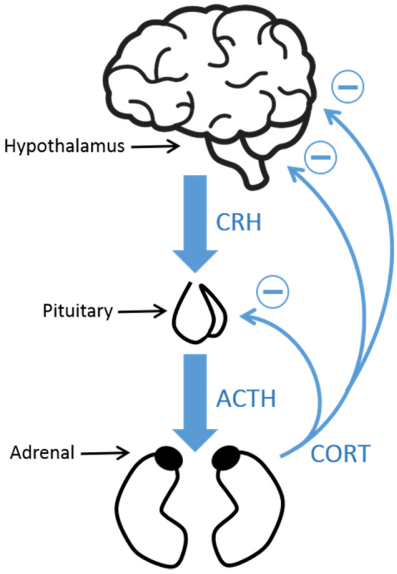

Figure 1. A basic summary of the hypothalamic-pituitary-adrenal (HPA) axis. CRH: Corticotropin-releasing hormone, ACTH: Adreno-corticotropic hormone, CORT: Corticosterone

1.3. Development of the HPA axis

At birth, most vertebrates are still strongly dependent on their mother for survival. At this time point, many areas of the brain are still developing and the stress response system is not fully mature yet. To discover how the stress response system develops, researchers have often used rodents as a model system, and evidence suggests that the development in humans follows a similar course (though on a different time scale) (Gunnar and Donzella, 2002, Sapolsky and Meaney, 1986). During the final days of gestation and at birth circulating GC levels in the fetus are high, as they are essential for healthy lung development of the fetus (Jaskoll et al., 1996), but decrease rapidly after delivery (Martin et al., 1977, Mastorakos and Ilias, 2003). In mice, CORT

levels then remain stable at low concentrations and fail to increase in response to most stressors until postnatal day (P)12 (Schmidt et al., 2003, Levine, 2002). This developmental phase is termed the stress hyporesponsive period (SHRP). During the SHRP, ACTH secretion is suppressed under basal condition and shows no measurable increase in response to stressors (Walker et al., 1986). In addition, the adrenal sensitivity to ACTH signaling is much reduced (Rosenfeld et al., 1992a). In contrast, CRH levels in the PVN are high from birth until P12, but this signal fails to stimulate a response from the HPA axis (Baram and Lerner, 1991, Schmidt et al., 2003). Similarly, MR are highly expressed from birth onwards, but GR levels are low at birth and increase steadily until P12 (Bohn et al., 1994, Rosenfeld et al., 1988, van Eekelen et al., 1991). After P12, animals show a transient rise in basal CORT levels, as the negative feedback loop comes "online" and GRs become more integrated into the hippocampus (Meaney et al., 1985, Schmidt et al., 2003). The regulation of HPA axis then gradually normalizes to adult levels. Evolutionarily, the purpose of the SHRP is most likely to protect the brain from the neurotoxicity of excessive GC exposure during a critical period of development.

In humans, the SHRP is less well described, due to several ethical and practical limitations concerning research in neonates. It is known that, at birth, human infants already show a reliable rise in cortisol in response to even minor stressors (Gunnar, 1992), but around three months of age, the adrenal sensitivity to ACTH becomes markedly reduced (Gunnar et al., 1996) and by 12 months of age, moderate stressors, such as inoculation shots or brief separation periods, fail to trigger a cortisol response, in spite of fearful or distressed behavioral reactions (Gunnar, 1998). Both human and animal data reveal clear evidence for a maternal regulation of the infants stress response system. For instance, insecurely attached children show elevated cortisol levels in response to a psychological challenge, when securely attached peers do not (Nachmias et al., 1996, Spangler and Grossmann, 1993). Also, maternally deprived rat pups show an HPA axis response to stressors during the SHRP, when non-deprived rats remain unresponsive (Stanton and Levine, 1990). Experiments have identified some individual components of maternal care, such as feeding, tactile stimulation, and temperature regulation, that are important regulators for the maintenance of the SHRP (Cirulli et al., 1992, Rosenfeld et al., 1993, Pauk et al., 1986, Bruder et al., 2011). However, no signal component on its own can fully recapitulate the suppressive effect of intact maternal care on the pups endocrine stress response (Levine, 2002). In summary, the SHRP is a critical phase in the development of the brain and the HPA axis, which is tightly regulated by maternal cues and can be disrupted only by severe stressors, such as by interfering with the mother-infant relationship.

1.4. Risk factors for Major Depressive Disorder

To better understand the etiology of MDD and its high incidence rate, much effort has been invested to delineate potential causes and risk factors. The identified factors can be roughly divided into internal (i.e. tied to the person's biology or to his/her choices and actions) and external factors (i.e. related to the environment).

1.4.1. Internal risk factors

1.4.1.1. Neuroendocrine vulnerability

A dysregulation of the neuroendocrine response to stressors is a key endophenotype associated with many affective disorders, such as schizophrenia and post-traumatic stress disorder (PTSD), as well as both melancholic/psychotic and atypical depression (Keller et al., 2006, Antonijevic, 2006, Gold and Chrousos, 1999, Gold and Chrousos, 2002). Interestingly, the deviation from the normal range of HPA axis function occurs in both directions, i.e. hypo-reactivity is often reported in PTSD and atypical depression patients (Yehuda, 1997, Gold and Chrousos, 2002), while hyper-reactivity is a common feature in melancholic depression (Heim et al., 2000a, Strohle and Holsboer, 2003). In schizophrenia patients, both hypo- and hyper-reactivity of the HPA axis have been described (Bradley and Dinan, 2010). The extent to which such a dysregulation of the stress response is cause or consequence of affective disorders is still an open question, but a lot of evidence indicates that a genetic vulnerability for HPA axis dysregulation may be an important predisposition for the development of MDD (Nestler et al., 2002). Indeed, resilience to depression has been proposed to depend on the ability of the HPA axis to respond appropriately to stressors of varying magnitudes and to be shut-off efficiently (Bale and Vale, 2003). Studies using animal models have shown that innate differences in stress reactivity moderate the susceptibility to adverse consequences of early-life stress (ELS) (McIlwrick et al., 2016, Rana et al., 2015). In humans, sex differences in the HPA axis responsiveness are commonly observed, probably related to the influence of reproductive hormones (Seeman, 1997, Young et al., 2001). Taken together with the fact that women are twice as frequently affected by depression than men (Bale, 2006), this supports the view that heightened stress sensitivity is an important risk factor for MDD.

The neuroendocrine changes associated with MDD include an increased release of CRH, blunted ACTH sensitivity, enlarged adrenal and pituitary glands, and changes in the expression of GR throughout the brain (Gold et al., 1984, Holsboer et al., 1987, Keck and Holsboer, 2001,

Nemeroff, 1996), suggesting that the HPA axis is critically involved in the pathophysiology of the disorder. Further evidence supporting the central role of the HPA axis in MDD is provided by the finding that patients, who respond to antidepressant treatment, show a normalization of their HPA axis function before symptom remission (Holsboer, 1983, Strohle and Holsboer, 2003). Two commonly used measures to assess HPA axis function are the combined dexamethasone/CRH test (Heuser et al., 1994, Ising et al., 2005) and the cortisol awakening response (CAR) (Kuehner et al., 2007). Strikingly, family-based linkage analysis revealed that first-degree relative of MDD patients show a slight, but significant, deviation in their HPA axis response compared to individuals with no family history of affective disorders (Holsboer et al., 1995), indicating that the vulnerability for HPA axis dysregulation is partly hereditary. Supporting this, studies investigating the genetic basis of the CAR in mono- and dizygotic twins unanimously showed that genetic, but also environmental factors, play a role in shaping the sensitivity of the HPA axis (see Dedovic and Ngiam, 2015 for review). In addition, personality traits, such as an inclination for hopelessness, worry, or neuroticism appear to be predictors of abnormal CAR and have been associated with both increased and decreased awakening cortisol levels (Kuehner et al., 2007, Portella et al., 2005). Taken together, HPA axis sensitivity is affected by both genetic predisposition and environmental influences, and a high stress sensitivity is linked to an increased risk for affective disorders.

1.4.1.2. Risk behaviour

Risk behaviour such as smoking, alcoholism, and other substance abuse show a strong statistical association with MDD and evidence suggests that co-morbid addiction and MDD can mutually exacerbate their disease progression (Chaiton et al., 2009, Danaei et al., 2009). For instance, substance abuse can affect stress sensitivity and stress coping by inducing a chronic activation of the HPA axis and upregulating the expression of CRH in the PVN and the amygdala, as these neurobiological processes form central components underlying the development of craving and drug seeking behaviour (Koob, 2010, Logrip et al., 2011). Furthermore, in the course of addiction, a blunting of the negative feedback loop of the HPA axis has been described (Koob and Kreek, 2007, Rasmussen et al., 2000) and in consequence, high circulating levels of GCs can sensitize CRH neurons in the extended amygdala and the medial PFC, leading to negative affective states and withdrawal symptoms (Koob, 2010, Shepard et al., 2000). Scientists agree that the tendency towards engaging in substance abuse behaviour has biological underpinnings (Nestler et al., 1993, Volkow and Baler, 2014), including a desensitization of the dopaminergic reward pathway and a vulnerability for HPA axis

dysregulation, which can also affect the risk for MDD (Stephens and Wand, 2012, Chu et al., 2014). In conclusion, overlapping factors seem to underlie the development of risk behaviors, such as substance abuse, and mental disorders, such as MDD. With aggravation of addictive symptoms several risk factors for MDD can become enhanced and, *vice-versa*, as progression of both disorders interferes with the common underlying neurobiological systems, controlling reward sensitivity, impulse control, and stress regulation.

1.4.1.3. Genetic factors

The inheritance of psychiatric disorders was already in the focus of research in the early nineteenth century (Slater, 1936, Kosters et al., 2015). Since then, family history studies and twin studies have provided ample evidence for a genetic component contributing to the risk for affective disorders, by showing that the incidence rates in first-degree relatives of patients with affective disorders are significantly higher than predicted based on the general population, and that monozygotic twins are more likely to share a mental disorder than dizygotic twins (Allen, 1976, Gershon et al., 1976, Gershon et al., 1971). Indeed, the heritability (i.e. the proportion of phenotypic variation that is explained by genetic variation) of MDD is estimated at ~40% (Burmeister et al., 2008, Sullivan et al., 2000) (which is relatively low, compared to e.g. autism spectrum disorder: ~90%, or schizophrenia: 70-85% (Burmeister et al., 2008)). However, genome-wide association studies (GWAS), including samples from thousands of patients, have not been able to identify genetic candidates that show strong main effects contributing to the risk for stress-related psychiatric disorders (Klengel and Binder, 2015). This "missing heritability" (Lee et al., 2013) may be partially due to the large heterogeneity in patients diagnosed with MDD, including different subtypes. Nevertheless, genetic association studies have identified some candidates, which may confer an increased risk. In particular, single nucleotide polymorphism (SNPs) in the DNA of genes involved in the serotonergic, the dopaminergic, and the neurotrophic system have been associated with increased affective disorders incidence (Antypa et al., 2016, Gatt et al., 2015, Levinson, 2006). Recently, a polymorphism on the gene coding for FKBP51, a co-chaperone protein regulating the affinity of the GR, has been implicated in MDD and in the response to antidepressant treatment (Binder et al., 2004, Gassen et al., 2015, Ising et al., 2008, Menke et al., 2013). However, none of the discovered SNPs have been unambiguously confirmed in GWA studies, suggesting that, while the detected polymorphisms may be associated with underlying traits contributing to the broad affective disorder phenotype, larger sample sizes and improved diagnostic classifications may be required to detect strong polygenic risk factors for MDD (Klengel and Binder, 2015,

Heinzmann et al., 2014). A further explanation for the "missing heritably" of MDD is provided by the discovery of epigenetics. Epigenetic mechanisms can modify the DNA without changing its actual code, for example by DNA methylation, histone modification, or microRNA interference. Epigenetics thus offers a mechanism for altering gene expression and regulation, while remaining undetected by traditional genomic tools.

1.4.2. External factors

1.4.2.1. Chronic Stress

The idea that extreme stress and depression are closely linked is widely accepted (Hammen, 2005). Supporting evidence comes from many case-control studies, which found that depressed patients report a significantly higher incidence rate of stressful life events prior to the disease than healthy controls (for review see Kessler, 1997, and Mazure, 1998). Importantly, this effect remains significant after correcting for a range of biases associated with the depression itself (e.g. personal responsibility in causing the events, mood congruent memory bias (Schwarz and Clore, 1983)). Furthermore, in the vast majority of depression patients, the disease onset was preceded by a severe negative life event, such as the death of a loved one, job loss, or divorce (Aseltine and Kessler, 1993, Carnelley et al., 1999, Dew et al., 1987), suggesting that adverse events may play an important role in the onset and development of MDD. Two factors, which seem to confer a particularly strong risk, are the uncontrollable nature and the chronic duration of certain stressors, like poverty, medical disability, or martial conflict (Kessler, 1979, Mazure, 1998). Overall, the experience of extended adverse conditions appears to provoke a chronic activation of the body's stress response system, without the necessary recovery phase and the return to physiological homeostasis, thereby increasing the allostatic load (McEwen, 2004). Over time, a high allostatic load can contribute to sustained changes in the cellular and molecular properties of brain cells (Joels et al., 2007), leading to a lasting dysregulation of the stress response system and to increased risk for MDD.

1.4.2.2. Perinatal stress

Perinatal depression affects 10-15% of childbearing women and, apart from being associated with increased morbidity and mortality for the mothers (Gavin et al., 2005), it can negatively impact on the future health of the child (Rahman et al., 2004, Talge et al., 2007). Stress during pregnancy has been associated with a lower birth weight of the infant and with an increased risk for hypertension, cardiovascular health deficits, type II diabetes, and psychopathology in

adulthood (Fumagalli et al., 2007, Goodman and Gotlib, 1999, Huizink et al., 2004, Meaney et al., 2007, Seckl, 2004). These adverse outcomes are thought to be a consequence of elevated levels of GC hormones in the mother during pregnancy, which can change the expression of stress-related genes in the placenta (Robinson et al., 1988) and pass through the placenta to the developing infant (Maccari et al., 2003). In unstressed conditions, only 10-20% of maternal GCs reach the fetus (Benediktsson et al., 1997), as the majority of GCs is rendered biologically inactive by a placental catalyst enzyme (11beta-hydroxysteroid dehydrogenase or 11β-HSD), which converts active cortisol to inactive cortisone (corticosterone to 11-dehydrocorticosterone in rodents)(Krozowski et al., 1999). Maternal stress is associated with both a rise in circulating maternal GCs and a downregulation of 11β-HSD in the placenta (Holmes et al., 2006, Seckl and Walker, 2001), allowing for increased crossing of active GCs to the developing infant. *In-utero* exposure to elevated levels of maternal stress hormones impacts on growth, morphology, and function of brain and peripheral tissue of the fetus, often with irreversible consequences (Duthie and Reynolds, 2013, Cottrell et al., 2012, Maniam et al., 2014).

After birth, the brain continues to develop and remains highly sensitive to environmental programming. In humans, both grey and white matter volumes increase significantly during the first 5 years of life as important brain areas expand, followed by a period of neuronal pruning and myelination that continues into late adolescence (Giedd et al., 1999, Paus et al., 1999). The exposure to adverse experiences or trauma during this sensitive time of brain development can lastingly impact on the child's future health (Carr et al., 2013, Heim and Binder, 2012, Mandelli et al., 2015). In an effort to define some critical insults on childhood wellbeing, the World Report on Violence and Health (WHO, 2002) has distinguished four different types of childhood maltreatment: sexual abuse, physical abuse, emotional and psychological abuse, and neglect. However, studies show that, in everyday life, these categories often overlap, i.e. children who are victims of one form of abuse are also more likely to be victims of another (Chartier et al., 2010, Dong et al., 2003, Felitti et al., 1998). Independently, all forms of childhood abuse have been associated with cognitive impairments (Gould et al., 2012, Nikulina and Widom, 2013), as well as with an increased risk for mental disorders, including MDD and PTSD (Afifi et al., 2009, Norman et al., 2012, Sadowski et al., 1999, Young and Widom, 2014, Kessler et al., 2010), and the accumulated experience of several adversities and traumas strengthens this relationship (Hill et al., 2001). All different types of ELS have repeatedly been associated with long-term changes in the regulation of the stress hormone system (Lupien et al., 2009). For instance,

children who experienced psychological neglect in orphanage care show a flattened diurnal cortisol profile later in life (Carlson and Earls, 1997) and childhood sexual abuse has been associated with elevated cortisol levels (Cicchetti and Rogosch, 2001). In addition to these neuroendocrine programming effects, animal studies have shown that ELS can lead to an accelerated maturation of neuronal morphology and regional brain circuitry, including the hippocampus, the amygdala, and the PFC (Bath et al., 2016, Moriceau et al., 2006), associated with a preterm shift to adult-like behaviour (Callaghan and Richardson, 2011). In line with these findings, results of a recent human imaging study revealed the early emergence of adult-like structural connectivity between amygdala and prefrontal cortex in children who were institutionalized in orphanages (Gee et al., 2013), showing that ELS leaves lasting imprints in the structure of emotion-processing networks in the brain.

One important mechanism by which ELS can lastingly influence brain and behaviour is through epigenetic programming of gene expression (Bale, 2011, Heim and Binder, 2012, Meaney et al., 2007, Murgatroyd, 2014). Epigenetic modification of DNA result in subtle changes in gene regulation, which can impact on neuronal morphology, plasticity, connectivity, and even brain structure, and thereby shape the behavioral phenotype (Klengel and Binder, 2015). For example, methylation of the GR promoter gene has been shown to be altered as a function of maternal care (Weaver et al., 2004), leading to enduring changes in GR expression and negative feedback regulation. Epigenetic modifications do not necessarily have to be revealed in changes of baseline gene expression, but can also lead to “poised states”, in which the targeted genes are rendered more sensitive, thus conferring enhanced vulnerability to future environmental hazards (Klengel and Binder, 2015). Overall, there is overwhelming evidence showing that adverse experiences during early-life can lead to neurobiological programming and to an increased risk for affective disorders later in life.

1.5. Gene × Environment interaction

Each of the risk factors described above is independently associated with an elevated risk for MDD. However, the individual contribution to the phenotype is relatively small, that is to say, many carriers of a specific risk allele show no symptoms of MDD, and many people experience severe or chronic stressors without developing an affective disorder. Over the lifespan, individuals with varying genetic predispositions are exposed to a great many environmental challenges, resulting in a large inter-individual variability of health-related outcomes (Klengel and Binder, 2015). As illustrated in Figure 2, gene × environment (G × E) interaction studies are investigating how innate and environmental factors together affect the chances for disease or resilience. Thus, a G × E interaction describes differences between genotypes in susceptibility to environmental stressors (Kendler and Eaves, 1986). Understanding these interactions will be essential to identify the biological mechanisms underlying vulnerability to psychiatric disorders and may benefit the development of personalized treatments based on individual history and genetic profile (Dempfle et al., 2008, Holsboer, 2008).

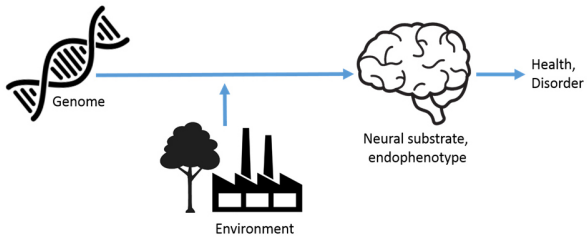

Figure 2. Gene × environment interaction paradigms assume that the effects of the environment depend on the genetic predisposition of the individual, shaping molecular processes, structure, and function of the brain, and thereby leading to variable health-related outcomes. (Inspired by Caspi and Moffitt, 2006)

G × E interaction studies require a well thought-through design, with the appropriate controls and statistical models to avoid misinterpretation of the data. For instance, gene × environment correlations, where the genotype influences the probability of exposure to certain risk environments, can lead to false positive findings (Karg and Sen, 2012). For example, divorce can increase the risk for depression, but it could equally be that people with a genetic risk for depression are more irritable and quarrelsome, thus promoting their chances for marital dispute and creating a correlation between the genotype and the environmental risk exposure (Jaffee and Price, 2008). However, due to the high etiological validity of G × E interaction models in psychiatric research, G × E interaction studies are becoming more frequent and have provided many valuable results. For instance, in a seminal longitudinal study, Caspi and colleagues found that maltreated children with a functional polymorphism in the gene encoding the neurotransmitter-metabolizing enzyme monoamine oxidase A (MAOA), which reduces the

expression of MAOA, were more likely to exhibit antisocial behaviour in adulthood than maltreated children who did not have this genotype (Caspi et al., 2002). Using a similar study design, it was demonstrated that a polymorphism in the serotonin transporter gene, 5-HTT, moderates the risk for developing MDD in individuals who experienced stressful life events in childhood or in adulthood (Caspi et al., 2003). Going one step further and investigating the underlying neurobiological mechanisms involved in G × E interactions, Klengel *et al.* found that in individuals who were exposed to ELS, an epigenetic modification of the gene coding for FKBP5, a functional regulator of GR sensitivity, was dependent on genotype. Specifically, carriers of the genetic risk allele showed increased demethylation at the transcription sites of the FKPB5 gene if they had experienced childhood abuse. In carriers of the protective allele, childhood abuse was not associated with FKBP5 methylation changes (Klengel et al., 2013). The epigenetic stimulation of FKBP5 transcription through demethylation leads to down-stream changes in the regulation of the HPA axis and confers a risk for later psychopathology (Klengel et al., 2013). These findings from human studies are supplemented by experiments using animal models, which have the advantage of high levels of experimental control, so that both genotype and exposure to risk factors can be experimentally manipulated. In particular, animal models of genetic susceptibility are a valuable tool to investigate the effect of specific environmental risk factors on the development of disease processes.

1.6. Animal models in psychiatric research

In the study of MDD much of our knowledge is derived from case studies in human patients and from epidemiological research. Human studies are evidently critical in the study of uniquely human disorders, but they have the drawback that, due to several important ethical and practical considerations, causality and mechanistic evidence at the level of neurobiological processes are extremely hard to obtain from human patients (Nestler and Hyman, 2010). Therefore, a lot of the research on the neurobiological underpinnings of MDD is conducted using animal models. However, MDD is a highly complex disease, affecting higher order cognition, mood, vegetative function, and overall health, and many of the core symptoms of MDD (e.g. sadness, guilt, or suicidal ideation) cannot be convincingly modelled in animals. Furthermore, to date, there are no objective diagnostic tests for MDD and the clinical diagnosis is given purely on the basis of phenomenology (Nestler and Hyman, 2010). Collectively, these impediments preclude the development of a fully convincing model of MDD in animals.

An opportunity to nonetheless make use of animal models in a meaningful way in the study of psychiatric disorders is granted by the focus on individual endophenotypes, which are accessible to be studied in animals (Hasler et al., 2004). Endophenotypes have been defined as "measurable components, unseen by the unaided eye, along the pathway between disease and distal genotype" (Gottesman and Gould, 2003). Thus, animal models in psychiatric research should not be understood as full disease models, but rather as models of selected endophenotypes associated with the disease. Rodents are popular model systems in the field of stress research, as they recapitulate many of the neuroendocrine changes observed in humans in response to acute and chronic stress (Tarantino et al., 2011). The approaches used to generate disease models include targeted genetic manipulations, selective breeding, optogenetics, brain lesions, and environmental manipulations. To constitute a powerful research tool, an animal model should present high levels of etiological, construct, face, and predictive validity (Chadman et al., 2009, Willner and Mitchell, 2002). Etiological validity relates to the origin or cause of the disease to be modelled, which should be similar in the model and in real-life cases. Construct validity refers to the process by which the phenotype was achieved, which should be plausibly related to the causal process of the phenotype in humans. Face validity implies that a model replicates key features of the target endophenotype. Predictive validity confirms that the animal model responds to treatment (e.g. pharmacotherapy) in the same way as human patients would. An optimal model should comply to a high degree with all of these criteria. However, some animal models can be beneficial and useful even when not all criteria are met completely (Belzung and Lemoine, 2011).

1.6.1. Genetic animal models

To delineate the role of specific genes and their contribution to physiology and behaviour in a bottom-up approach, genetic engineering, i.e. the direct manipulation of the genome by targeting specific candidate genes, is a powerful research technique promising high construct validity (Nestler and Hyman, 2010). Using molecular genetic manipulations, scientists can for example generate "transgenic" lines, where DNA sequences are inserted and result in functional changes downstream, or "knock-in/knock-out" lines, where the expression of specific genes is altered in a controlled manner, thus creating animals carrying selected genetic variants of interest. In general, the higher the penetrance of a genetic variant in humans (i.e. the proportion of individuals carrying a particular variant that also express the associated phenotype), the more likely it is that a mouse model carrying this variant will also produce the desired

phenotype (Cox, 2015). Unfortunately, for most affective disorders, few or no alleles matching this criterion have been identified (Klengel and Binder, 2015). In addition, in different individuals the genetic pathway leading to a disease phenotype may vary, and *vice versa*, the same genetic variant may lead to different phenotypes depending on its interaction with other genes, epigenetic factors, and environmental influences (Nestler and Hyman, 2010, Sullivan et al., 2000). This greatly complicates the process of constructing and validating genetic animal models for MDD. However, genetic models are employed with great success to recreate specific endophenotypic alterations associated with MDD. For example, impaired or increased feedback inhibition of the HPA axis has been genetically engineered in animal models by knocking out or overexpressing the GR in selected brain regions (Howell and Muglia, 2006, Müller and Holsboer, 2006) or by interfering with the CRH system (Dedic et al., 2012, Wang et al., 2013). Genetically manipulated mice have also been employed to explore the contribution of various monoamine and neuropeptide systems, the neurotrophic system, and the immune system in endophenotypes associated with MDD (for review see Barkus, 2013, and Urani et al., 2005).

A different approach to investigating the genetic component of psychiatric disorders using a more top-down design is selective breeding. That is, animals exhibiting a specific trait are used to study a particular endophenotype of interest. To generate such a model, animals are screened and selected for breeding based on the presence or absence of a defined genetic trait, leading over time to the stable expression of this trait in the resulting population. Examples of selectively bred animal models relevant to affective disorders are the Flinders sensitive and resistant rat lines (Overstreet and Russell, 1982), the congenitally learned-helpless rats (Vollmayr and Henn, 2001), the Fawn Hooded rats (Tschopp and Zucker, 1972), the Wistar-Kyoto rats (Okamoto and Aoki, 1963, Okamoto et al., 1966), the swim low-active and high-active rats (Weiss et al., 1998), and the high-anxiety and low-anxiety rats and mice (Kromer et al., 2005, Landgraf et al., 1999).

As a dysregulation of the HPA axis is a central feature in the pathophysiology of both melancholic/psychotic and atypical depression, a mouse model for extremes in stress reactivity was generated (Touma et al., 2008) in order to further elucidate the neurobiological parameters underlying these disease endophenotypes. The so-called Stress Reactivity (SR) mouse model consists of a high reactivity (HR) and a low reactivity (LR) breeding line, with a third intermediate reactivity (IR) line providing an internal “normal” reference. To generate this model, a founder generation of 100 male and 100 female outbred CD1 mice, aged ~8 weeks,

was screened and selected for breeding based on their plasma CORT increase in response to a standardized psychological stressor (15 min restraint). In each new generation this screening and selection procedure was repeated to maintain the three breeding lines and expand the phenotype, leading to, today, thirty generations of the SR mouse model. HR animals are proposed to model the neuroendocrine endophenotype associated with melancholic/psychotic depression (hyper-responsive HPA axis), while LR animals present a neuroendocrine endophenotype reminiscent of atypical depression (hypo-responsive HPA axis). Extensive phenotyping of the SR mouse model has brought to light several further parallels between the HR and LR mouse lines and their corresponding subtypes of MDD, including changes in bodyweight (Heinzmann et al., 2014, McIlwrick et al., 2016, Touma et al., 2008, Touma et al., 2009), locomotor activity and stress-coping behaviour (Knapman et al., 2010a, McIlwrick et al., 2016, Surget et al., 2016, Touma et al., 2008), cognitive function (Knapman et al., 2010a, Knapman et al., 2010b, Knapman et al., 2012, McIlwrick et al., 2016, Surget et al., 2016), sleep architecture (Fenzl et al., 2011), as well as in circadian rhythms of stress hormone secretion and feedback sensitivity of the HPA axis (Heinzmann et al., 2014, Touma et al., 2009, Touma et al., 2008). The SR mouse model thus offers a promising starting point to explore of the role of HPA axis dysregulation and its sequelae in the etiology and pathophysiology of MDD, by modelling genetic predispositions for extremes in stress reactivity including several endophenotypes of MDD.

1.6.2. Environmental animal models

1.6.2.1. Environmental stress paradigms in adulthood

It has long been recognized that the environment can have a lasting influence on a person's behaviour and wellbeing. One of the first experimental demonstrations of the profound effect of an environmental stressor on affective behaviour was provided in the early 80s, when Katz and colleagues described a paradigm in which a mouse was subjected to painful foot shocks and showed increasingly passive and anhedonic behaviour in consequence (Katz, 1982). Following this observation, the experiment was extended by using a sequence of different milder physical stressors, making the model more ethical and etiologically valid, and it was shown that the resulting anhedonia in the animals was reversible by chronic antidepressant treatment (Willner et al., 1987). Since then, the so-called chronic unpredictable mild stress model has become highly popular and is generally accepted as a valid model of behavioral depression in rodents

(Hill et al., 2012, Surget et al., 2008, Yalcin et al., 2008). A similar depression-like phenotype can also be ascertained by using the chronic social defeat paradigm (Nestler and Hyman, 2010). In this social stress paradigm, a mouse is exposed to daily bouts of repeated social defeat by a larger, aggressive resident mouse. After the defeat, the animals are physically separated, but sensory contact remains through a perforated dividing wall, thus intensifying the chronic nature of the stressor (Golden et al., 2011). The depression-like symptoms in mice after chronic social defeat include anhedonia, social withdrawal, and metabolic disturbance, which can be ameliorated with chronic antidepressant treatment (Bourke et al., 2014, Murata et al., 2015, Razzoli et al., 2011). Both the chronic unpredictable mild stress and the chronic social defeat paradigms show good levels of construct, face, and predictive validity and have been used as valuable tools in several studies to investigate the neurobiological processes underlying affective disorders (Hill et al., 2012, Hollis and Kabbaj, 2014).

1.6.2.2. Perinatal environmental stress paradigms

The perinatal period is a critical phase in the etiology of several affective disorders. To investigate how stress exposure during this phase impacts on future health and coping, several pre- and postnatal stress paradigms have been developed. Prenatally, the most common methods include repeated restraint of the dam during gestation or repeated injections of GC hormones to mimic a chronic stress response (Maccari and Morley-Fletcher, 2007, Seckl, 2004, Seckl and Meaney, 2004). To model ELS in the postnatal period, maternal separation has frequently been employed, with a range of differing parameters (e.g. varying the duration of deprivation, removing the dam from the nest *versus* removing the pups, leaving the litter intact *versus* isolating each pup, using a heating pad during the absence of the dam or not, etc.) (Millstein and Holmes, 2007, Nishi et al., 2014, Plotsky and Meaney, 1993, Weaver et al., 2007). These manipulations have generated convincing data showing that maternal separation can lead to a lasting dysregulation of the HPA axis (Avishai-Eliner et al., 1995, van Oers et al., 1997), affect learning and memory (Lehmann et al., 1999), alter stress-coping behaviour (Aisa et al., 2007), and influence a range of neurobiological processes (Brunton, 2015, Gracia-Rubio et al., 2016, Nishi et al., 2014, Rosenfeld et al., 1992b). However, all variations of the maternal separation paradigm are to some extent subject to rising criticism that is concerned with the effects of starvation or irregular nursing and thermal stressors affecting the pups during the separation periods (Molet et al., 2014a). To minimize the influence of these confounding factors, a novel postnatal stress paradigm was developed, which attempts to create a more naturalistic

model of ELS. In this so-called limited nesting and bedding material paradigm, dams and their litters are kept in "impoverished" housing conditions during a 7-day period in the early postnatal phase, achieved by strongly reducing the amount of cage bedding and nest building material. In both rats and mice, this environmental manipulation has been reported to reliably induce adverse changes in maternal behaviour, such as increased erratic and unpredictable sequences of maternal care and frequent exits from the nest area (Avishai-Eliner et al., 2001b, Rice et al., 2008). The limited nesting and bedding material paradigm therefore creates a situation of unpredictable und uncontrollable ELS for the pups, reminiscent of childhood neglect and adverse care in humans, making it a valuable tool to investigate the effects of ELS in rodents.

1.1. Aims of this thesis

Previous research has demonstrated a high degree of validity of the SR mouse model as a genetic animal model for affective disorders. Several key endophenotypes of melancholic/psychotic and atypical depression are recapitulated in the HR and the LR mouse lines, respectively, making the SR mouse model a promising tool for the investigation of G × E interactions underlying affective disorders.

In the presented work, we aimed to complement the existing data by assessing the predictive validity of the SR mouse model. Further, the main objective of this thesis was to investigate the G × E interaction of ELS and genetic predisposition for extremes in stress reactivity using the SR mouse model to advance our understanding of how ELS interacts with a specific genetic vulnerability by shedding light onto the behavioral, neuroendocrine, and molecular consequences at different stages of development. Such knowledge has the potential to increase our understanding of the etiology and pathophysiology of MDD, and may contribute to the development of novel intervention or treatment approaches for affective disorders, which take genetic risk factors into account.

Thus, in an initial study, we exposed animals of the three SR mouse lines to chronic antidepressant treatment by daily fluoxetine injections, and tested the effect of this pharmacological manipulation on a range of parameters for endophenotypes of MDD (Chapter 2). We used a battery of behavioral assays to evaluate the animals' locomotor activity, stress-coping, and anxiety-related behaviour. Furthermore, the animals' spatial learning and memory performance was tested, and we employed neuroendocrine tests to assess alterations in their

stress reactivity and HPA axis feedback regulation. In addition, changes in hippocampal neurogenesis were investigated with immunohistochemistry. The results of this study add further validity to the SR mouse model as a genetic animal model for affective disorders.

In the reality of human patients, and as described in the introduction above, the development of MDD often results from an interaction of genetic and environmental factors. To model this clinical situation, we exposed animals of the SR mouse model to ELS with the objective of assessing the consequences of this G × E interaction at the level of emotional behaviour, cognitive function, neuroendocrine regulation, and gene expression over the animals' life span (Chapters 3 and 4).

As a means to induce ELS, we established the limited nesting and bedding material paradigm (Rice et al., 2008) in our mouse model and monitored the resulting changes in maternal behaviour in dams of the HR, IR, and LR lines (Chapter 3). Our aim was to assess (a) whether, as predicted, maternal care was adversely affected by the ELS paradigm, and (b) if there were any significant differences in the effects of the ELS paradigm on maternal behaviour between dams of the three SR mouse lines. The latter assessment was of particular importance, as line differences in maternal care could explain later differences observed in the pups.

Once this ground work had been laid, we examined how the ELS paradigm influenced behaviour, neuroendocrine development, and gene regulation in the pups (Chapter 3). We used an adapted version of the stress reactivity test to measure the pups' HPA axis response to a stressor at different time points until weaning. As a supplementary measure of HPA axis activity, we also collected the adrenals from pups at different stages of postnatal development. To assess changes in their emotional behaviour, we recorded the pups' ultrasonic vocalizations (USVs) and their locomotor activity during the first week of life, as well as their stress-coping behaviour on the day of weaning. The animals' physiological development was monitored throughout the experiment by weighing them regularly.

In a next step, we asked whether the long-term consequences of ELS exposure differ between mice of the three SR breeding lines. We therefore repeated several of the previous assessments when the animals had reached early adulthood, including tests for emotional behaviour, stress-coping, stress reactivity and recovery, as well as gene expression measurements in relevant areas of the brain (Chapter 3).

Finally, we aimed to understand how the discovered consequences of ELS and the differences between the three mouse lines developed over time into late adulthood. Furthermore, as reduced cognitive function has been linked to adverse early-life experience and MDD, we also wanted to investigate if cognitive impairments emerged in any of the three SR mouse lines as a function of ELS. Therefore, we employed a battery of cognitive tests and evaluated the cognitive performance of ELS-exposed and standard-housed animals in early and late adulthood. In addition, we replicated previous findings regarding changes in the regulation of the HPA axis in early adulthood and were able to show that these consequences of ELS were long-lasting, but differed between the three mouse lines as a function of their genetic predisposition (Chapter 4).

CHAPTER 2:

Antidepressant treatment differentially affects the phenotype of high and low stress reactivity mice

2.1. Declaration of author contributions

All listed authors contributed to this manuscript: Alexandre Surget, Petra Van Nieuwenhuijzen, Jan Michael Heinzmann, Alana Knapman, Silja McIlwrick, Willy Paul Westphal, Chadi Touma, and Catherine Belzung.

The study was designed by Alexandre Surget, Chadi Touma, and Catherine Belzung. The acquisition of the data was accomplished by Petra Van Nieuwenhuijzen, Jan Michael Heinzmann, Alana Knapmann, and Willy Paul Westphal. Data analysis and statistics were done by Alexandre Surget and Silja McIlwrick. Alexandre Surget drafted the manuscript, which was revised by Catherine Belzung, Chadi Touma and Silja McIlwrick. All authors approved to all modifications and approval of the final version of the manuscript.

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Antidepressant treatment differentially affects the phenotype of high and low stress reactive mice

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ABSTRACT

Modelling key endophenotypes can be a powerful approach to gain insight into mechanisms underlying the aetiology and pathophysiology of neuropsychiatric disorders. Based on evidence of stress hormone system dysregulations in depression, the Stress Reactivity (SR) mouse model has been generated by a selective breeding approach for extremes in HPA axis reactivity, resulting in high (HR), intermediate (IR) and low (LR) reactive mice. The characterisation of their phenotypic alterations has highlighted many similarities of HR and LR mice with the melancholic and atypical depression, respectively. We therefore aimed to examine whether the antidepressant fluoxetine (10 mg/kg/day i.p., 4–5 weeks) can ameliorate the phenotypic characteristics of HR and LR mice in neuroendocrine functions (HPA axis basal activity, stress reactivity, negative feedback), emotional reactivity/coping-strategy (open field, forced swim tests), spatial learning/memory (Morris water-maze) and hippocampal neurogenesis. Line differences in HPA axis reactivity were maintained under fluoxetine treatment. However, we observed fluoxetine effects on glucocorticoid-induced negative feedback, stress-coping behaviours, cognitive functions and neurogenesis. Specifically, our results revealed line-dependent consequences of fluoxetine treatment: (1) an amelioration of the 'melancholic-like' features of HR mice (reversing the negative feedback resistance, the hyperactive coping style and the memory deficits; increasing hippocampal neurogenesis); (2) an exacerbation of the phenotypic deviations of LR mice (increasing their pronounced negative feedback and passive coping style). Thus, these findings support the predictive validity of antidepressant treatment in the HR mouse line and emphasize the translational value of the SR mouse model for the development of therapeutic strategies based on endophenotype-driven classifications.

1. Introduction

Animal models are essential to explore neural circuits and dynamic neurobiological processes that are not directly accessible from human subjects. However, the lack of knowledge on major depressive disorders (MDDs) limits the capacity to objectively assess the quality of the models and question their validity (van der Staay et al., 2009). Moreover, considering the multifactorial origins of depression along with its symptomatic variability and the subjective nature of some symptoms (Ostergaard et al., 2011), trying to recapitulate the full depressive syndrome in animals is certainly unrealistic. In order to overcome these limitations, an alternative approach focusing on endophenotypes has increasingly gained support (Hasler et al., 2004). Endophenotypes are assumed to represent more basic phenomena that are related either to a single component of the clinical symptomatology or a biological trait associated with MDDs. As more elementary phenotypes, they are supposed to involve fewer genes, to underlie more straightforward pathophysiological processes and to improve the translation from

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preclinical to clinical research.

Selective breeding is a valuable approach to isolate specific endophenotypes. It has recently been used to select CD-1 mice for extremes in hypothalamic-pituitary-adrenocortical (HPA) axis reactivity, thereby establishing the so-called 'stress reactivity' (SR) mouse model (Touma et al., 2008). Indeed, dysfunction of the stress hormone system is one of the most consistent physiological alterations reported in MDDs (de Kloet et al., 2005; Nemeroff et al., 1984; Stetler and Miller, 2011). In particular, HPA axis abnormalities are commonly observed in depressed patients and can be characterized by altered circadian activity, aberrant glucocorticoid (GC) release in response to stressors, and/or an impaired negative feedback (Holsboer, 2000; Holsboer and Ising, 2010). It is noteworthy that the directionality of HPA axis dysregulations depends on the MDD subtypes: hyperactivity and disturbed negative feedback are frequently found in melancholic depression, while hypoactivity and exaggerated negative feedback have been linked to atypical depression (Antonijevic, 2006; Gold and Chrousos, 2002). In the SR mouse model, three breeding lines with different levels of corticosterone (CORT) release in response to a psychological stressor (15 min restraint) have been established: the high (HR), intermediate (IR) and low (LR) reactivity mice. In addition to their neuroendocrine disturbances, HR and LR mice display a number of other phenotypic similarities with MDDs (Heinzmann et al., 2014; Knapman et al., 2010b; Touma et al., 2008; Touma et al., 2009). For instance, increased HPA axis activity and decreased glucocorticoid-induced negative feedback in the HR line are associated with lower body weight, disturbed sleep architecture, cognitive deficits, increased emotional reactivity and hyperactive stress-coping behaviour. Such impairments are reminiscent of many characteristics of the melancholic subtype of depression. In contrast, the LR mice recapitulate some features associated with atypical depression: exacerbated HPA axis negative feedback, higher body weight, reduced emotional reactivity and passive coping style. Altogether, these previous works enabled the identification of many phenotypic consequences of the genetic predisposition for extremes in HPA axis reactivity. However, the question whether the different neuroendocrine and behavioural phenotypes of HR and LR mice can be reversed by antidepressant treatments had not been addressed yet.

Accordingly, this study aimed to assess the effects of antidepressant treatments in the SR mouse model. We investigated whether the selective serotonin reuptake inhibitor (SSRI) fluoxetine can ameliorate the HPA axis disturbances and other important phenotypic characteristics (emotional reactivity, spatial learning/memory) observed in HR and LR mice. Neuroendocrine functions were assessed via three distinct measures of HPA axis activity: basal activity, stress responsiveness and glucocorticoid-induced negative feedback as assessed in the combined dexamethasone/corticotropin-releasing hormone (Dex/CRH) test. The examination of emotional reactivity was based on stress-coping behaviour in the Forced Swim test (FST) as well as anxiety-related behaviours and exploratory drives in the Open Field test. Hippocampus-dependent spatial learning and memory were evaluated in the Morris water-maze (MWM). Finally, since hippocampal neurogenesis has been found to be reduced in depression (Lucassen et al., 2010) and critically involved in antidepressant response and regulation of the HPA axis (Surget et al., 2011), we also examined cell proliferation (Ki67+) and levels of immature neurons (doublecortin-positive, DCX+) in the dentate gyrus (DG) of the hippocampal formation.

2. Methods

2.1. Animals and housing conditions

The study used adult male mice from generations XV and XVII of the SR mouse model, which consists on three independent mouse lines derived from the CD-1 mouse strain and selectively bred for extreme HPA axis reactivity (see details about this approach in Touma et al., 2008). The mice were housed in groups of four animals in transparent polycarbonate cages (38 × 22 × 15 cm) with wood chips as bedding and wood shavings as nesting material. At the age of about eight weeks, HPA axis responsiveness to a stressor was assessed by means of the stress reactivity test (SRT) described below (see 2.3). The mice used in the experiments described below were 3–5 months of age and single housed (cage 23 × 16 × 14 cm) at least ten days before performing the experiments in order to avoid potential dominance hierarchy effects. Housing and experimental rooms were kept under standard laboratory conditions (12/12 h light/dark cycle, lights on 8:00 h; temperature: 22 ± 1 °C; relative humidity: 55 ± 10%). Commercial mouse diet (Altromin GmbH, Lage, Germany) and tap water were available ad libitum. All conducted experiments were in accordance with the current regulations covering animal experimentation (European Communities Council Directive 86/609/EEC) and approved by the appropriate local authorities.

2.2. Experimental design

A first cohort of mice was used to investigate the effects of chronic fluoxetine treatment on HPA activity and emotional reactivity (Fig. 1). Briefly, HR, IR and LR lines received a daily treatment with fluoxetine or vehicle (0.9% NaCl) for a total of 5 weeks. Fluoxetine was administered i.p. and at the concentration of 10 mg/kg/day based on previous experiments (Surget et al., 2008; Yalcin et al., 2008). After 25 days of treatment, baseline plasma CORT level was assessed from blood samples ('initial sample'). After 28 days of treatment, emotional reactivity was examined in a combined behavioural test comprising a 10-min open field test and a 6-min FST, which was immediately followed by a blood sampling to assess HPA axis reactivity to the swim stress ('reaction sample'). After 32 days of treatment, another blood sampling was carried out to obtain baseline plasma CORT values ('untreated sample') for the combined Dex/CRH test. Finally, after 35 days of treatment, the combined Dex/CRH test was performed.

A second cohort of mice was used to investigate the effects of chronic fluoxetine on spatial memory and hippocampal neurogenesis (Fig. 5a). HR, IR and LR mice received a daily treatment with fluoxetine (10 mg/kg/day, i.p.) or vehicle (0.9% NaCl) for a total of 33 days. After 26 days of treatment, all mice were subjected to the MWM test, consisting of a 4-day training stage followed by a probe trial. Four days later, the brain was collected to assess hippocampal cell proliferation/neurogenesis by immunohistochemistry.

2.3. Stress reactivity test (SRT)

The SRT is described in detail elsewhere (Touma et al., 2008). Briefly, the test consists of a 15-min restraint period and two tail blood samplings, one immediately before and one right after the restraint stressor. The animals' plasma CORT increase in response to the SRT served as criterion for selecting the animals over generations for the respective experimental groups of the three mouse lines.

2.4. Blood sampling

Blood sampling was performed as described previously (Touma et al., 2008). Blood samples obtained from the animals' ventral tail vessel were collected in EDTA-coated tubes (Microvette, Sarstedt, Nürnbrecht, Germany). Trunk blood was collected in EDTA-coated tubes (KABE Labortechnik GmbH, Nürnbrecht-Elsenroth,

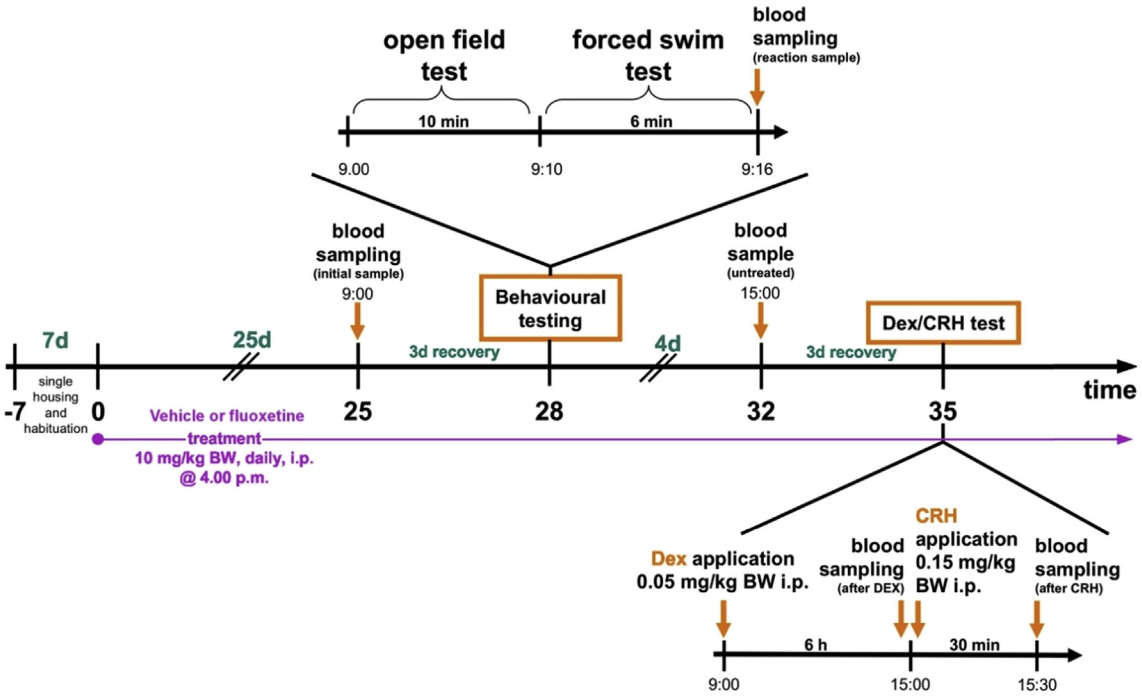

Fig. 1. Schematic representation of the experiment designed to assess chronic fluoxetine effects on emotional reactivity and HPA axis functions. Time scale is expressed in days. Abbreviations: BW, body weight; Dex, dexamethasone; CRH, corticotropin-releasing hormone.

Germany) equipped with 10 μl of the protease inhibitor Aprotinin (Bayer Vital GmbH, Leverkusen, Germany). All blood samples were immediately cooled on ice and centrifuged at 4000 g for 10 min at 4 °C. The plasma was transferred into fresh tubes (SafeSeal, Sarstedt, Nürnbrecht, Germany) and stored at −20 °C until further analysis.

2.5. Open field test

The Open Field test was performed to assess locomotor activity and anxiety-related behaviours in a novel environment. As previously described (Varadarajulu et al., 2011), each mouse was placed in the centre of a circular arena (60 cm in diameter, surrounded by 40 cm high walls) and tested for 5 min under dimly lit conditions (~15 lux). The total distance travelled and the time the animal spent in the inner zone (30 cm diameter) were measured. Total distance and avoidance of the inner zone are commonly used to assess locomotor activity and anxiety-related behaviours, respectively (Prut and Belzung, 2003).

2.6. Forced swim test

The FST was used to measure stress-coping behaviour. It involves placing each mouse for 6 min in an inescapable and aversive situation in a glass cylinder (12 cm diameter, 24 cm height) filled two-thirds with 23 °C warm water. After an initial period of vigorous activity ('swimming' and 'struggling') in an attempt to escape (swimming is characterized by relatively strong movements of the limbs and the tail, while 'struggling' also includes breaking the water surface with the front paws or trying to climb up the walls of the beaker), the animals intermittently adopt immobile postures ('floating') interspersed with bouts of swimming. The total time spent floating (defined as ceasing to move altogether, making only those movements necessary to keep the head above water) during the test was scored by a trained observer and has been proposed to reflect a state of despair or an alteration in coping strategy from active to passive.

2.7. Morris water-maze test

The Morris water maze (MWM) consisted of a circular pool (90 cm in diameter × 30 cm in height) filled with water (21 ± 1 °C) made opaque by addition of black polypropylene 4-mm granules at a height of 14 cm. The pool was virtually subdivided in four equal quadrants. Animals had to learn the location of a submerged platform (5 × 5 cm) placed in the centre of one quadrant, in order to escape the water. Several extra-maze cues (posters and objects) could serve to locate the platform. All mice were trained for 4 consecutive days and received 4 trials per day, each time randomly starting from a different start point (four in total) with an inter-trial interval of 6 min. If the mice were unable to find the platform within the 60 s trial, they were gently guided there by the experimenter. After 5 s on the platform, the mice were placed back into their homecage. Each trial was videotracked and analysed using Ethovision (Noldus, Netherlands). Twenty-four hours after the final training trial mice were tested in a 1-min probe trial to assess the spatial retention memory. During the probe trial the platform was removed from the pool and the time the animals spent in each quadrant was recorded.

2.8. The combined Dex/CRH test

The combined Dex/CRH test was performed as described previously (Heinzmann et al., 2014; Touma et al., 2011). Briefly, a reference blood sample was collected by an incision in the ventral tail vessel at 15:00, three days prior to the actual test ('untreated' value). On the experimental day, at 09:00, HR, IR and LR mice were injected ip with a relatively low dose of Dex (0.05 mg/kg). At 15:00, a second blood sample was drawn from the tail vessel ('after Dex' value), immediately followed by an injection of CRH (0.15 mg/kg,

ip). Thirty minutes later, the mice were sacrificed and trunk blood was collected ('after CRH' value). All blood samples were stored frozen at −20 °C until plasma CORT concentrations were analysed.

2.9. CORT radioimmunoassay

Plasma corticosterone (CORT) was measured as described before (Touma et al., 2008), using a commercially available CORT radioimmunoassay (RIA) kit (MP Biomedicals) following the manufacturer's protocol with slight modifications, i.e. using half of the recommended volume of all reagents. Ten microliters of plasma of each sample were applied to the assay and all samples were analysed in duplicate. Furthermore, the same pool samples (one in the upper and one in the lower concentration range) were run in every assay as an internal standard to control for intra- and inter-assay variations, which were both below 10% in this study. Radiolabelled samples were measured by a gamma counter (Wallac Wizard 1470 automatic gamma counter, Perkin Elmer life science, Rodgau, Germany). CORT concentrations were calculated by means of a seven point standard curve. Double estimations were accepted if the coefficient of variation was below 10%. The detection limit of the CORT RIA was 1 ng/ml plasma.

2.10. Immunohistochemistry

Immunohistochemistry was performed as previously described (Tanti et al., 2013). Mice were anesthetised with ketamine/xylazine (120 and 10 mg/kg, respectively) and transcardially perfused with saline for 2 min followed by 4% paraformaldehyde (PFA)/0.1 mol phosphate-buffered saline (PBS; pH = 7.4) for 5 min. Brains were collected and postfixed overnight in 4% PFA at 4 °C and then cryoprotected in a 20% sucrose solution for 48 h. Serial coronal sections through the hippocampus were cut (35 μm) and consecutive sections were placed sequentially in four vials containing freezing solution (Glycerol, Ethylene glycol and PB) and stored at −20 °C until processing. Sections were rinsed 3× in PB, followed by incubation in 3% H2O2 for 30 min. Sections were blocked in 1% normal Horse serum and incubated in DCX (1:500) for 36 h or Ki-67 (1:500) for 48 h. Following incubations, sections were rinsed in PB 3×, then incubated in the secondary anti-body, biotynilated anti-rat for DCX or anti-rabbit for ki-67 for 2 h. Sections were rinsed in PB (3×), incubated for 1hr in ABC −kit, washed (PB, 3×) and DAB was used for visualisation. Stained sections were mounted on gel coated slides, dehydrated in alcohol, cleared with claral and coverslipped. DCX and Ki-67 positive cells were quantified in every 4th section. The coordinates and boundaries used to dissociate the dorsal and ventral hippocampus were based on previous publications (Tanti et al., 2012): −1.06 mm to −2.06 mm and −3.08 mm to −3.80 mm from Bregma for dorsal and ventral hippocampus, respectively.

2.11. Statistics

Data were analysed with factorial or repeated-measures ANOVA using 'line', 'antidepressant treatment', 'Dex treatment', 'sampling time point' (in the combined Dex/CRH test) and/or 'training day' (in the MWM test) as factors where appropriate. Significant main effects or interactions were followed up with posthoc tests including the Bonferroni methods for multiple comparisons. A *t*-test was used for the probe trial of the MWM in order to compare observed distributions to a theoretical distribution and to compare fluoxetine effects within each line. A Pearson correlation coefficient was used to determine the correlation between performances in the MWM and neurogenesis or cell proliferation in the DG.

3. Results

3.1. Effects of chronic fluoxetine treatment on HPA axis function

Three different functions of the HPA axis were examined: basal CORT levels, stress responsiveness and HPA axis regulation as assessed in the combined Dex/CRH test.

After 25 days of chronic fluoxetine treatment, baseline CORT levels as assessed from blood samples collected at 09:00 ('initial sample', Fig. 1), revealed low values in all three mouse lines (Fig. 2a). It is noteworthy, however, that HR, IR and LR mice already differed significantly for this measure ($F_{44,2} = 11.518$, $p < 0.001$). Specifically, HR mice exhibited a significantly higher plasma CORT level than IR mice ($p = 0.003$), while the lowest concentration was found in LR animals (HR vs LR: $p < 0.001$, IR vs. LR: $p = 0.470$). These differences were independent of the fluoxetine treatment, i.e. no significant differences were found between vehicle- and fluoxetine-treated animals in any of the three mouse lines ($F_{44,1} = 1.049$, $p = 0.311$; Fig. 2a).

On day 28, another blood sampling was carried out immediately after the behavioural tests (10-min OF + 6-min FST) ('reaction sample', Fig. 1). The results demonstrated that the three lines displayed a robust increase of plasma CORT concentrations in response to the forced swim stressor (Fig. 2b). However, substantial differences in the rise of CORT levels were found between HR, IR and LR mice ($F_{44,2} = 206.660$, $p < 0.001$, post hoc tests: all $p < 0.001$). HPA axis reactivity was the highest in HR mice with a CORT increase clearly over 400 ng/ml. In contrast, the CORT rise in LR mice was lower than 100 ng/ml, while intermediate values were found in the IR line (mean increase: about 200 ng/ml). Chronic fluoxetine treatment did not affect these differences between the lines and did not change HPA axis responsiveness ($F_{44,1} = 0.277$, $p = 0.601$; Fig. 2b).

On day 32, blood samples were collected at 15:00 in order to obtain a reference value for the Dex/CRH test ('untreated', Fig. 1). The three lines, whether treated with fluoxetine or not, did not differ for this measure ($F_{43,2} = 1.884$, $p = 0.164$; Fig. 2c). On day 35, the combined Dex/CRH test was carried out. Dex was injected and plasma samples were collected six hours later, immediately before the CRH injection and 30 min afterwards. Overall, the test revealed a strong main effect of 'sampling time point' (i.e. 'untreated', 'after Dex' and 'after CRH') ($F_{86,2} = 676.881$, $p < 0.001$), an interaction of 'sampling time point' and 'line' ($F_{86,4} = 134.805$, $p < 0.001$), an interaction of 'sampling time point' and 'treatment' ($F_{86,4} = 4.338$, $p = 0.016$), as well as a strong trend for a three way interaction of 'sampling time point', 'line', and 'treatment' ($F_{86,4} = 2.369$, $p = 0.059$).

Posthoc analyses revealed that animals of all three lines showed a significant Dex-induced suppression of CORT levels ('untreated' vs. 'after Dex': $p < 0.001$ in HR, IR and LR mice). Interestingly, however, in the HR and the IR mouse line, the Dex-induced suppression of CORT was significantly weaker in the vehicle-compared to the fluoxetine-treated animals ('after Dex': HR vehicle vs fluoxetine: $p = 0.002$; IR vehicle vs fluoxetine: $p = 0.005$), showing that chronic fluoxetine treatment led to a stronger glucocorticoid-induced negative feedback in these animals. There was no difference in Dex-induced suppression between vehicle- and fluoxetine-treated animals in the LR line ($p = 0.951$), presumably due to floor effects. The 'after CRH' value, however, revealed that fluoxetine-treated LR mice had a dampened CRH-induced CORT secretion compared to vehicle-treated LR animals ($p = 0.004$), indicating a very strong Dex-induced suppression, which hampered the activation of the stress hormone secretion in fluoxetine-treated animals. Animals of the HR and IR line did not show a significant difference between vehicle and fluoxetine treatment in their 'after CRH' CORT levels ($p = 0.732$ and $p = 0.222$). The plasma samples

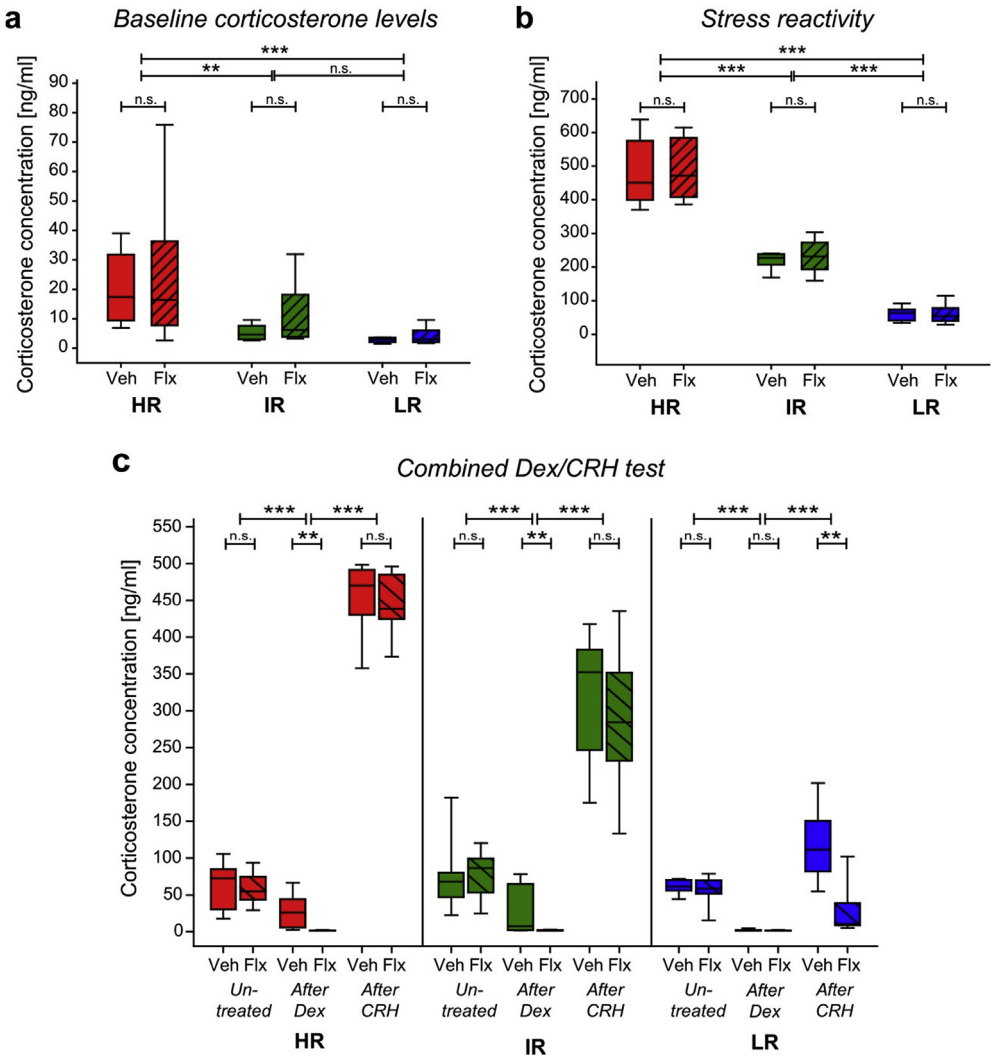

Fig. 2. Chronic fluoxetine effects on HPA axis activity and regulation in the SR mouse model. Effects on HPA axis functions were assessed through plasma corticosterone concentration for (a) basal HPA axis activity (treatment day 25; Flx, 10 mg/kg/day, ip), (b) stress reactivity measured immediately following behavioural testing (treatment day 28), and (c) in the combined Dex/CRH test (treatment day 35). Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes) as well as 10% and 90% percentiles (whiskers), n = 8–9 per group. Results of the posthoc group comparison are indicated above the appropriate boxes (n.s., $p > 0.1$; *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$). Abbreviations: CRH, corticotropin-releasing hormone; Dex: dexamethasone; Flx, fluoxetine-treated mice; HR, high reactive mice; IR, intermediate reactive mice; LR, low reactive mice; Veh, vehicle-treated mice.

collected after the injection of CRH showed a marked effect of line on CORT levels ($F_{43,2} = 163.364$, $p < 0.001$), reminiscent of the line effect seen in the stress reactivity measurements described above. Taken together, the results of the combined Dex/CRH test indicate an augmentation of the glucocorticoid-induce negative feedback of the HPA axis by chronic fluoxetine treatment in all three SR lines.

3.2. Effects of chronic fluoxetine treatment on emotional reactivity

The effects of fluoxetine on emotional behaviour were assessed in the Open Field test and the FST (Fig. 1). In the Open Field test, mice were allowed to freely explore an empty arena for 10 min. Mice typically tend to avoid bright and open spaces of unfamiliar environments, hence a decrease of the exploratory drive toward the inner zone of the Open Field is commonly used as an indicator for anxiety-related behaviours (Prut and Belzung, 2003). Overall, HR mice showed a slightly increased locomotor activity compared to LR animals ($F_{44,2} = 4.116$, $p = 0.023$; post hoc test: $p = 0.018$; Fig. 3a). However, in all three SR mouse lines, fluoxetine treatment did not change the total distance travelled by the animals during the test ($F_{44,1} = 1.313$, $p = 0.258$; Fig. 3a), nor the time they spent in the inner zone ($F_{44,1} = 0.769$, $p = 0.377$; Fig. 3b).

Immediately after the Open Field test, the mice were subjected to a 6 min FST (Fig. 1). In this test, animals are exposed to an aversive situation from which they cannot escape, restricting their behavioural response to two different coping alternatives: active (struggling and swimming) or passive (floating). The FST is commonly used to assess stress-coping behaviour and to screen antidepressant drugs (Cryan and Holmes, 2005). Our results revealed significant differences between the SR lines in their stress-coping behaviour (struggling time: $F_{44,2} = 9.214$, $p < 0.001$, Fig. 3c; floating time: $F_{44,2} = 13.984$, $p < 0.001$, Fig. 3d). HR mice spent clearly more time struggling compared with both IR ($p = 0.010$) and LR mice ($p < 0.001$). No difference in struggling time was found between IR and LR mice ($p = 0.482$). Consistent with this result, the time spent floating was markedly lower in HR mice compared with both IR ($p = 0.001$) and LR mice ($p < 0.001$), while the latter two lines again did not differ significantly for this measure ($p = 0.286$). Strikingly, the data showed a main effect of fluoxetine treatment on both struggling time ($F_{44,1} = 8.251$, $p = 0.006$) and floating time

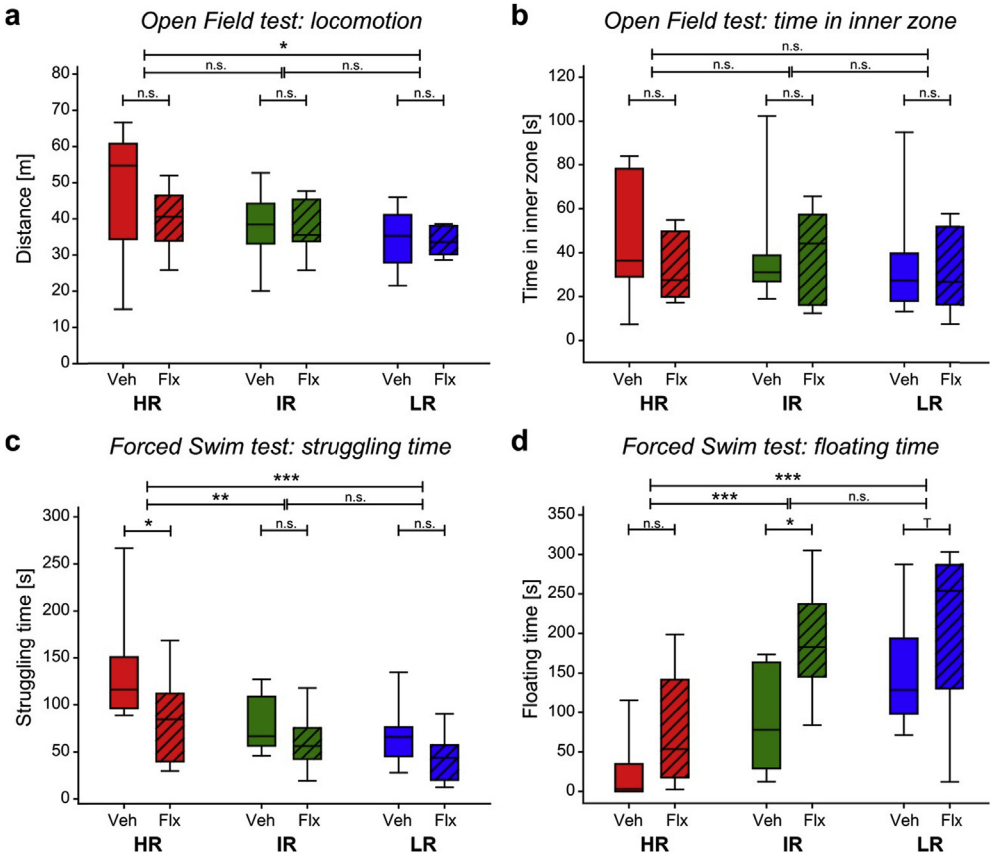

Fig. 3. Chronic fluoxetine effects on emotional reactivity and stress-coping behaviour in the SR mouse model. The figure illustrates the results obtained from the Open Field test (10 min) and the Forced Swim test (6 min) (treatment day 28; Flx, 10 mg/kg/day, ip): (a) the total distance travelled during the Open Field test; (b) the time spent exploring the inner zone of the Open Field; (c) the time spent floating and (d) the time spent struggling during the Forced Swim test. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes) as well as 10% and 90% percentiles (whiskers), n = 8–9 per group. Results of the posthoc group comparison are indicated above the appropriate boxes (n.s., $p > 0.1$; T, $p < 0.1$; *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$). Abbreviations: Flx, fluoxetine-treated mice; HR, high reactive mice; IR, intermediate reactive mice; LR, low reactive mice; Veh, vehicle-treated mice.

($F_{44,1} = 12.316$, $p = 0.001$). Posthoc within-line comparisons revealed that the chronic fluoxetine treatment significantly decreased struggling time in the HR line ($p = 0.011$; Fig. 3c), and increased floating time in IR and LR animals ($p = 0.008$ and $p = 0.092$; Fig. 3d).

3.3. Effects of chronic fluoxetine treatment on spatial learning and memory

A significant decrease of the distance to reach the escape platform was evidenced along the training period in all three lines (training effect HR: $F_{3,30} = 11.11$, $p < 0.001$; IR: $F_{3,30} = 24.62$, $p < 0.001$; LR: $F_{3,24} = 11.12$, $p < 0.001$). However, differences appeared when looking at the different treatment groups within each line, with a significant effect of treatment found in the HR line only ($F_{1,30} = 26.01$, $p < 0.01$). Vehicle-treated mice from the IR and LR lines displayed a significant decrease of the distance to escape between day 1 and day 4, indicative of an effective learning (Fig. 4b). Vehicle-treated mice from the HR line, however, were unable to significantly reduce this distance during the same timeframe, suggestive of learning deficits in the HR line. On the other hand, all 3 lines significantly decreased the distance to reach the platform when treated with fluoxetine. Posthoc comparisons confirmed the memory-enhancing effects of fluoxetine in HR mice, with a significant reduction of the distance swum to reach the platform on day 4 compared with vehicle-treated HR mice ($p < 0.05$, Fig. 4b). No significant differences due to chronic fluoxetine treatment were observed in IR and LR mice.

Subsequent to the 4-day training period, a probe trial was carried out. The percentage of time spent in the target quadrant (where the platform was located during the training) was computed in order to evaluate spatial reference memory. The effects observed during the learning phase were corroborated by the results in the probe trial. All experimental groups, except for the HR vehicle-treated mice, spent a significantly higher than chance percentage of time in the target quadrant (>25%) (Fig. 4c). Once again, the memory-enhancing effects of fluoxetine in the HR line were evidenced in the within-line comparison, with a significant augmentation of the time spent in the target quadrant compared with vehicle-treated HR mice. No fluoxetine effects were observed in IR and LR mice.

3.4. Effects of chronic fluoxetine treatment on hippocampal neurogenesis

Following the MWM, fluoxetine treatment was continued for another 4 days before brain collection. The density of proliferative Ki67-expressing cells and immature DCX-expressing neurons were assessed in the dorsal and ventral DG (dDG and vDG) of the hippocampus.

The examination of DG cell proliferation revealed significant differences between the three SR lines (Fig. 5a) in both the dDG

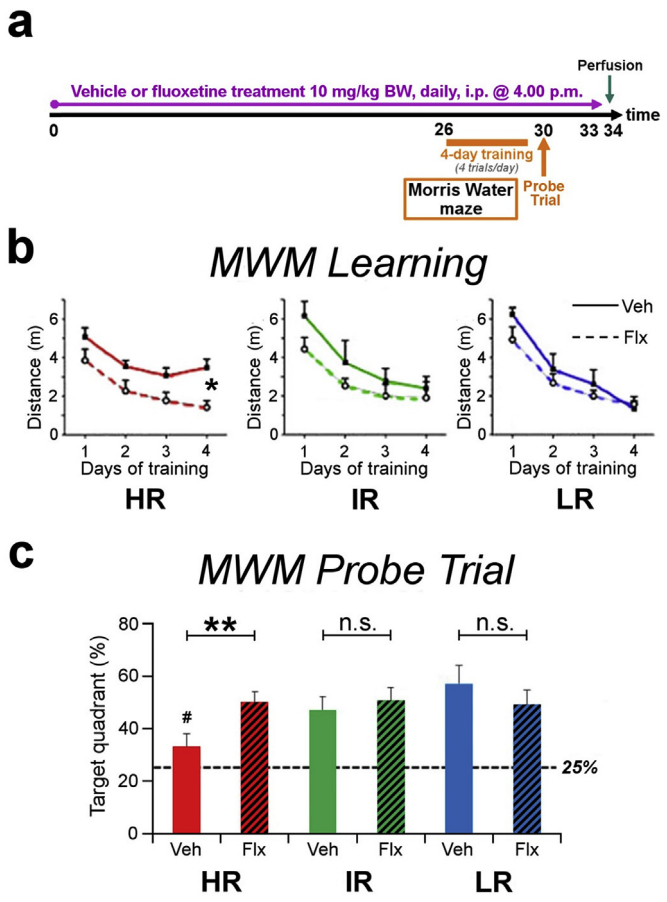

Fig. 4. Chronic fluoxetine effects on the spatial learning and memory in the SR mouse model. (a) Schematic representation of the experiment. The time scale is expressed in days. (b) MWM learning: represents the total distance to reach the escape platform each day during the 4-day training stage, n = 5–6 per group. *p < 0.05, Veh-vs Flx-treated mice. (c) MWM Probe trial: represents the percentage of time spent in the target quadrant (where the platform was located during training). Data are given as mean + S.E.M., n = 5–6 per group. Results of the posthoc group comparison are indicated: n.s. p > 0.1, **p < 0.05, **p < 0.01, Veh-vs Flx-treated mice. # indicates the experimental group with a time spent in target quadrant that was *not* significantly different from chance (25% dashed line). Abbreviations: BW, body weight; Flx, fluoxetine-treated mice; HR, high reactive mice; IR, intermediate reactive mice; LR, low reactive mice; MWM, Morris Water maze; Veh, vehicle-treated mice.

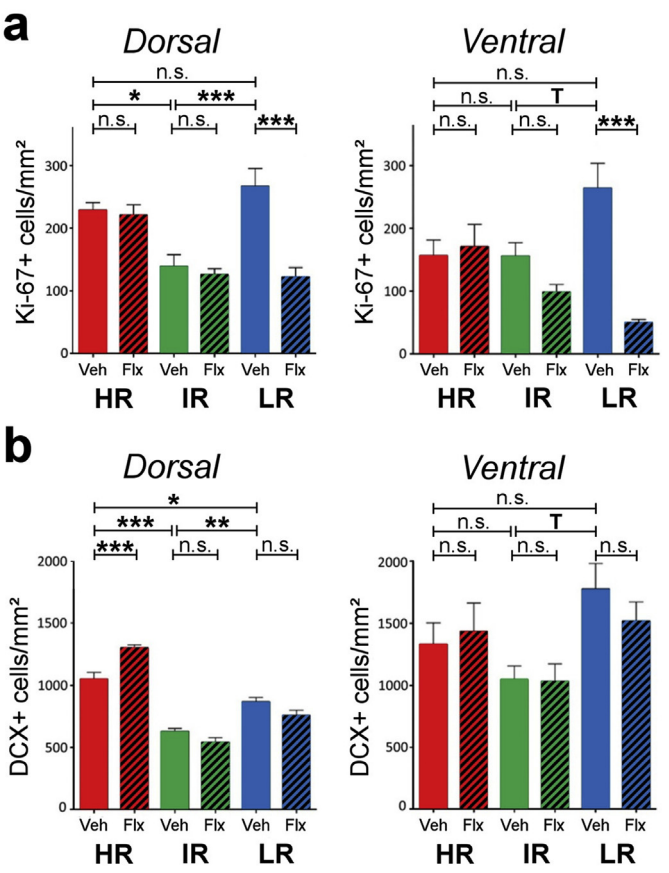

Fig. 5. Chronic fluoxetine effects on cell proliferation and neurogenesis in the dentate gyrus in the SR mouse model. (a) Density of Ki67-labelled proliferative cells in the granular layer of the dentate gyrus in the dorsal and ventral hippocampus. (b) Density of doublecortin-labelled immature neurons in the granular layer of the dentate gyrus in the dorsal and ventral hippocampus. Data are given as mean + S.E.M., n = 4–5 per group. Results of the posthoc group comparison are indicated above the appropriate bars (n.s., p > 0.1; T, p < 0.1; *p < 0.05, **p < 0.01; ***p < 0.001). Abbreviations: DCX, doublecortin; Flx, fluoxetine-treated mice; HR, high reactive mice; IR, intermediate reactive mice; LR, low reactive mice; Veh, vehicle-treated mice.

(line effects $F_{2,22} = 16.25$, $p < 0.001$; treatment effects $F_{1,22} = 15.72$, $p < 0.001$; line x treatment $F_{2,22} = 9.49$, $p < 0.01$) and the vDG (treatment effects $F_{1,22} = 16.39$, $p < 0.001$; line x treatment $F_{2,22} = 9.56$, $p < 0.01$). HR and LR mice exhibited a significantly higher density of Ki67 + cells than IR mice in the dDG. In addition to the dDG, LR mice also displayed a trend for higher density of Ki67 + cells in the vDG. Strikingly, chronic fluoxetine treatment was able to reverse these effects in the LR line and reduced cell proliferation of LR mice in both dDG and vDG. No fluoxetine effects were found in the 2 other lines.

Significant differences in the density of immature DCX-expressing neurons (Fig. 5b) were observed in both the dDG (line effects $F_{2,23} = 135.66$, $p < 0.001$; line x treatment $F_{2,23} = 15.43$, $p < 0.001$) and the vDG (line effects $F_{2,23} = 5.94$, $p < 0.01$). An increased density of DCX + neurons in the dDG was found in HR mice compared with IR mice. Interestingly, chronic fluoxetine treatment in HR mice was able to further increase this density in the dDG. By contrast, the density of DCX + cells in the dDG did differ between LR and IR mice but no significant fluoxetine effects were found in these lines. In the vDG, we found a trend for higher densities of DCX + cells in LR but not in HR mice, compared with IR mice. No significant fluoxetine effects on DCX + cell density were found in the vDG. Finally, a significant correlation was observed between the memory performance in the MWM and the density of DCX + neurons in the dDG in HR mice (but not in the vDG or with Ki67 + cell density). In IR and LR mice, the density of Ki67 + cells or DCX + neurons in the dDG or vDG was not found to correlate with memory performance in the MWM (Suppl. Tab. 1).

4. Discussion

Our results confirmed several phenotypic alterations previously found in the SR mouse model on neuroendocrine functions, stress-coping strategies and spatial memory. Remarkably, we identified distinct phenotypic consequences of a chronic antidepressant treatment with fluoxetine in HR and LR mice (Fig. 6). More precisely, HR mice exhibited a disturbed HPA axis negative feedback in the Dex/CRH test, a hyperactive coping style in the FST and cognitive impairments in the MWM, mirroring some hallmarks of the melancholic subtype of MDDs. Chronic fluoxetine treatment improved all of these disturbances in addition to increasing neurogenesis in the dorsal hippocampus. By contrast, instead of reversing the 'atypical' features of LR mice, fluoxetine treatment tended to exacerbate their phenotypic deviation, i.e. amplifying the already pronounced HPA axis negative feedback and enhancing the

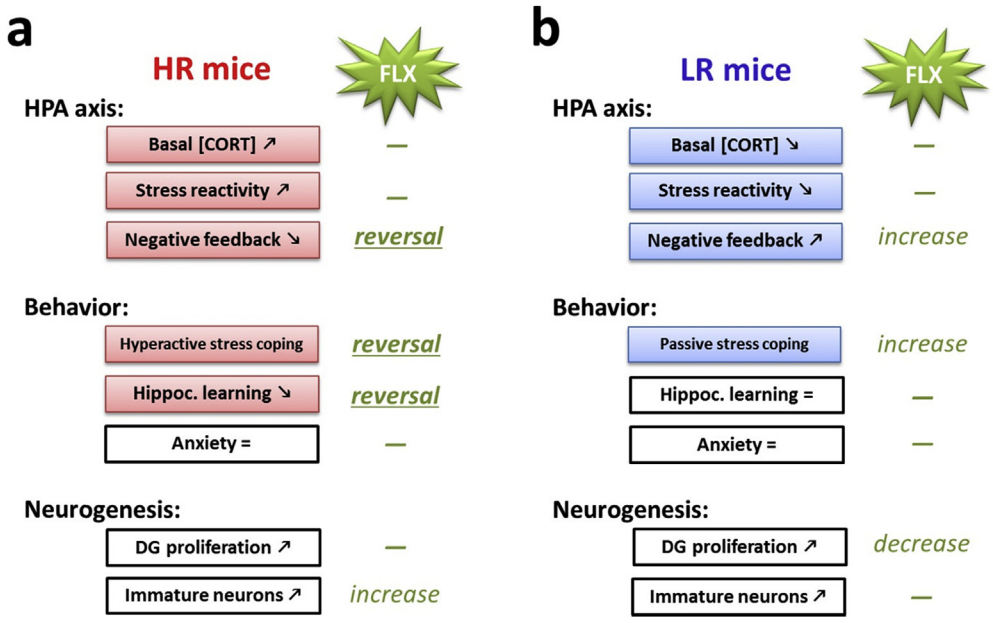

Fig. 6. Summary of the phenotypic characteristics observed in HR and LR mice (relative to IR) and the effects induced by chronic fluoxetine treatment. The results obtained in (a) HR and (b) LR mice recapitulated some symptomatic features of melancholic (red-filled boxes) and atypical (blue-filled boxes) depression, respectively. Chronic fluoxetine treatment (10 mg/kg/day, i.p. for 4–5 weeks) ameliorated the melancholic-like profile of HR mice but exacerbated phenotypic alterations of LR mice. The phenotypic characteristics described in each box correspond to the results of vehicle-treated mice of the HR and LR lines compared to vehicle-treated IR mice. Effects of chronic fluoxetine treatment are indicated in green at the left of each box and correspond to the within-line comparison of fluoxetine-treated mice versus vehicle-treated mice. A bar (–) indicates a lack of fluoxetine effects on the phenotype. Abbreviations: CORT, corticosterone; DG, dentate gyrus; FLX, fluoxetine-treated mice; HR mice, high reactive mice; IR mice, intermediate reactive mice; LR mice, low reactive mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

passive coping behaviour in the FST.

4.1. Fluoxetine treatment did not affect baseline activity and stress responsiveness of the HPA axis

HPA axis dysregulation is one of the most consistent biological endophenotypes reported in MDDs and inspired the development of the SR mouse model (Touma et al., 2008). We therefore aimed to assess whether a chronic antidepressant treatment could affect three different functions of the HPA axis: CORT basal levels, reactivity to stressors and feedback inhibition.

Basal CORT measurements revealed only minor line differences and no additional effect of fluoxetine treatment. No difference was observed between IR and LR mice while HR mice displayed significantly higher basal CORT concentrations at 9:00 (Fig. 2a). This effect was not observed in samples collected at 15:00, i.e. in the 'untreated sample' of the Dex/CRH test (Fig. 2c), which is likely due to the circadian variation of CORT secretion as previously described (Touma et al., 2008). On the other hand, our results confirmed clear line differences in HPA axis reactivity between HR, IR and LR mice (Fig. 2b). These differences were present in the vehicle-treated animals and were maintained also under fluoxetine treatment. Because extremes in HPA axis reactivity served as the selection criterion used in the selective breeding approach to establish the SR mouse lines, such robust differences in stress reactivity were expected. As described previously (Touma et al., 2008), the response to selection in the SR mouse model was rapid (i.e. from the first breeding generation), strong and stable over generations, indicating a pronounced genetic basis for this endophenotype. This finding is also in line with selection studies on CORT secretion in other species (Evans et al., 2006; Pottinger and Carrick, 1999; Satterlee and Johnson, 1988). Accordingly, it could be expected that such a phenotype with a strong genetic basis would be hard to affect by a relatively short pharmacological treatment with SSRIs, such as fluoxetine, that do not directly target the HPA axis.

4.2. Fluoxetine treatment improved the HPA axis negative feedback

We also assessed the glucocorticoid-induced negative feedback of the HPA axis, whose dysfunction represents one of the most consistent alterations of the HPA axis in MDD (Holsboer and Ising, 2010). This was tested in the combined Dex/CRH test, a potent tool to detect dysregulations of the HPA axis, including negative feedback mechanisms (Ising et al., 2007). Dex, acting as a glucocorticoid receptor (GR) agonist, has a suppressive effect on HPA axis activity, setting the inhibitory feedback loop into action. In contrast, CRH, the principal ACTH secretagogue, elicits HPA axis activation and ultimately the release of CORT from the adrenals. The prior action of Dex could potentially counterbalance the CRH effects in the Dex/CRH test and limit the CORT release, depending on the strength of the feedback inhibition. Accordingly, both measures 'after Dex' and 'after CRH' provide a dual readout reflecting the magnitude of the negative feedback. Our findings demonstrated that the three SR mouse lines significantly differ in this test. HR mice exhibited a diminished negative feedback with a reduced DEX-induced CORT suppression and a pronounced CRH-induced CORT rise (see also Heinzmann et al., 2014). These features are reminiscent of the melancholic subtype of depression, whose patients display HPA feedback resistance (Gold and Chrousos, 2002). Interestingly, the feedback resistance of HR mice was counteracted by fluoxetine, as shown by the reestablishment of a robust Dex-induced CORT suppression. This result is particularly relevant for MDDs since the normalization of HPA axis negative feedback by antidepressants has been shown to precede or parallel successful remissions in patients and animal models (Ising et al., 2007; Law et al., 2016; Surget et al., 2011). Evidence from preclinical and clinical studies suggests that this normalization by antidepressants involves alterations of GR functions and it is possible that similar changes

occurred in HR mice. Indeed, GR activation in various brain areas regulates HPA axis activity (Ulrich-Lai and Herman, 2009), and antidepressants enhance GR expression and function (Okugawa et al., 1999; Pariante et al., 1997). The contribution of alternative mechanisms has also been suggested, involving for instance the modulation of blood-brain barrier (BBB) permeability. Indeed, steroid transporters, such as the multidrug resistance 1 P-glycoprotein (Mdr1-Pgp) strongly regulate the accessibility of glucocorticoids to cells of the central nervous system (CNS). Remarkably, antidepressants can inhibit steroid transporters, resulting in facilitated glucocorticoid penetration into the brain and a potential mechanism for HPA axis feedback enhancement (Mason et al., 2008; Pariante et al., 2003).

Mechanisms that promote GR functions and glucocorticoid access to the brain are therefore assumed to strengthen the feedback inhibition on the HPA axis (Anacker et al., 2011a). However, in contrast to HR mice, LR mice exhibit an increased sensitivity to glucocorticoids. It is therefore unclear if and how the potential antidepressant mechanisms described above would be beneficial to LR mice and could reverse an exaggerated HPA axis negative feedback. Actually, our results indicate that fluoxetine appears to enhance the negative feedback in all three SR lines, including in the LR mice. The pronounced negative feedback of LR mice (already maximal 'after Dex') was not alleviated but exacerbated by fluoxetine treatment (as revealed by the sustained CORT suppression 'after CRH' stimulation). Our results can be compared with clinical conditions where similar antagonistic HPA dysregulations occur in melancholic vs. atypical depression (Antonijevic, 2006; Gold and Chrousos, 2002). Interestingly, these two MDD subtypes also exhibit differences in antidepressant responses. Symptoms in the melancholic subtype appear to be treated more successfully by SSRIs and tricyclic antidepressants (TCAs), while atypical depression is more likely to respond to monoamine oxidase inhibitors (MAOIs) (Baghai et al., 2008; Hirschfeld, 1999; Thase et al., 1995). These actions are associated with contrasting effects on the HPA axis. SSRIs and TCAs reduce HPA axis activity and improve negative feedback essentially by increasing GR expression in HPA axis feedback-related forebrain regions (Heydendael and Jacobson, 2008; Mukherjee et al., 2004). By contrast, MAOIs tend to decrease GR expression and facilitate noradrenergic stimulation from the *locus coeruleus* to brain nuclei activating the HPA axis, thereby dampening the inhibitory feedback and promoting HPA activation (Heydendael and Jacobson, 2009; Ziegler et al., 1999). Altogether, these results raise the possibility that different classes of antidepressants may have distinct effects on HPA axis activity and distinct effectiveness to treat different MDD subtypes. Such a scenario might also apply to the SR mouse model, with fluoxetine treatment being appropriate to improve a reduced HPA axis negative feedback, as previously shown in other depression models (Surget et al., 2011). Overall, these studies suggest there would be a considerable interest to stratify patients and determine therapeutic strategies according to their endophenotypes, particularly HPA axis related-functions. In this context, the SR mouse model may represent a valuable tool for the identification and development of novel therapeutic targets designed for specific endophenotypes and patient groups.

4.3. Fluoxetine treatment altered stress-coping behaviours in the FST

Our results did not show any significant line- or fluoxetine-effects on anxiety-related behaviours in the Open Field test but revealed that the three SR mouse lines adopted markedly different coping behaviours when exposed to the more stressful forced swimming situation. In the Open Field test, the absence of line differences for anxiety-related measures (Fig. 3b) and the slight increase in locomotor activity of HR mice (Fig. 3a) are consistent with previous findings in the SR mouse model (Heinzmann et al., 2014; McIlwrick et al., 2016; Touma et al., 2008). The lack of fluoxetine effects was also expected because such antidepressant treatments have been shown to have very little, if any, effects in this test, particularly on anxiety-related behaviours (for review see Prut and Belzung, 2003). In the FST, HR mice engaged in a hyperactive stress-coping strategy encompassing a pronounced agitated behaviour (struggling) and hardly any immobility (floating), while LR mice expressed more passive stress-coping behaviours with high levels of immobility, which is generally interpreted as 'behavioural despair' (Cryan and Mombereau, 2004). The distinct behavioural phenotypes of HR and LR mice in the FST were already reported in earlier generations of the SR mouse models (Knapman et al., 2010a; Touma et al., 2008). It has been proposed that they mimic different stress-coping strategies often seen in melancholic vs. atypical depression, similar to signs of restlessness and agitation vs. signs of retreat and apathy, respectively (Touma, 2011). Strikingly, chronic fluoxetine treatment significantly altered the animals' stress-coping behaviour in the FST. It promoted the development of more passive strategies in all three SR lines, *i.e.* reducing the hyperactive coping behaviour of HR mice and increasing the floating time in IR and LR mice. Interestingly, fluoxetine effects in the FST thoroughly paralleled those in the Dex/CRH test: fluoxetine enhancing both HPA axis negative feedback and passive stress-coping behaviours in the three lines. These results are rather consistent with other models targeting genes involved in HPA axis regulation. For instance, CRH overexpression or GR depletion in the CNS led to HPA axis hyperactivity along with reduced immobility in the FST reminiscent of the phenotypes observed in HR mice (Lu et al., 2008; Tronche et al., 1999). Together with these previous findings, our study reveals that conditions leading to long-lasting HPA axis overreactivity are associated with hyperactive/agitated stress-coping behaviours, and that both HPA axis overreactivity and hyperactive stress-coping strategies can be alleviated by chronic fluoxetine treatments.

4.4. Fluoxetine treatment reversed cognitive deficits in HR mice

There is solid evidence of an inverted-U-shaped relationship between HPA axis activity and explicit learning/memory, suggesting that long-lasting HPA axis abnormalities are linked to cognitive deficits in MDD patients, particularly in those MDD subtypes presenting adrenocortical hyperactivation, such as psychotic and melancholic depression (Lupien and McEwen, 1997; Rimmele et al., 2013). The SR mouse model is therefore a valuable tool to explore relationships between depression-related phenotypes, HPA axis dysfunctions and cognitive deficits. While IR and LR mice performed well in the MWM test, HR mice were unable to significantly improve their performance during the four days of training and showed deficits to remember the target location in the probe trial, indicating spatial learning and memory deficits. Strikingly, the chronic fluoxetine treatment led to an enhanced performance of HR mice in the MWM, without affecting IR and LR animals. These results are paralleled by previous findings demonstrating cognitive deficits of HR mice in other behavioural paradigms (Y-maze, reversal learning, novel object recognition test) exploring executive functions and hippocampus- and prefrontal cortex-dependent learning and memory (Knapman et al., 2010a, 2010b; 2012). Interestingly, cognitive impairments have also been documented in depressed patients, encompassing deficits in learning, memory and executive functions dependent on brain regions such as the hippocampus and prefrontal cortex (Austin et al., 2001). Moreover, these deficits appear to be related to the presence of an aberrant

HPA axis activity/regulation (Reppermund et al., 2007). Although differences in CORT release may contribute to the contrasting test performance observed between the SR mouse lines or treatment groups, it is believed that more persistent cognitive deficits (as seen in MDDs) depend on profound neurobiological and plastic changes in critical brain areas such as the hippocampus (Pittenger and Duman, 2008). Consistent with this view, reduced neuronal integrity and decreased brain-derived neurotrophic factor (BDNF) levels have been found in the hippocampus of HR mice (Knapman et al., 2010a, 2012). In addition, spine density in the CA1 hippocampal field has been shown to be lower in HR mice compared to LR mice (Pillai et al., 2012).

4.5. Fluoxetine treatment enhanced hippocampal neurogenesis in HR mice

Hippocampal neurogenesis is another neurobiological process suggested to be involved in MDDs, since studies highlighted its pivotal role in: (1) behavioural effects of antidepressants (Santarelli et al., 2003), (2) HPA axis negative feedback (Snyder et al., 2011; Surget et al., 2011) and (3) hippocampus-dependent memory (Dupret et al., 2008; Snyder et al., 2005). These three functions were all altered by the fluoxetine treatment in our study pointing toward hippocampal neurogenesis as a critical factor for the alterations observed at least in HR mice after fluoxetine treatment. However, a complex picture emerged from our cell proliferation/neurogenesis results. HR and LR mice displayed higher densities of Ki67 + cells in the dorsal DG than IR mice, an effect entirely counteracted by chronic fluoxetine treatment in LR animals. Because Ki67 immunohistochemistry labels undifferentiated proliferative cells only, we also assessed the density of DG cells expressing DCX, a marker specific of newborn immature neurons and hence a more accurate method/tool to estimate adult neurogenesis. Again, both HR and LR mice displayed higher densities of immature neurons in the dorsal hippocampus compared to IR mice. Interestingly, however, fluoxetine treatment did markedly increase the density of DCX + neurons in the dorsal hippocampus only in HR mice. A large body of evidence highlighted a negative correlation between CORT levels and hippocampal neurogenesis (Cameron and Gould, 1994), which supports the results obtained in the LR mice but makes the relatively high level of immature neurons in HR mice somewhat unexpected. This relationship, however, is far from being linear. For instance, GR activation and circadian variations of glucocorticoids are necessary for the neurogenic effects of antidepressants (Anacker et al., 2011b; Huang and Herbert, 2006). Only prolonged high CORT levels by exogenous administration or repeated CORT upsurges induced by chronic stress procedures led to consistent decreases of DG cell proliferation and neurogenesis (Cameron and Gould, 1994; David et al., 2009; Surget et al., 2011). Apart from a slightly higher diurnal CORT trough, HR mice exhibit a relatively normal range of daily CORT levels under normal, non-stressed conditions (Touma et al., 2009), which is probably sufficient to maintain neurogenesis rates equal or higher than in IR mice. However, considering their HPA axis hyperreactivity to stressors, HR mice are expected to be particularly vulnerable to chronic stress conditions and it would be interesting to determine whether hippocampal neurogenesis of HR mice is especially impacted under such conditions, as observed in other mouse models of depression (Surget et al., 2011; Van Bokhoven et al., 2011). Moreover, the neurogenesis-promoting effects of fluoxetine in HR mice are in line with results obtained in other animal models of depression (David et al., 2009; Surget et al., 2011). Interestingly, the antidepressant-induced increase of hippocampal neurogenesis appears to be necessary to reverse HPA axis abnormalities and behavioural alterations in these models. Whether a proneurogenic action is required for the antidepressant effects of fluoxetine in HR mice is hard to determine at this point; we can however speculate that hippocampal neurogenesis may at least contribute to the memory-enhancing effects of fluoxetine in HR mice, as suggested by our results and neurogenesis correlations with MWM performance.

4.6. Conclusion

In summary, our study is the first demonstrating phenotypic alterations induced by chronic antidepressant treatments in the SR mouse model, affecting neuroendocrine functions, stress-coping behaviour, spatial learning and memory, and hippocampal neurogenesis. Remarkably, fluoxetine enhanced both HPA axis negative feedback and passive stress-coping behaviours in all three SR lines, resulting in an amelioration of the 'melancholic-like' features of HR mice and an exacerbation of the phenotypic deviations of LR mice. Thus, our findings underscore the predictive validity of chronic antidepressant treatments in the HR line and suggest that the SR mouse model represents a valuable tool for the identification of novel therapeutic targets and drug development. Future studies will have to determine whether other classes of antidepressant drugs (e.g. MAOIs) can improve the phenotype of LR mice, which would further support the translational value of the SR mouse model to investigate therapeutic strategies in melancholic vs. atypical depression subtypes, and thus promote the modern concept of endophenotype-driven classification and personalized medicine in biological psychiatry.

Conflicts of interest

The authors report no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.07.007.

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SUPPLEMENTAL INFORMATION

Antidepressant treatment differentially affects the phenotype of high and low stress reactive mice

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Supplemental Table 1. Correlation matrix between the percentages of time spent in the target quadrant during the MWM probe trial and the densities of Ki67+ or DCX+ cells in the dorsal and ventral dentate gyrus.

	Target quadrants (%)		
	HR	**IR**	**LR**
dKi67+	-0.2687	0.2041	0.5377
vKi67+	-0.0079	-0.0174	0.0295
dDCX+	0.7977**	0.3315	0.5342
vDCX+	0.1991	0.1282	0.0292

Values represent Pearson correlation coefficients. ** indicates a significant correlation at $p < 0.01$ (n=9-10 per group). Ki67+ and DCX+ cells in the dorsal hippocampus are indicated by dKi67+ and dDCX+. Ki67+ and DCX+ cells in the ventral hippocampus are indicated by vKi67+ and vDCX+.

CHAPTER 3:

Genetic predisposition for high stress reactivity amplifies effects of early-life adversity

3.1. Declaration of author contributions

All listed authors contributed to this manuscript: Silja McIlwrick, Alexandra Rechenberg, Mariana Matthes, Jessica Burgstaller, Thomas Schwarzbauer, Alon Chen, and Chadi Touma.

The study was designed by Silja McIlwrick and Chadi Touma. Silja McIlwrick performed the experiments and prepared the manuscript. Alexandra Rechenberg assisted with the sample preparation for qPCR. Mariana Matthes assisted with the USV experiment. Jessica Burgstaller assisted with the analysis of the maternal behaviour and USV recordings. Thomas Schwarzbauer assisted with the recording of maternal behaviour. Chadi Touma edited the manuscript and supervised the study. Alon Chen gave advice regarding the format of the manuscript and data presentation. All authors approved to all modifications and approval of the final version of the manuscript.

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Genetic predisposition for high stress reactivity amplifies effects of early-life adversity

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ABSTRACT

A dysregulation of the hypothalamus-pituitary-adrenocortical (HPA) axis and the experience of early-life adversity are both well-established risk factors for the development of affective disorders, such as major depression. However, little is known about the interaction of these two factors in shaping endophenotypes of the disease. Here, we studied the gene-environment interaction of a genetic predisposition for HPA axis dysregulation with early-life stress (ELS), assessing the short-, as well as the long-lasting consequences on emotional behavior, neuroendocrine functions and gene expression profiles. Three mouse lines, selectively bred for either high (HR), intermediate (IR), or low (LR) HPA axis reactivity, were exposed to one week of ELS using the limited nesting and bedding material paradigm. Measurements collected during or shortly after the ELS period showed that, regardless of genetic background, ELS exposure led to impaired weight gain and altered the animals' coping behavior under stressful conditions. However, only HR mice additionally showed significant changes in neuroendocrine stress responsiveness at a young age. Accordingly, adult HR mice also showed lasting consequences of ELS, including hyperactive stress-coping, HPA axis hyperreactivity, and gene expression changes in the *Crh* system, as well as downregulation of *Fkbp5* in relevant brain regions. We suggest that the genetic predisposition for high stress reactivity interacts with ELS exposure by disturbing the suppression of corticosterone release during a critical period of brain development, thus exerting lasting programming effects on the HPA axis, presumably via epigenetic mechanisms. In concert, these changes lead to the emergence of important endophenotypes associated with affective disorders.

1. Introduction

Major Depressive Disorder (MDD) is one of the most prevalent and costly psychiatric disorders (Ferrari et al., 2013; Greenberg et al., 2015). Among the large number of patients diagnosed with MDD, there exist smaller clinical subgroups, which can be distinguished by their opposing vegetative symptoms (for example: motor agitation or retardation, insomnia or hypersomnia, weight loss or gain) (Antonijevic, 2006; Gold, 2014; Gold and Chrousos, 1999, 2002; Lamers et al., 2012). In addition, patients suffering from these different subtypes of MDD also show opposite symptoms regarding the function of their hypothalamus-pituitary-adrenocortical (HPA) axis, one of the main neuroendocrine systems controlling the body's stress response (Joëls and Baram, 2009; Nemeroff, 1996; Sapolsky et al., 1984). Specifically, the atypical depression subtype is associated with blunted cortisol release in response to stressors, while patients with the psychotic or melancholic depression subtype show stress hyper-reactivity with extreme cortisol release and a flattened profile in their diurnal glucocorticoid secretion (Antonijevic, 2006; Gold and Chrousos, 1999, 2002). Thus, due to its central role in the systemic regulation of the stress response, a dysfunctional HPA axis may be a critical factor in the etiology of both depression subtypes (Gold, 2014; Holsboer, 1999; Lamers et al., 2012). However, a genetic predisposition for high or low HPA axis reactivity alone is probably not sufficient to cause a psychiatric disorder; severe negative experiences or other stressful environmental factors are thought to play an equally important role (Provençal and Binder, 2015). Until today, it remains poorly understood how a genetic predisposition for HPA axis dysregulation and environmental stressors interact, and what the short- and long-term consequences are.

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The Stress Reactivity (SR) mouse model offers a good starting point to investigate this gene × environment (G × E) interaction. This genetic animal model consists of three independent, CD1-derived mouse lines, selectively bred for either high (HR), intermediate (IR), or low (LR) HPA axis reactivity in response to a psychological stressor, thereby mirroring the HPA axis dysregulation endophenotypes described in the melancholic/psychotic and the atypical depression subtypes, respectively (Heinzmann et al., 2014; Touma et al., 2008, 2009). Compared to the IR line, which serves as reference line, HR mice have lower bodyweight, show disturbed circadian activity patterns and increased REM sleep, display hyperactive stress-coping behavior and show cognitive deficits, corresponding to symptoms of melancholic/psychotic depression. In contrast, LR mice have increased bodyweight, show passive coping behavior, as well as intact sleep and cognitive function, akin to symptoms of atypical depression (Fenzl et al., 2011; Heinzmann et al., 2014; Knapman et al., 2010a,b, 2012; Touma et al., 2008, 2009). Thus, the SR mouse model is an appropriate tool to investigate the interaction of a genetic vulnerability for disturbances in the stress hormone system and environmental stressors.

Exposure to early-life stress (ELS) is a well-established environmental risk factor for affective disorders (Baram et al., 2012; Heim et al., 2002; Kessler et al., 2005; Penza et al., 2003). During the early postnatal period, the central nervous system is still highly plastic, so that the environment can profoundly and lastingly shape the brain and the neuroendocrine system (Everson-Rose et al., 2003; Lehmann et al., 2002; Oomen et al., 2010a,b; Plotsky and Meaney, 1993; Wilson et al., 2005). Recent evidence from animal models and human data suggests that this process involves epigenetic modifications at several target sites, including the genes coding for the glucocorticoid receptor (*Gr*), vasopressin (*Avp*), corticotropin-releasing hormone (*Crh*), and FK506 binding protein 5 (*Fkpb5*) (Bockmühl et al., 2015; Klengel et al., 2012; Provençal and Binder, 2015; Radtke et al., 2015; Weaver et al., 2004; Zimmermann et al., 2015). A dominant factor in a mouse pup's early-life environment is the dam. Via her maternal behavior, she has a profound influence on her pups' brain development (Caldji et al., 2004; Meaney, 2001). For example, the amount of maternal licking and grooming can change the behavior of the offspring by altering their brain function and HPA axis responsiveness (Champagne et al., 2003; Francis et al., 1999; Liu et al., 1997; Sarro et al., 2014).

Also in humans, the mother acts as a powerful regulator of the development of the infant's physiological regulatory systems, including the HPA axis (Als et al., 2004; Hane and Fox, 2006) and the quality of the mother-child relationship is highly predictive of the child's trajectory regarding mental and cognitive health (Belsky and Fearon, 2002; Bowlby, 1958; Masur et al., 2005). For the development of a secure attachment relationship maternal sensitivity and responsiveness are of critical importance (McElwain and Volling, 2004). Fragmentation and unpredictability of maternal care are therefore important triggers of ELS and predictors of mental health deficits in children. In rodents such fragmented and unpredictable maternal care can be experimentally induced by reducing the amount of the nesting and bedding material available to nursing dams (Molet et al., 2014; Rice et al., 2008), thus permitting controlled studies of the consequences of ELS.

In our study, we combined the genetic predisposition for HPA axis hyper- or hypo-reactivity of the SR mouse lines with exposure to ELS to investigate the interaction of these two risk factors at the level of physiology, behavior, neuroendocrine function and gene expression in the brain. We aimed to (i) study the long-lasting consequences of this G × E interaction, including key endophenotypes of affective disorders, and (ii) examine the short-term effects of ELS-exposure, which may be involved in mediating the long-term outcomes. Therefore, we exposed mice of the three SR mouse lines to ELS and studied the pups and the adult animals using a battery of tests for emotional behavior, HPA axis reactivity and recovery, as well as expression profiles of candidate genes in relevant brain nuclei. Additionally, factors influencing the animals' stress experience during the ELS paradigm, such as maternal behavior, nest temperature, and nest quality were monitored throughout the experiment. This experimental design allowed us to detect endophenotypes associated with the development of long-lasting consequences of ELS, which can contribute to the ongoing search for intervention targets after early-life adversity.

2. Methods

All presented work is in accordance with the accepted standards of humane care and use of experimental animals and was approved by the appropriate local authority. The supplementary material provides additional descriptions of methods and experimental procedures.

2.1. Animals and housing conditions

In all experiments, animals from the SR mouse model were used (Touma et al., 2008). A detailed description of the breeding procedure used to generate and maintain this mouse model is provided in the supplementary material, section 1.1. Animals were housed under standard laboratory conditions (stable 12 h light/dark cycle (lights on at 8 a.m.), 22 ± 2 °C, 55 ± 10% humidity, standard diet chow and water *ad libitum*).

2.2. Experimental design

We used a 3 × 2 factorial design: Three SR breeding lines (HR, IR, and LR) and two environmental conditions (early-life stress (ELS) and standard housing (STD)), resulting in a total of six experimental groups. The data presented here was generated from five independent cohorts of experimental animals, derived from breeding generations XXIII–XXVIII of the SR mouse model, focusing on either the long-lasting effects of ELS in adulthood (cohorts I and II) or on the short-term effects in the pups (cohorts III, IV and V).

2.3. Breeding of experimental cohorts

For each cohort, 48 breeding pairs were used, i.e. 16 male-female pairs per mouse line. The cages of pregnant primiparous females were inspected daily at 5 p.m. for the delivery of pups and the day a litter was discovered was defined as postnatal day 0 (P0). On P2, litters were culled to seven pups, consisting of six males and one female, when possible. Only litters with a total number of at least six pups and including at least four males were included in the study to ensure a comparable early-life situation for pups from different litters.

2.4. Early-life stress paradigm

Dams and their offspring were randomly assigned to either the ELS or the STD condition ($N = 8$ dams per line and condition). We used a stress paradigm based on limiting the resources of nesting and bedding material, described by Rice et al. (2008). This paradigm creates a chronic ELS environment that has been described as more naturalistic and creating fewer metabolic side-effects than repeated maternal-separation (Molet et al., 2014). Briefly, on P2, dams assigned to the ELS condition were placed, together with their litter, into a polycarbonate type II cage fitted with an aluminum grid floor (mesh dimensions 0.4 × 0.9 cm, catalog no. 57398; McNichols Co., Tampa, U.S.A). A reduced amount of sawdust bedding material (~20 g) was spread underneath the aluminum grid and half a

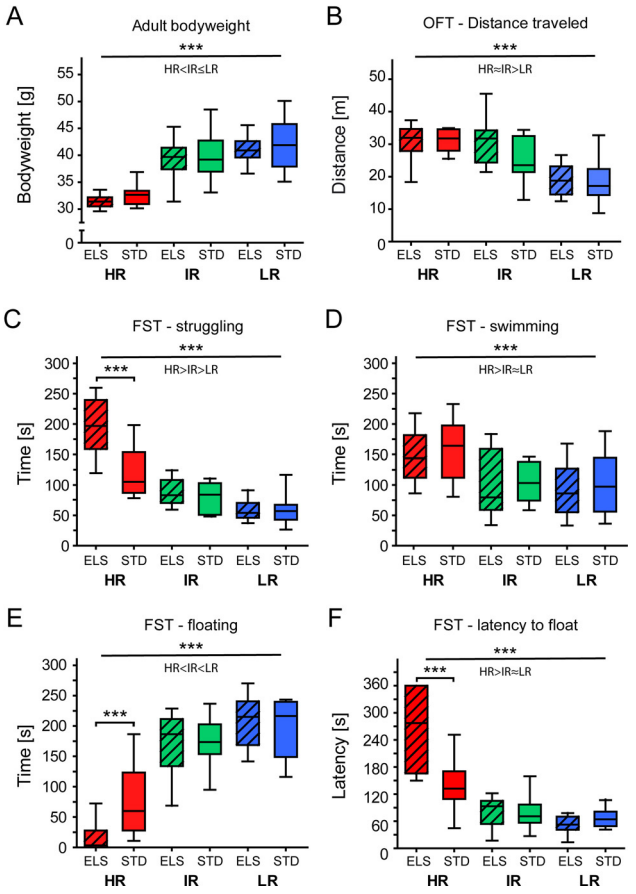

Fig. 1. Bodyweight and behavior in adult animals. Data from adult high (HR), intermediate (IR), and low (LR) reactivity mice raised in early-life stress (ELS) or standard (STD) housing conditions are presented as boxplots showing the median (horizontal line in the boxes), 25–75% (boxes) and 10–90% (whiskers). (A) The animals' bodyweight at 10 weeks of age showed a main effect of mouse line ($F_{2,114}$ = 89.253, p < 0.001, post-hoc tests: HR vs IR and HR vs LR: p < 0,001; IR vs LR: p = 0.059), N = 19–21 per group. (B) Distance traveled in the Open Field Test (OFT) showed a main effect of line ($F_{2,54}$ = 23.372, p < 0.001, post-hoc tests: HR vs IR: p = 0.409, HR vs LR and IR vs LR: p < 0.001; N = 8–11 per group). (C) Time spent struggling in the Forced Swim Test (FST) showed a main effect of line ($F_{2,114}$ = 87.201, p < 0.001, post-hoc tests: HR vs IR and LR: p < 0.001, IR vs LR: p = 0.006), a main effect of condition ($F_{1,114}$ = 19.971, p < 0.001) and an interaction effect ($F_{2,114}$ = 12.933, p < 0.001, post-hoc tests: HR ELS vs STD: p < 0.001, IR ELS vs STD p = 0.309, LR ELS vs STD: p = 0.994; N = 18–21 per group). (D) Time spent swimming in the FST showed a main effect of line ($F_{2,114}$ = 13.411, p < 0.001, post-hoc tests: HR vs IR and HR vs LR: p < 0.001, IR vs LR: p = 1.0; N = 18–21 per group). (E) Time spent floating in the FST showed a main effect of line ($F_{2,114}$ = 96.723, p < 0.001, post-hoc tests: HR vs IR and HR vs LR: p < 0.001, IR vs LR: p = 0.019), a main effect of condition ($F_{1,114}$ = 4.272, p = 0.041) and an interaction ($F_{2,114}$ = 4.807, p = 0.010, post-hoc tests: HR ELS vs STD: p < 0.001, IR ELS vs STD: p = 0.91, LR ELS vs STD: p = 0.723; N = 18-21 per group). (F) The latency to the first floating bout showed a main effect of line ($F_{2,114}$ = 82.112, p < 0.001, post-hoc tests: HR vs IR and HR vs LR: p < 0.001, IR vs LR: p = 0.231), a main effect of condition ($F_{1,114}$ = 13.985, p < 0.001) and an interaction ($F_{2,114}$ = 17.725, p < 0.001, post-hoc tests: HR ELS vs STD: p < 0.001, IR ELS vs STD: p = 0.795, LR ELS vs STD: p = 0.425; N = 18-21 per group). Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line (≈ p ≥ 0.1, ≤/≥, p < 0.1, </> p < 0.05). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001).

nestlet (~5 g) (Nestlets NES3600, Ancare, Bellmore, U.S.A.) was provided for nest building. Mice assigned to the STD housing condition were placed into polycarbonate type II cages with normal amounts of sawdust bedding (~100 g) and two nestlets (~20 g). Supplementary Fig. 1 shows examples of the ELS and STD housing conditions. The animals were left undisturbed for seven days. On P9, dams and pups were weighed and moved to standard housing cages. On P25, one or two male sibling pairs from each litter were weaned and pair-housed in standard cages until adulthood (cohort I and II).

2.5. Long-lasting effects of ELS

2.5.1. Bodyweight

Two weeks before the behavioral phenotyping, the test animals were weighed and single housed in order to avoid dominance hierarchy effects.

2.5.2. Behavioral parameters

All behavioral tests were conducted during the light phase (between 9 and 12 a.m.), when corticosterone levels are in the circadian trough (Ishida et al., 2005). Between tests, mice were given at least 48 h of rest to avoid carry over effects from one test to another, as advised for repeated behavioral testing (McIlwain et al., 2001).

2.5.2.1. Locomotor and exploratory activity. The Open Field Test (OFT) was used to detect differences in locomotor and exploratory activity. Briefly, each mouse (12–14 week old males, cohort I) was placed into the center of a circular arena (60 cm diameter), evenly illuminated with 15 lx, and left to explore for 5 min (for details see Varadarajulu et al., 2011). The test was video recorded, and a tracking software (ANYmaze, Stoeling Co, Wood Dale, U.S.A.) was used to analyze the animal's behavior. After testing, the animal was returned to its home cage and the OF apparatus was cleaned (soapy water and ethanol solution) and dried to leave no odor cues for the subsequent animal.

2.5.2.2. Anxiety-related behavior. The Dark-Light Box Test was used to assess anxiety-related behavior. This test is based on the animals' natural aversion for brightly lit spaces and on their innate tendency to explore novel environments (Crawley and Goodwin, 1980). Briefly, each mouse (12–14 week old males, cohort I) was placed into a small dark compartment (15 × 20 × 25 cm, <10 lx), which was connected with a larger, brightly lit compartment (30 × 20 × 25 cm, >700 lx) through a short tunnel. The latency for the animal to enter the brightly lit compartment, the number of entries, and the time it spent there were extracted with the help of a tracking software (ANYmaze, Stoeling Co, Wood Dale, U.S.A.).

2.5.2.3. Stress-coping behavior. Stress-coping behavior was assessed using the Forced Swim Test (FST) and the Tails-Suspension Test (TST). Importantly, animals were assigned to one or the other of these tests so that no mouse was tested in both the FST and the TST. Briefly, in the FST, each mouse (12–14 week old males, cohorts I and II) was placed into a glass beaker (12 cm diameter, 24 cm high) filled with warm water (23 °C) and its behavior was video recorded for 6 min. The mouse was then removed from the water, gently dried and placed back into its home cage. In the analysis, three behaviors were quantified: (1) struggling: strong movements with front and hind paws, breaking through the water surface; (2) swimming: movement with all four paws, and (3) floating: no limb movement, or minimal movement to prevent sinking. All videos were scored by the same trained observer, who was blind to the animals' breeding line and experimental condition.

In the TST, each mouse (12–14 week old males, cohort I) was suspended by its tail using tape, in such a way that it could not escape. The test, lasting for 6 min, was video-recorded for later scoring by a trained observed. Two behaviors were quantified: (1) struggling: active mobility, including any escape attempts; (2) immobility: passive hanging or swinging with not attempt to escape.

2.5.3. HPA axis function

We used the stress reactivity test (SRT) to measure basal and stress-induced corticosterone levels in the adult animals (see

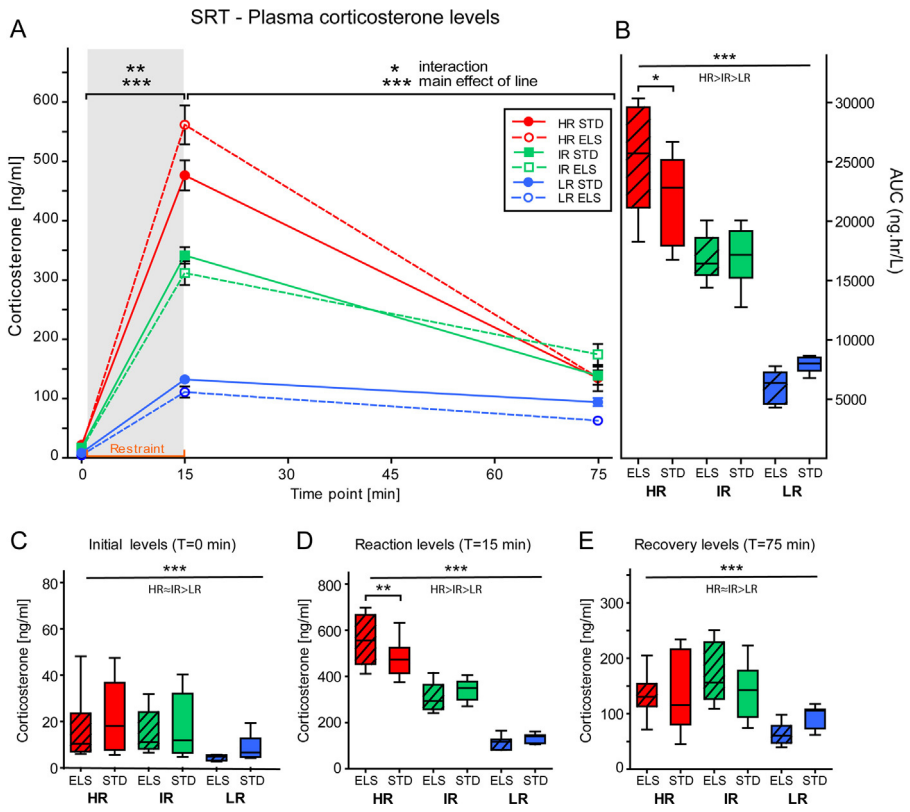

Fig. 2. Neuroendocrine stress responsiveness in adult animals. The neuroendocrine response of the HPA axis in the Stress Reactivity Test (SRT) of adult high (HR), intermediate (IR), and low (LR) reactivity mice raised in early-life stress (ELS) or standard (STD) housing conditions is presented as line plot, showing means and SEM, and boxplots, showing the median (horizontal line in the boxes), 25–75% (boxes) and 10–90% (whiskers), $N = 8$–11 per group. (A) The increase in plasma corticosterone levels after 15 min restraint showed a main effect of line ($F_{2,54} = 199.722$, $p < 0.001$, post-hoc tests: all $p < 0.001$) and an interaction of line and condition ($F_{2,54} = 5.743$, $p = 0.005$, post-hoc tests: HR ELS vs STD: $p < 0.002$, IR ELS vs STD: $p = 0.322$, LR ELS vs STD: $p = 0.526$). The recovery of plasma corticosterone levels showed a main effect of line ($F_{2,54} = 76.544$, $p < 0.001$, post-hoc tests: all: $p < 0.001$) and an interaction of line and condition ($F_{2,54} = 3.536$, $p = 0.036$, post-hoc tests: HR ELS vs STD: $p = 0.016$, IR ELS vs STD: $p = 0.225$, LR ELS vs STD: $p = 0.860$). (B) The area under the curve (AUC) showed a main effect of line ($F_{2,54} = 180.231$, $p < 0.001$, post-hoc tests: all $p < 0.001$) and an interaction of line and condition ($F_{2,54} = 4.340$, $p = 0.018$, post-hoc tests: HR ELS vs STD: $p = 0.012$, IR ELS vs STD: $p = 0.862$, LR ELS vs STD: $p = 0.148$). (C) Initial corticosterone levels showed a main effect of line ($F_{2,54} = 6.485$, $p = 0.003$, post-hoc tests: HR vs IR: $p = 0.949$, HR vs LR: $p = 0.003$, IR vs LR: $p = 0.044$). (D) Corticosterone reaction levels (after 15 min of restraint) showed a main effect of line ($F_{2,54} = 192.243$, $p < 0.001$, post-hoc test: all $p < 0.001$), as well as an interaction ($F_{2,54} = 5.001$, $p = 0.010$, post-hoc tests: HR ELS vs STD: $p = 0.004$, IR ELS vs STD: $p = 0.303$, LR ELS vs STD: $p = 0.464$). (E) Corticosterone recovery levels (60 min after termination of the stressor) showed a main effect of line ($F_{2,54} = 14.432$, $p < 0.001$, post-hoc tests: HR vs IR: $p = 0.512$, HR vs IR and vs LR: $p < 0.001$), and a trend for an interaction ($F_{2,54} = 2.484$, $p = 0.094$, post hoc tests: HR ELS vs STD: $p = 0.964$, IR ELS vs STD: $p = 0.095$, LR ELS vs STD: $p = 0.153$). Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line ($\approx$ $p \geq 0.1$, $\leq/\geq$, $p < 0.1$, </> $p < 0.05$). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The grey shaded area indicates the period of restraint.

Touma et al., 2008 for details). Briefly, a sample of blood was obtained from each animal (14–16 week old males, cohort II) through a small incision in the ventral tail vessel within 2 min after disturbing the cage (initial sample). The animal was then submitted to a 15 min restraint period in a 50 ml plastic tube, with holes for ventilation and an aperture in the cap for the tail, followed by a second incision and blood sample collection (reaction sample). For a recovery period of 60 min, the animal was returned to its home cage, whereafter a third blood sample was taken (recovery sample). All blood samples were kept on ice until further processing.

2.5.4. Candidate gene expression

The activity of the HPA axis is dependent on the expression and function of multiple genes in several brain regions, including the paraventricular nucleus of the hypothalamus (PVN), the dorsal and ventral hippocampus, the basolateral amygdala (BLA) and the pituitary. To investigate the interaction of early-life adversity and genetic predisposition for extremes in stress reactivity, we assessed the expression of several candidate genes in these brain nuclei using quantitative real-time polymerase chain reaction (qPCR). A detailed description is provided in the supplementary material (1.2.).

2.6. Short-term effects of ELS

2.6.1. Bodyweight

Pups were weighed on P2, P9, P17 and P25 when cages where changed. To minimize handling of the pups, litters were weighed as units and the weight reported here is the average weight per pup per litter.

2.6.2. Behavioral parameters

2.6.2.1. Ultrasonic vocalization. Mouse pups isolated from their mothers emit ultrasonic vocalization (USV) calls (Portfors, 2007) and previous research has shown that ELS can alter pups' vocalization behavior (Laloux et al., 2012; Zimmerberg et al., 2003). We therefore included a USV test as a measure of early behavioral effects of the ELS paradigm. The USV test was conducted on P3 and P7 between 2 and 4 p.m. Briefly, one randomly chosen male pup (cohort III) was removed from each litter, weighed and placed onto a glass dish (20 cm diameter) in a noise protected chamber with a sensitive microphone (Avisoft-SASLab Pro, Glienicke, Germany) for 5 min to record the USV calls. Before and after the test the animals body surface temperature was measured using a non-invasive infrared thermometer (Visual IR Thermometer VT02, FLUKE, Eind-

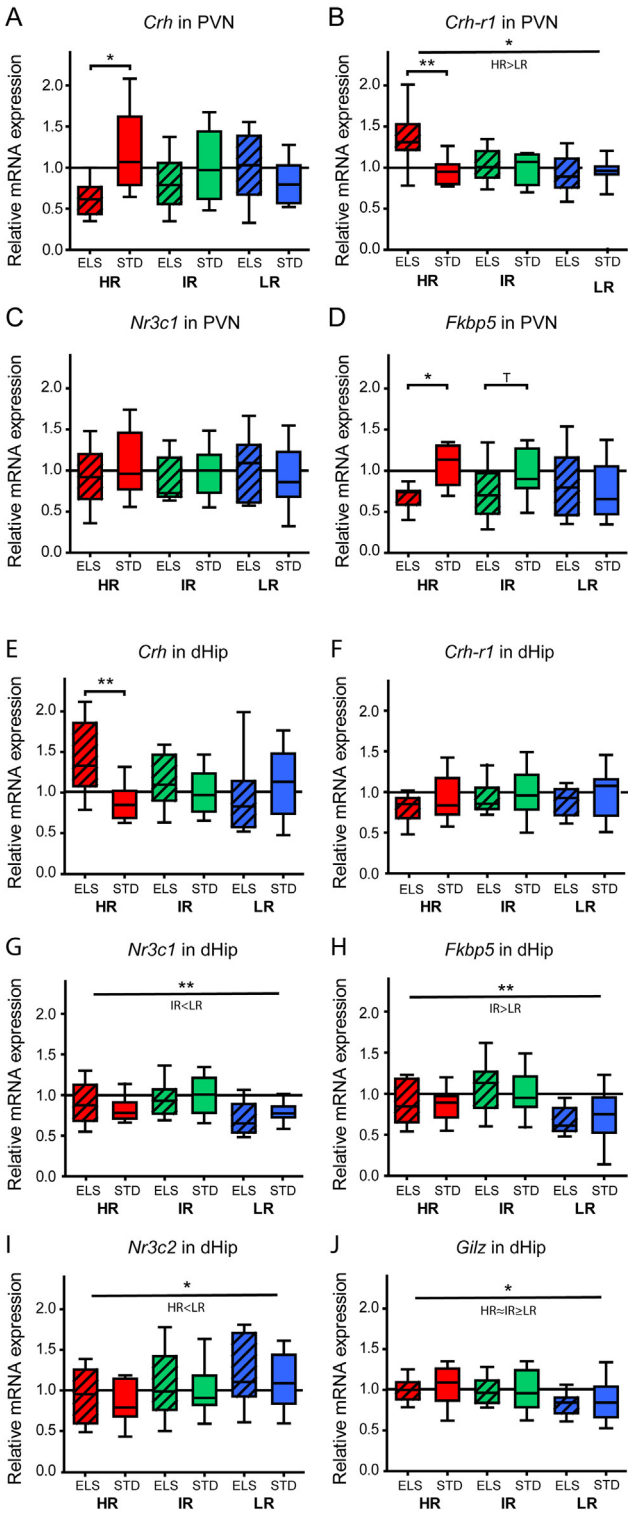

Fig. 3. Candidate gene expression in adult animals. Relative gene expression in the paraventricular nucleus (PVN) and the dorsal Hippocampus (dHip) of adult high (HR), intermediate (IR), and low (LR) reactivity mice raised in early-life stress (ELS) or standard (STD) housing conditions is presented as boxplots, showing the median (horizontal line in the boxes), 25–75% (boxes) and 10–90% (whiskers), $N = 8$–11 per group. (A) The expression of *Crh* was affected by an interaction of line and condition ($F_{2,54} = 3.592$, $p = 0.034$, post-hoc tests: HR ELS vs STD: $p = 0.010$, IR ELS vs STD: $p = 0.267$, LR ELS vs STD: $p = 0.280$). (B) The expression of *Crh-r1* showed a main effect of line ($F_{2,54} = 4.219$, $p = 0.020$, post-hoc tests: HR vs IR: $p = 0.256$, HR vs LR: $p = 0.026$, IR vs LR: $p = 0.934$), a main effect of condition ($F_{1,54} = 4.349$, $p = 0.042$), and an interaction of line and condition ($F_{2,54} = 4.977$, $p = 0.010$, post-hoc tests: HR ELS vs STD: $p = 0.001$, IR ELS vs STD: $p = 0.851$, LR ELS vs STD: $p = 0.713$). (C) Expression of *Nr3c1* showed no effect of line or condition. (D) The expression of *Fkbp5* showed a main effect of condition ($F_{1,54} = 5.094$, $p = 0.028$) and a trend for an interaction of line and condition ($F_{2,54} = 2.762$, $p = 0.072$, post-hoc tests: HR ELS vs STD: $p = 0.011$, IR ELS and STD: $p = 0.074$, LR ELS and STD: $p = 0.580$). (E) The expression of *Crh* showed an interaction of line and condition ($F_{2,53} = 4.662$, $p = 0.014$, post-hoc tests: HR ELS vs STD: $p = 0.004$, IR ELS vs STD: $p = 0.517$, LR ELS vs STD: $p = 0.212$). (F) Expression of *Crh-r1* showed no effect of line or condition. (G) The expression of *Nr3c1* showed a main effect of line ($F_{2,53} = 6.141$, $p = 0.004$, post-hoc tests: HR vs IR: $p = 0.299$, HR vs LR: $p = 0.242$, IR vs LR: $p = 0.003$). (H) The expression of *Fkbp5* showed a main effect of line ($F_{2,53} = 8.531$, $p = 0.001$, post-hoc tests: HR vs IR: $p = 0.174$, HR vs LR: $p = 0.121$, IR vs LR: $p < 0.001$). (I) The expression of *Nr3c2* showed a main effect of line ($F_{2,53} = 3.430$, $p = 0.040$, post-hoc tests: HR vs IR: $p = 0.479$, HR vs LR: $p = 0.027$, IR vs LR: $p = 0.601$). (J) The expression of *Gilz* showed a main effect of line ($F_{2,54} = 3.692$, $p = 0.031$, post-hoc tests: HR vs IR: $p = 1.0$, HR vs LR: $p = 0.045$, IR vs LR: $p = 0.087$). Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line ($\approx$ $p \geq 0.1$, $\leq/\geq$, $p < 0.1$, $</>$ $p < 0.05$). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

hoven, Netherlands; Suppl. Fig. 4D). A detailed description of the testing protocol is provided in the supplementary material (1.3.)

2.6.2.2. Locomotor and exploratory activity. During the 5 min USV tests on P3 and P7, the behavior of the male pups (cohort III) was recorded using a light sensitive camera installed in the set-up (see above). The distance traveled during the testing session was manually scored with the help of grid lines on the surface of the glass dish (squares sized 2×2 cm). Every line crossing (defined as head and forelegs across the line) was counted as one distance unit to assess the pup's locomotor and exploratory activity.

2.6.2.3. Stress-coping behavior. To investigate the effects of ELS on the pups coping strategies in a stressful situation we used the FST as described above (see 2.5.2.3.). For this test, one or two male pups per litter (cohort V) were removed from the nest in the morning of P25 (before weaning) and individually submitted to 6 min of forced swimming.

2.6.3. Neuroendocrine parameters

2.6.3.1. Relative adrenal weight. Adrenals were dissected from one pup per litter on P13 and P25 (reliable removal of the adrenals in younger animals was not possible). Briefly, one randomly chosen male pup (cohort III) was removed from the nest, weighed and quickly decapitated. Both kidneys, with the adrenals attached, were dissected and placed in labelled Eppendorf tubes in PBS solution. Subsequently, the adrenals were dissected, remaining fat tissue was removed, and adrenals were weighed on a microscale (0.01 mg readability, 40SM-200A Precisa, Abbott, Chicoago, U.S.A.). The relative weight of both adrenals was calculated by dividing the adrenal weight by the respective pup's bodyweight.

2.6.3.2. HPA axis function. To assess the short-term effects of ELS on HPA axis function in pups, a modified version of the SRT was conducted on P9 and P25. Briefly, two male pups (cohort IV) were removed from each litter: The first was immediately decapitated and trunk blood was obtained (baseline sample; time between removal from the nest and decapitation was always below 2 min). The second pup was placed into an empty cage with fresh bedding for 15 min (novelty exposure), whereafter it was decapitated and trunk blood was collected (reaction sample). To investigate the possibility of preexisting differences between the groups, basal plasma samples were also collected from one male pup per litter on P2.

2.6.4. Candidate gene expression

To investigate the short-term effects of ELS on the expression of HPA axis related genes, we measured the expression of several candidate genes in the PVN using qPCR. A detailed description is provided in the supplementary material (1.4.).

2.7. Effects of the ELS paradigm on dams and nesting environment

Differences in the response of HR, IR, and LR dams to the ELS paradigm could influence the resulting phenotypes in the pups by changing their early-life environment. To detect any line-specific difference, we assessed several relevant parameters of the pups' early-life environment, including maternal behavior, maternal bodyweight, nest temperature, and nest quality from P2 until weaning. A detailed description of the methods used is provided in the supplementary material (1.5.).

2.8. Corticosterone measurement

All blood samples were centrifuged at 4 °C and plasma samples were stored at −20 °C until further analysis. Corticosterone concentration was measured using a radioimmunoassay kit (DRG Instruments GmbH, Marburg, Germany) with 10 μl of plasma per dilution and duplicates for each sample (for details see Touma et al., 2008). The intra- and inter-assay coefficients of variation were both below 10%.

2.9. Statistical analysis

All statistical analyses were done using PASW statistics 18. Data was analyzed in a two-way analysis of variance (ANOVA) with "experimental condition" × "mouse line" as fixed factor and Bonferroni corrected post-hoc *t*-tests when appropriate. All data points that were collected repeatedly from the same animal were analyzed using a repeated-measures ANOVA, with "time point" as a within-subjects variable, and "experimental condition" and "mouse line" as between-subjects factors. Bonferroni corrected post-hoc tests were calculated when appropriate. Whenever a variable was assessed in more than one experimental cohort, a covariate "cohort" was included into the statistical model. Statistical significance was accepted for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), while $p < 0.1$ (T) was considered a trend.

3. Results

3.1. Long-lasting effects of ELS

3.1.1. Bodyweight

Being raised in the ELS condition did not affect the animals' bodyweight in adulthood (Fig. 1A). Nevertheless, there was a main effect of mouse line ($F_{2,114} = 89.253$, $p < 0.001$), confirming previous findings (Heinzmann et al., 2014; Touma et al., 2008), showing that HR mice were lighter than IR and LR animals, and LR mice tended to be heavier than IR animals.

3.2. Locomotor and explorative activity

The adult mice showed no effect of ELS on any of the OFT readouts (total distance traveled; time, number of entries and distance traveled in the inner zone) (Suppl. Fig. 2A–C). However, overall, LR mice traveled shorter total distances ($F_{2,54} = 23.372$, $p < 0.001$, Fig. 1B), made less entries to the inner zone ($F_{2,54} = 3.480$, $p = 0.038$) and traveled shorter distances in the inner zone ($F_{2,54} = 6.046$, $p = 0.004$) than HR and IR animals (Suppl. Fig. 2A and C). These differences in locomotor activity may lead to differences in energy expenditure and could thus contribute to the differences in bodyweight between the three mouse lines.

3.3. Anxiety-related behavior

ELS housing had no significant effect on the animals behavior in the dark-light box test. However, the data showed that, overall, HR mice tended to be more anxious than IR and LR mice, indicated by a lower number of entries to, less time spent in, and a greater latency to enter into the lit zone (Suppl. Fig. 2D–F).

3.4. Stress-coping behavior

The animals' stress-coping behavior in the FST showed an interaction effect of mouse line and condition on the time spent struggling ($F_{1,114} = 12.933$, $p < 0.001$, Fig. 1C) and the time spent floating ($F_{2,114} = 4.807$, $p = 0.01$, Fig. 1E). Post-hoc tests revealed that ELS-raised HR mice struggled significantly longer ($p < 0.001$), and floated significantly less ($p < 0.001$) than STD-raised HR mice. The behavior of IR and LR animals was not changed by their ELS-experience. The latency to the first floating episode also showed a significant interaction of line and condition ($F_{2,114} = 17.725$, $p < 0.001$), with ELS-raised HR mice starting to float significantly later than HR STD animals ($p < 0.001$), but showed no difference in IR and LR mice (Fig. 1F). Overall, HR ELS mice showed an even more hyperactive stress-coping style than the already highly active HR STD animals.

The FST results also revealed some profound differences in stress-coping behavior between the three SR mouse lines, confirming previous findings (Touma et al., 2008; Knapman et al., 2010a,b; Heinzmann et al., 2014). There was a main effect of line on the time spent struggling ($F_{2,114} = 87.201$, $p < 0.001$, Fig. 1C), the time spent swimming ($F_{2,117} = 13.441$, $p < 0.001$, Fig. 1D), and the time spent floating ($F_{2,1142114} = 96.723$, $p < 0.001$, Fig. 1E), as well as on the latency to the first floating episode ($F = 82.112$, $p < 0.001$, Fig. 1F).

The results of the TST are in line with the FST data (Suppl. Fig. 2G–I). Specifically, there was a trend for an interaction of mouse line and condition on the time spent struggling ($F_{2,54} = 3.740$, $p = 0.058$, post-hoc tests: HR: $p = 0.029$, IR and LR: $p > 0.1$), as well as a main effect of mouse line ($F_{2,54} = 5.987$, $p = 0.004$), with LR animals showing less active coping behavior than HR and IR mice.

3.5. Neuroendocrine parameters

Unsurprisingly, since the responsiveness in the stress reactivity test is the selection criterion for breeding the SR mouse lines, the plasma corticosterone increase in this test revealed a strong main effect of line ($F_{2,54} = 199.722$, $p < 0.001$). In addition, the HPA axis response showed an interaction of mouse line and condition ($F_{2,54} = 5.743$, $p = 0.005$) and post-hoc tests revealed that in ELS-exposed HR mice the increase in plasma corticosterone after restraint was even higher than in HR STD mice ($p = 0.002$, Fig. 2A). In IR and LR animals, the ELS-experience did not affect corticosterone release.

The area under the curve (AUC) also showed an interaction of mouse line and condition ($F_{2,51} = 4.340$, $p = 0.018$), demonstrating that HR ELS mice were exposed to a higher cumulative amount of corticosterone during the test than HR STD animals ($p = 0.012$, Fig. 2B). Conversely, ELS exposure did not influence this parameter in IR and LR mice.

The absolute initial, reaction and recovery levels of plasma corticosterone are presented in Fig. 2C–E. There were significant differences in corticosterone levels between the three lines at all three time points, which were especially pronounced immediately after the restraint stressor.

3.6. Candidate gene expression

In the PVN, the expression of *Crh* mRNA showed an interaction of mouse line and condition ($F_{2,54} = 3.592$, $p = 0.034$). Post-hoc tests revealed that the relative expression of *Crh* was reduced in ELS-exposed HR mice compared to HR STD animals ($p = 0.010$), while

the IR and LR mice appeared to be resilient to this ELS-induced impact on gene expression (Fig. 3A).

A similar interaction of line and condition was found regarding the expression of *Crh-r1* ($F_{2,54} = 4.977$, $p = 0.010$). The *Crh-r1* mRNA was significantly upregulated in the PVN of ELS-exposed HR mice compared to HR STD animals ($p = 0.001$), but not in IR and LR mice (Fig. 3B).

The expression levels of *NR3c1* in the PVN were similar in all three mouse lines and showed no effect of ELS-exposure (Fig. 3C). However, the relative expression of *Fkbp5* mRNA indicated a trend for an interaction of line × condition ($F_{2,54} = 2.762$, $p = 0.072$). Post-hoc tests revealed that ELS-exposed HR mice had significantly lower levels of *Fkbp5* than STD-housed HRs ($p = 0.011$), and IR ELS mice showed a trend in the same direction ($p = 0.074$), while LR ELS and STD mice showed no differential expression (Fig. 3D).

Interestingly, in the dorsal hippocampus, *Crh* mRNA was upregulated by ELS only in HR mice (interaction of line × condition: $F_{2,53} = 4.662$, $p < 0.014$, post-hoc tests HR: $p < 0.004$, IR and LR: $p \geq 0.1$, Fig. 3E). No other of the investigated candidate genes showed a significant regulation by ELS-exposure in the dorsal hippocampus. However, several genes showed expression differences between the three mouse lines (Fig. 3G–J).

In the ventral hippocampus, the BLA, and the pituitary we detected no ELS-induced changes in gene expression, but some line differences. An overview of the gene expression profiles in the analyzed brain areas is provided in supplementary Table 2.A-E and gene expression levels in the pituitary are illustrated in supplementary Fig. 3.

3.7. Short-term effects of ELS

3.7.1. Bodyweight

The bodyweight development of the pups between P2 and P25 is presented in Fig. 4A. The analysis revealed that ELS-exposed pups in all three mouse lines gained significantly less weight than STD-housed pups ($F_{1,119} = 25.554$, $p < 0.001$). Post-hoc tests showed that this difference in weight gain was significant on P9 ($p < 0.001$), P17 ($p = 0.002$), and P25 ($p = 0.011$), demonstrating that the ELS paradigm had a substantial effect on the development of pups in all lines. Overall, HR pups were lagging slightly behind IR and LR pups in bodyweight until weaning ($F_{2,119} = 7.805$, $p = 0.001$). Importantly, on P2, i.e. before the ELS exposure, there was no difference in bodyweight between pups of all three mouse lines or between conditions (Fig. 4B). However, on P9, i.e. after seven days of ELS or STD housing, the bodyweight showed a substantial difference between ELS and STD-housed pups ($F_{1,174} = 145.863$, $p < 0.001$, Fig. 4C).

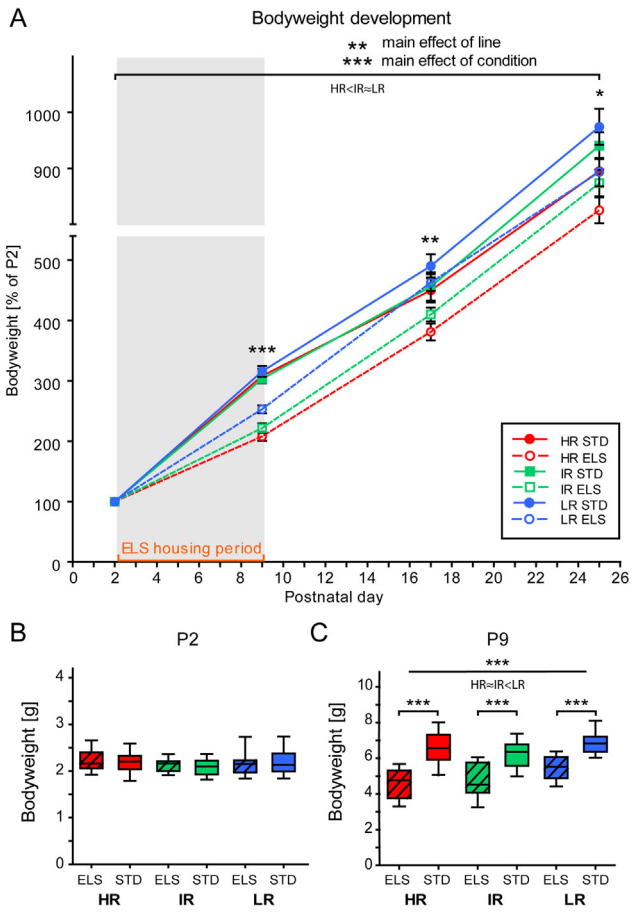

Fig. 4. Bodyweight development in young animals before weaning. The bodyweight of high (HR), intermediate (IR), and low (LR) reactivity mouse pups raised in early-life stress (ELS) or standard (STD) housing conditions measured from postnatal day (P) 2 until weaning (P25) is presented as line plots, showing means and SEM, and boxplots, showing the median (horizontal line in the boxes), 25–75% (boxes) and 10–90% (whiskers). (A) Weight gain in percent from P2 to P25. A repeated-measures ANOVA showed that weight gain was affected by a main effect of line ($F_{2,119} = 7.805$, $p = 0.001$, post-hoc tests: HR vs IR: $p = 0.067$, HR vs LR: $p = 0.001$, IR vs LR: $p = 0.356$) and by a main effect of condition ($F_{1,119} = 25.554$, $p < 0.001$, post-hoc tests overall time points: HR ELS vs STD: $p = 0.002$, IR ELS vs STD: $p = 0.001$, LR ELS vs STD: $p = 0.029$; $N = 19–23$ per group). (B) The bodyweight of the pups before the start of the ELS paradigm did not differ between the three lines or between STD and ELS litters ($N = 30–33$ per group). (C) Bodyweight on P9 showed a main effect of line ($F_{2,175} = 9.381$, $p < 0.001$, post-hoc tests: HR vs IR: $p = 1.0$, HR vs LR: $p = 0.006$, IR vs LR: $p < 0.001$) and a main effect of condition ($F_{1,175} = 145.863$, $p < 0.001$, post-hoc tests: all $p < 0.001$; $N = 27-32$ per group). Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line (≈ $p \geq 0.1$, ≤/≥, p<0.1, </> $p < 0.05$). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The grey shaded area indicates the period of ELS or STD housing.

3.9. Behavioral parameters

3.9.1. Ultrasonic vocalizations, body temperature, and exploratory activity

When isolated from their nest and littermates on P3, pups from the STD condition made more USV calls than ELS-condition pups ($F_{1,36} = 16.409$, $p < 0.001$). However, post-hoc tests showed that the difference was only significant in the HR and the IR lines (HR: $p = 0.008$, IR: $p = 0.009$), but not in LR pups (Fig. 5 A). Furthermore, the ELS-exposed HR pups called at a lower peak frequency ($F_{2,36} = 3.020$, $p = 0.061$, post-hoc tests: HR: $p = 0.001$, IR and LR: $p > 0.1$, Suppl. Fig. 4A), made shorter calls ($F_{2,36} = 8.314$, $p = 0.001$, post-hoc tests: HR: $p = 0.002$, IR: $p > 0.1$, LR: $p = 0.021$, Suppl. Fig. 4C), and had a longer inter-call interval than HR STD pups ($F_{1,36} = 7.779$, $p = 0.008$, post-hoc tests: HR: $p = 0.027$, IR and LR: $p > 0.1$, Suppl. Fig. 4D). Both before and after the USV recording on P3, pups in the ELS condition had a significantly lower body surface temperature than STD-housed pups (before: $F_{1,36} = 37.456$, $p < 0.001$; after: $F_{1,36} = 12.811$, $p = 0.001$, Suppl. Fig. 5). However, although ELS pups were less warm than STD pups, they experienced a less pronounced drop in surface temperature during the USV test ($F_{1,36} = 22.934$, $p < 0.001$, Suppl. Fig. 5), probably due to a floor effect created by the heating pad in the USV test set-up. The exploratory activity, measured by the number of line crossings during the USV test, demonstrated that ELS-condition pups were significantly less active than STD-housed pups ($F_{1,36} = 20.660$, $p < 0.001$, post-hoc tests: HR: $p = 0.004$, IR: $p = 0.008$, LR: $p = 0.055$, Fig. 5B).

On P7, i.e. after five days of ELS or STD housing, no significant effect of housing condition on the number of USV calls was observed (Fig. 5C and Suppl. Fig. 4E–H), and only a marginally reduced number of exploratory line crossings was counted in ELS mice ($F_{1,35} = 6.246$, $p = 0.017$, post-hoc tests: HR and LR: $p > 0.1$, IR: $p = 0.038$, Fig. 5D). The pups' body surface temperature before and

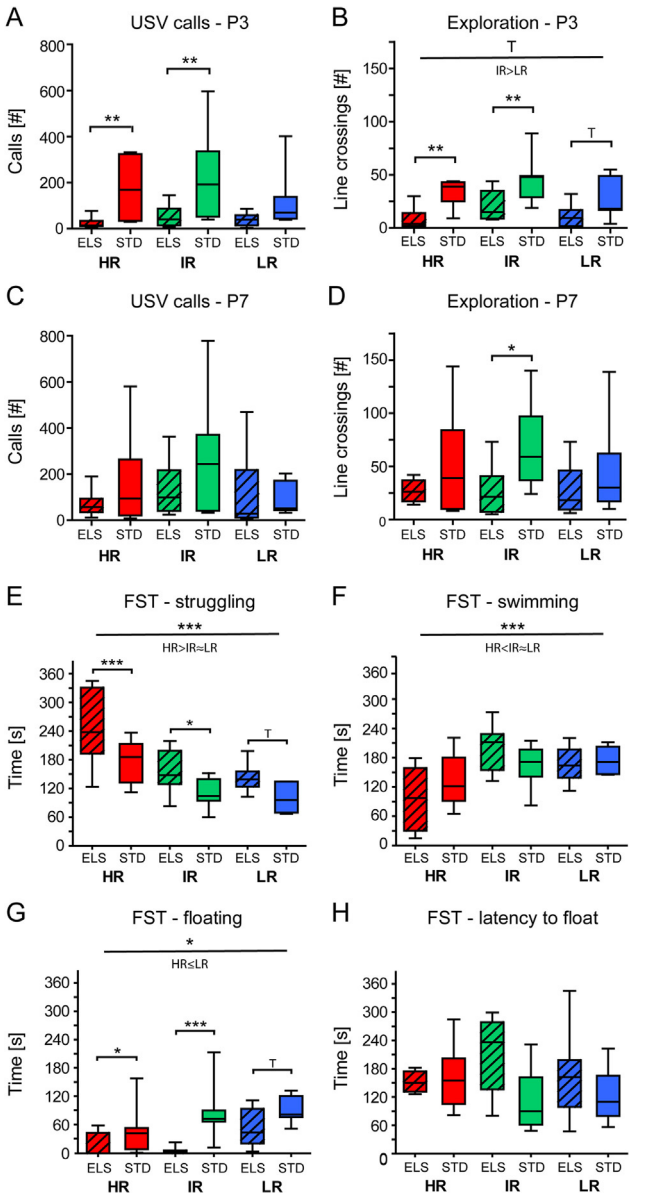

Fig. 5. Behavior in young animals before weaning. Ultrasonic vocalizations (USV) measured on postnatal day (P) 3 and 7, and stress-coping behavior tested on P25, of high (HR), intermediate (IR), and low (LR) reactivity mouse pups raised in early-life stress (ELS) or standard (STD) housing conditions are presented as boxplots, showing the median (horizontal line in the boxes), 25–75% (boxes) and 10–90% (whiskers), $N = 6$–10 per group. (A) The number of USV calls emitted by pups on P3 showed a main effect of condition ($F_{1,36} = 16.409$, $p < 0.001$, post-hoc tests: HR ELS vs STD: $p = 0.008$, IR ELS vs STD: $p = 0.009$, LR ELS vs STD: $p = 0.172$). (B) The number of line crossings during the USV test on P3 showed a trend for a main effect of line ($F_{2,36} = 2.963$, $p = 0.064$, post-hoc tests: HR vs IR: $p = 0.148$, HR vs LR: $p = 1.0$, IR vs LR: $p = 0.044$) and a main effect of condition ($F_{1,36} = 20.660$, $p < 0.001$, post-hoc tests: HR ELS vs STD: $p = 0.004$, IR ELS vs STD: $p = 0.008$, LR ELS vs STD: $p = 0.055$). (C) There was no effect of line or condition on the number of USV calls on P7. (D) The number of line crossings during the USV test on P7 showed a main effect of condition ($F_{1,35} = 6.246$, $p = 0.017$, post-hoc tests: HR ELS vs STD: $p = 0.251$, IR ELS vs STD: $p = 0.038$, LR ELS vs STD: $p = 0.337$). (E) The time spent struggling in the Forced Swim Test (FST) showed a main effect of line ($F_{2,50} = 20.062$, $p < 0.001$, post-hoc tests: HR vs IR and HR vs LR: $p < 0.001$, IR vs LR: $p = 1.0$) and a main effect of condition ($F_{1,50} = 16.432$, $p < 0.001$, post-hoc tests: HR ELS vs STD: $p = 0.008$, IR ELS vs STD $p = 0.022$; LR ELS vs STD: $p = 0.058$) (F) The time spent swimming in the FST showed a main effect of line ($F_{2,50} = 10.266$, $p < 0.001$, post-hoc tests: HR vs IR: $p < 0.001$, HR vs LR: $p = 0.005$, IR vs LR: $p = 1.0$). (G) The time spent floating in the FST showed a main effect of line ($F_{2,50} = 3.676$, $p = 0.033$, post-hoc tests: HR vs IR: $p = 1.0$, HR vs LR: $p = 0.094$, IR vs LR: $p = 0.235$) and a main effect of condition ($F_{1,50} = 23.958$, $p < 0.001$, post-hoc tests: HR ELS vs STD: $p = 0.036$, IR ELS vs STD: $p < 0.001$, LR ELS vs STD: $p = 0.062$). (H) The latency to the first floating bout showed no effect of line or condition. Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line ($\approx$ $p \geq 0.1$, $\leq/\geq$, $p < 0.1$, $</>$ $p < 0.05$). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

after the USV test also showed no significant difference between lines and conditions, apart from a trend for increased cooling during the test in the STD- compared to the ELS-housed animals ($F_{1,35} = 3.282$, $p = 0.079$, Suppl. Fig. 5).

3.9.2. Stress-coping behavior

Stress-coping behavior of the pups was assessed on P25 using the FST. The results showed that, similar to the FST in adult mice, there was a main effect of mouse line on the animals' coping style. Specifically, HR pups struggled longer than IR and LR pups ($F_{2,50} = 20.062$, $p < 0.001$, Fig. 5E) and floated less ($F_{2,50} = 3.676$, $p = 0.033$, Fig. 5G).

Regarding the short-term impact of ELS on stress-coping behavior, the results showed that ELS-exposed animals in all lines struggled longer than STD-raised mice ($F_{1,50} = 16.432$, $p < 0.001$, post-hoc tests: HR: $p = 0.008$, IR: $p = 0.022$, LR: $p = 0.058$). However, the interaction of line and condition on struggling duration, that had been highly significant in the adult mice, did not reach significance in the pups ($F_{2,50} = 0.106$, $p = 0.897$, Fig. 5E). Accordingly, ELS-exposed pups of all lines spent less time floating, i.e. showed less passive coping behavior, compared to STD-raised pups ($F_{1,50} = 23.958$, $p < 0.001$, post-hoc tests: HR: $p = 0.036$, IR: $p < 0.001$, LR: $p = 0.062$, Fig. 5G). The latency to the first floating episode, however, was unaffected by mouse line or condition (Fig. 5H).

3.10. Neuroendocrine parameters

3.10.1. Relative adrenal weight

On P13, i.e. shortly after the end of the ELS exposure, the relative adrenal weight was significantly increased in HR and IR ELS pups compared to the respective STD-housed pups, while no such effect was present in the LR line (interaction of line × condition: $F_{2,35} = 8.601$, $p = 0.001$, post-hoc tests: HR: $p < 0.001$, IR: $p = 0.025$, LR: $p \geq 0.1$). Moreover, the relative adrenal weight showed a main effect of mouse line ($F_{2,35} = 6.645$, $p = 0.004$). Overall, HR and IR mice had a higher relative adrenal weight than LR mice (Fig. 6A).

On P25, the effects of ELS on relative adrenal weight were no longer statistically significant, while the relative difference between the three mouse lines was more pronounced than on P13 ($F_{2,25} = 9.966$, $p = 0.001$, Fig. 6B). The latter confirms previous findings in adult HR/IR/LR mice (Heinzmann et al., 2014; Touma et al., 2008).

3.10.2. HPA axis reactivity

On P2, basal corticosterone levels were similarly low in all six experimental groups (Suppl. Fig. 6). On P9, i.e. after one week of ELS or STD housing, ELS-exposed HR pups had elevated basal corticosterone levels compared to STD pups, while pups of the IR and LR mouse line showed no difference associated with ELS (interaction of mouse line × condition: $F_{(2,40)} = 8.093$, $p = 0.001$, post-hoc tests: HR: $p < 0.001$, IR and LR: $p \geq 0.1$). The basal corticosterone levels also revealed a main effect of mouse line ($F_{2,40} = 27.807$, $p < 0.001$, Fig. 6C).

After a 15-min novelty exposure, the stress-induced levels of corticosterone confirmed the main effect of mouse line ($F_{2,40} = 27.222$, $p < 0.001$, Fig. 6C). However, within each of the three lines, the stress-induced corticosterone levels of ELS and STD-raised pups were on a similar level.

Before weaning on P25, the pups' basal corticosterone levels only slightly differed between lines and conditions (line:

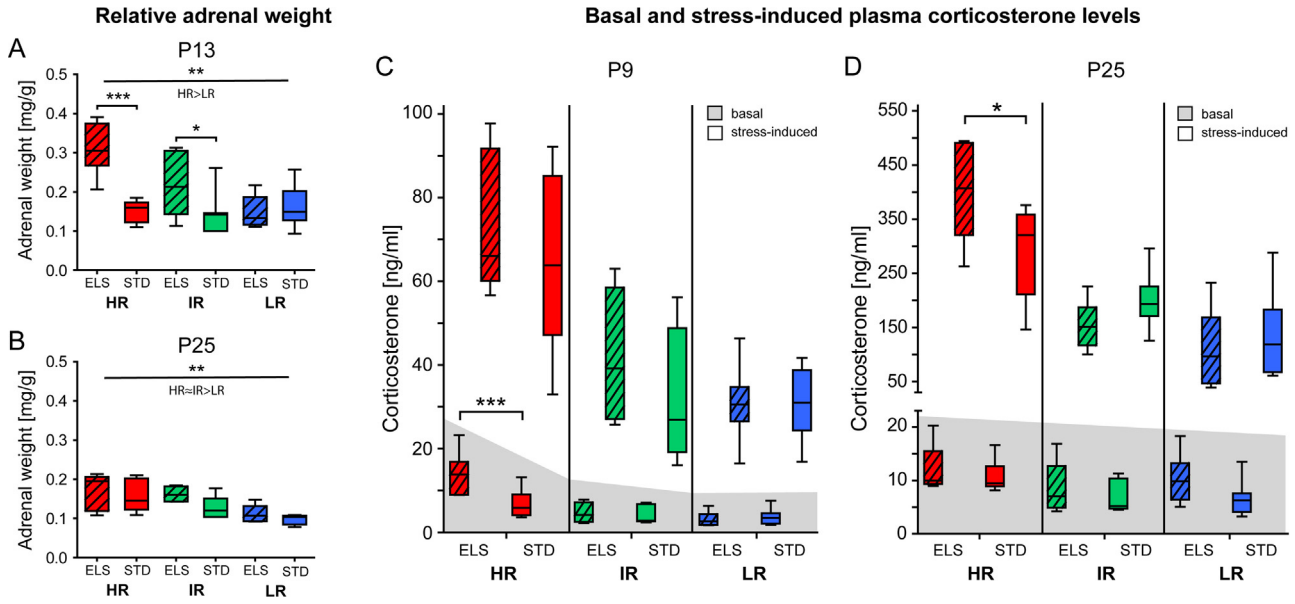

Fig. 6. Neuroendocrine stress responsiveness in young animals before weaning. Parameters of neuroendocrine responsiveness of the HPA axis in high (HR), intermediate (IR), and low (LR) reactivity mouse pups raised in early-life stress (ELS) or standard (STD) housing conditions, measured on postnatal day (P) 9, 13 and 25, are presented as boxplots, showing the median (horizontal line in the boxes), 25–75% (boxes) and 10–90% (whiskers). (A) The relative adrenal weight on P13 showed a main effect of line ($F_{2,35} = 6.645$, $p = 0.004$, post-hoc tests: HR vs IR: $p = 0.123$, HR vs LR: $p = 0.006$, IR vs LR: $p = 0.771$), as well as a main effect of condition ($F_{1,35} = 17.988$, $p < 0.001$) and an interaction of line and condition ($F_{2,35} = 8.601$, $p = 0.001$, post-hoc tests: HR ELS vs STD: $p < 0.001$, IR ELS vs STD: $p = 0.025$, LR ELS vs STD: $p = 0.659$; $N = 6$–8 per group). (B) The relative adrenal weight on P25 showed a main effect of line ($F_{2,25} = 9.966$, $p = 0.001$, post-hoc tests: HR vs IR: $p = 0.348$, HR vs LR: $p = 0.001$, IR vs LR: $p = 0.041$) and a trend for a main effect of condition ($F_{1,25} = 3.022$, $p = 0.094$, post-hoc tests: HR ELS vs STD: $p = 0.615$, IR ELS vs STD: $p = 0.103$, LR ELS vs STD: $p = 0.439$; $N = 4$–6 per group). Panels (C) and (D) show the basal and stress-induced (15 min novelty exposure) levels of plasma corticosterone in pups on P9 and on P25, respectively. Note the different Y-axis scale in these panels. (C) The basal plasma corticosterone levels on P9 showed a main effect of line ($F_{2,40} = 27.807$, $p < 0.001$, post-hoc tests: HR vs IR: $p < 0.001$, HR vs LR: $p < 0.001$, IR vs LR: $p = 0.979$), a main effect of condition ($F_{1,40} = 8.796$, $p = 0.005$) and an interaction of line and condition ($F_{2,40} = 8.093$, $p = 0.001$, post-hoc tests: HR ELS vs STD: $p < 0.001$, IR ELS vs STD: $p = 0.690$, LR ELS vs STD: $p = 0.760$; $N = 7$–8 per group). The stress-induced plasma corticosterone levels on P9 only showed a main effect of line ($F_{2,40} = 27.222$, $p < 0.001$, post-hoc tests: HR vs IR: $p < 0.001$, HR vs LR: $p < 0.001$, IR vs LR: $p = 0.721$; $N = 7$–8 per group). (D) The basal plasma corticosterone levels on P25 showed a trend for a main effect of line ($F_{2,34} = 2.862$, $p = 0.071$, post-hoc tests: HR vs IR: $p = 0.095$, HR vs LR: $p = 0.239$, IR vs LR: $p = 1.0$), and a trend for a main effect of condition ($F_{1,34} = 3.027$, $p = 0.091$, post-hoc tests: HR ELS vs STD: $p = 0.612$, IR ELS vs STD: $p = 0.396$, LR ELS vs STD: $p = 0.096$; $N = 5$–8 per group). The stress-induced plasma corticosterone levels on P25 showed a main effect of line ($F_{2,32} = 30.724$, $p < 0.001$, post-hoc tests: HR vs IR: $p < 0.001$, HR vs LR: $p < 0.001$, IR vs LR: $p = 0.182$) and an interaction of line and condition ($F_{2,32} = 4.053$, $p = 0.027$, post-hoc tests: HR ELS vs STD: $p = 0.016$, IR ELS vs STD: $p = 0.256$, LR ELS vs STD: $p = 0.575$; $N = 5$–8 per group). Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line ($\approx$ $p \geq 0.1$, $\leq/\geq$, $p < 0.1$, </> $p < 0.05$). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The grey shaded area indicates the basal samples.

$F_{2,34} = 2.862$, $p = 0.071$, condition: $F_{1,34} = 3.027$, $p = 0.091$, post-hoc tests: all $p \geq 0.1$). However, after the 15-min novelty exposure, ELS-reared HR pups had significantly higher corticosterone levels than STD-raised HR pups, while IR and LR pups showed no significant difference between the ELS and STD groups (interaction of mouse line × condition: $F_{2,32} = 4.053$, $p = 0.027$, post-hoc tests: HR: $p = 0.016$, IR and LR: $p \geq 0.1$, Fig. 6D). In addition, a strong main effect of mouse line appeared in response to the novelty-stress ($F_{2,32} = 30.724$, $p < 0.001$), with HR pups reaching significantly higher stress-induced corticosterone levels than both IR and LR mice.

3.11. Candidate gene expression

On P9, the expression of *Crh* mRNA in the PVN showed an interaction of mouse line and condition ($F_{2,36} = 2.598$, $p = 0.088$). Similar to the effect observed in adult mice, post-hoc tests revealed that the relative expression of *Crh* was reduced in ELS-exposed HR mice compared to STD animals ($p = 0.051$), while the IR and LR mice showed no such effect (Suppl. Fig. 7A). The expression levels of *Crh-r, Nr3c1* and *Fkbp5* mRNA in the PVN were not significantly different between ELS and STD housed pups on P9 (Suppl. Fig. 7B–D). In the dorsal hippocampus there were no significant differences in the expression levels of *Crh, Crh-r, Nr3c1*, *Fkbp5, Nr3c2,* and *Gilz* between conditions or between lines (Suppl. Fig. 7E–J).

3.12. Effects of the ELS paradigm on dams and nesting environment

The between-lines comparison revealed no significant differences between HR, IR and LR dams in any of the quantified parameters of maternal behavior and nesting environment. However, in all three lines, the ELS paradigm lead to more fragmented maternal behavior, reflected by an increased number of exits from the nest area on P3 ($F_{1,42} = 34.634$, $p < 0.001$, post-hoc test: HR: $p = 0.004$, IR: $p < 0.001$, LR: $p = 0.042$, Suppl. Fig. 8A). In addition, ELS nests were of poorer quality, indicated by lower nest scores ($F_{1,36} = 70.226$, $p < 0.001$, post-hoc tests: all $p < 0.001$, Suppl. Fig. 9E) and the temperature in ELS nests was lower than in STD nests ($F_{1,32} = 64.612$, $p < 0.001$, post-hoc tests: HR: $p = 0.002$, IR and LR: $p < 0.001$, Suppl. Fig. 9C). A more detailed description of the results regarding the effects of the ELS paradigm on the maternal behavior and the nesting environment is provided in the supplementary material (2.1.).

4. Discussion

The aim of the presented study was to investigate a G × E interaction in an established mouse model of affective disorders with a genetic predisposition for extremes in HPA axis reactivity and to describe the short-term, as well as the lasting consequences of ELS on emotional behavior, neuroendocrine function, and gene expres-

sion profiles. We detected a range of short-term effects in pups of all three mouse lines, affecting bodyweight development, USV calling and explorative behavior, as well as stress-coping behavior. However, only offspring of HR animals showed substantial functional changes in neuroendocrine parameters shortly after ELS exposure. Long-lasting consequences of ELS also emerged primarily in HR animals, affecting their stress-coping behavior, HPA axis function, and the expression of HPA axis-related genes. In contrast, IR and LR mice showed no measurable evidence of enduring consequences of the ELS exposure, though some short-term effects had appeared in IR pups. Thus, our results suggest that a genetic predisposition for a dysregulation of the HPA axis may be a critical factor influencing the vulnerability to programming effects of early-life adversity, and that a neuroendocrine dysregulation during early development may set the stage for the emergence of endophenotypes of affective disorders later in life. We have presented data supporting these suggestions and will discuss the results in detail below.

4.1. Physiological consequences of ELS

In HR, IR and LR pups, ELS caused a delay in bodyweight gain (Fig. 4) that was independent of genetic predisposition for extremes in stress reactivity, suggesting that metabolic factors are underlying this developmental phenotype. However, the effect was transient, since by week 12, animals raised in the ELS condition had caught up in bodyweight with the STD-housed mice (Fig. 1A). Possible causes of the initial developmental delay in ELS pups include a reduced nursing time (Ivy et al., 2008), lower milk quality or poor absorption of nutrients from the milk by the pups (Yam et al., 2015), and an increased metabolic expenditure of the pups due to heat loss (Harshaw and Alberts, 2012). In line with previous studies (Brunson et al., 2005; Rice et al., 2008), our analysis of maternal behavior revealed that the ELS paradigm led to fragmented maternal care in all three mouse lines (Suppl. Fig. 8A). In addition, we for the first time showed that nests in the ELS housing condition were colder than STD nests (Suppl. Fig. 9C), suggesting that thermoregulation plays an important role in the developmental phenotype induced by this ELS paradigm. Counterintuitively, dams in the ELS condition spent significantly more time on their nest than STD housed dams (Suppl. Fig. 8B), thus, theoretically, allowing more time for nursing. Unfortunately, our video recordings were not detailed enough to enable a quantitative analysis of the nursing behavior in the nest. A possible explanation for the dams' behavior could be that the grid floor in ELS cages is rather cold and hard, and hence no attractive alternative to resting on the nest.

4.2. Behavioral consequences of ELS

During the ELS-paradigm, on P3, pups in the ELS condition showed significantly altered USV behavior. These animals made fewer USV calls, of shorter duration, and with lower peak frequency than STD housed pups (Fig. 5A, and Suppl. Fig. 4). Furthermore, ELS-housed pups showed less exploratory behavior during the USV test (Fig. 5B) and displayed a hyperactive stress-coping behavior in the FST compared to STD animals (Fig. 5E and G). However, these short-term consequences of ELS were only significant in HR and to some extent in IR pups (LR ELS pups showed only a trend for reduced exploration and actually made longer USV calls), indicating that the behavioral short-term effect of ELS are partially moderated by the animals' genetic predisposition for high HPA axis reactivity to stressors.

In adulthood, ELS-reared HR mice showed a clearly hyperactive stress-coping style in the FST compared to STD-housed HR mice (Fig. 1C–F), while IR and LR animals no longer displayed ELS-related changes in stress-coping behavior. This data was further supported by similar results in the TST (Suppl. Fig. 2G–I). The baseline locomotor activity under less stressful conditions (in the OFT) showed that, while HR and IR mice moved around more than LR mice, there was no difference between the STD- and the ELS-reared groups (Fig. 1.B and Suppl. Fig. 2A–C). Similarly, there was no indication for ELS-induced changes in anxiety-related behavior (Suppl. Fig. 2D–F). These data indicate that the pronounced hyperactivity displayed by ELS HR mice in the FST and the TST is not a general trait of these animals, but rather an extreme behavioral response to the aversive testing situation, which might be a behavioral correlate of an extreme HPA axis response.

In the context of antidepressant drug screening, struggling is regarded as an active way of coping in the FST and the TST, while floating (or immobility) is classically interpreted as a sign of behavioral despair (Petit-Demouliere et al., 2004; Porsolt et al., 1977). However, this interpretation has rightfully been debated (Borsini and Meli, 1988; O'Neill and Valentino, 1982) and it has been pointed out that for example chronic stress, a trigger for depression-like behavior, can lead to increased struggling in the FST (Platt and Stone, 1982), i.e. produce an "antidepressant-like" effect. These contradicting findings make it clear that the interpretation of struggling and immobility in tests of stress-coping behavior is not straightforward. Regarding the very high persistence and intensity of the escape-related struggling activity observed in HR mice, particularly HR ELS animals, we propose that this behavior indicates a hyperactive stress-coping style, which resembles agitation rather than an adaptive coping behavior (for further discussion, see also Touma el al. 2008).

4.3. Neuroendocrine consequences of ELS

ELS-exposed HR pups had a significantly increased relative adrenal weight, as well as elevated basal and stress-induced corticosterone levels compared to STD-housed HR pups (Fig. 6A,C,D). In contrast, ELS had a milder effect on IR mice and did not at all affect these parameters of HPA axis function in LR offspring. When interpreting these findings it is important to know that from ~P1-12 mouse pups go through a so-called "stress-hypo-responsive period" (SHRP) (Levine, 1994; Schmidt et al., 2003). This period is characterized by a blunted HPA axis response and altered gene regulation, and its likely evolutionary function is to protect the developing brain from the impact of glucocorticoids (Levine, 1994; Lupien et al., 2009; Schmidt et al., 2003). Thus, our results indicate that, particularly in HR pups, ELS-exposure caused an activation of the stress-hormone system during the SHRP, which managed to override the HPA axis suppression, so that elevated glucocorticoid levels during this critical period of development exerted epigenetic programming effects, leading to a lasting dysregulation of neuroendocrine functions. The IR and LR mouse lines were somewhat protected from these downstream effects (or recovered rapidly from their temporary impact, in the case of IR mice), demonstrating that the enduring effects of ELS depend on an interaction of genetic and environmental factors.

The stress reactivity test results in adult animals confirm this interpretation by revealing that a brief restraint period led to an extreme HPA axis response in HR ELS mice, with corticosterone levels surpassing even the already very high levels of HR STD mice (Fig. 2A and D), while adult IR and LR mice showed no ELS-associated differences in HPA axis function. Importantly, the feedback regulation of the HPA axis was not impaired by the ELS experience, since the recovery levels of corticosterone (taken 60 min after the end of the restraint) did not differ between ELS and STD mice in all three lines (Fig. 2A and D). However, the enlarged AUC (Fig. 2B) demonstrates that HR ELS animals are probably exposed to an increased amount of stress hormones throughout their life. Previous research has shown that such chronic excess of glucocorticoid exposure can lead to dendritic atrophy (Magariños

and McEwen, 1995; Woolley et al., 1990), reduce neurogenesis and the survival of immature neurons (Lucassen et al., 2015; Wong and Herbert, 2004), increase the risk for affective disorders (Bebbington et al., 1993; McEwen, 2005), and impair cognitive performance (Aisa et al., 2007; McEwen and Sapolsky, 1995). Interestingly, studies in the SR mouse model revealed that dendritic arborization in the hippocampus of young adult STD HR mice was not different from the arborization in IR and LR animals, while spine density was increased in LR mice (Pillai et al., 2012). It remains to be investigated to what extent the ELS exposure and the downstream neuroendocrine alterations (most likely leading to increased glucocorticoid exposure throughout life) may impact on structural measures of hippocampal integrity and cognitive function.

4.4. Consequences of ELS on gene expression

The above described long-lasting consequences of early-life adversity co-occurred with specific alterations on the level of gene expression (Fig. 3). These mainly affected the CRH system, a neuropeptide system that functions as the primary regulator of the neuroendocrine stress responses (Vale et al., 1981), and has repeatedly been described to play a central role in mediating the effects of early-life adversity (Brunson et al., 2001; Meaney et al., 1996; Wang et al., 2012). In our study, we observed a down-regulation of *Crh* mRNA in the PVN of HR mice after ELS both in adult mice (Fig. 3A) and in pups on P9 (Suppl. Fig. 7A), which is in accordance with findings reported by Avishai-Eliner et al. (2008) and Rice et al. (2008), who described similar effects of the limited nesting and bedding material ELS paradigm in Sprague-Dawley rat and C57Bl/6 mice. In adult animals, prolonged activation of the HPA axis generally leads to CRH depletion and compensatory up-regulation of the *Crh* mRNA in the PVN, which can be suppressed by glucocorticoid receptor activation directly in the PVN (Yi et al., 1993), or via inhibitory GABAergic pathways from the hippocampus (De Kloet et al., 1998; Plotsky et al., 1987). However, a compensatory *Crh* up-regulation does not occur during the stress-hypo-responsive period in pups (Avishai-Eliner et al., 1995), and negative feedback by glucocorticoids has been suggest as potential mechanism for this (Brunson et al., 2001). Thus, abnormally high glucocorticoid levels during the stress-hypo-responsive period, as observed in HR ELS mice, could trigger a lasting down-regulation of hypothalamic *Crh* mRNA via high levels of glucocorticoid-induced feedback in the PVN and increased activation of GABAergic innervation from the hippocampus.

In the PVN of adult HR ELS mice the down-regulation of *Crh* was accompanied by an upregulation of the *Crhr1* receptor (Fig. 3B), suggesting an internal compensatory mechanism acting within this nucleus, and by an up-regulation of *Crh* in the dorsal hippocampus (Fig. 3E). During the stress response, CRH is released from limbic brain structures, such as the hippocampus and the amygdala, into the extracellular space and acts as a neuromodulator in the interaction of limbic and endocrine systems (Brunson et al., 2002; Chen et al., 2004). Excess CRH-R1 stimulation, however, causes spine loss and dendritic atrophy in the hippocampus (Chen et al., 2008). Thus, our data suggests that aged HR ELS mice are likely to suffer from cognitive deficits and hippocampal degeneration symptoms, but this remains to be investigated.

A further important candidate gene that showed a significant change in its expression associated with ELS was *Fkbp5*. Fkbp51 (the protein of the *Fkbp5* gene) indirectly regulates the sensitivity of the glucocorticoid receptor (Binder, 2009; Grad and Picard, 2007; Touma et al., 2011), and elevated *Fkbp5* expression has been proposed as a potential pathogenetic factor for affective disorders (Binder, 2009; Klengel et al., 2012; O'Leary et al., 2011). Chronic mild stress can trigger an up-regulation of *Fkbp5* expression in the ventral hippocampus and prefrontal cortex (Guidotti et al., 2012). Using our ELS paradigm, we found a significant down-regulation of basal *Fkbp5* expression in the PVN of adult HR ELS mice, and a trend in the same direction in IR ELS animals (Fig. 3D), but no changes in other brain areas (Suppl. Table 2) and no changes on P9 (Suppl. Fig. 7D and H). Under basal conditions, *Fkbp5* expression in the PVN is relatively low, but the expression is transiently increased by an acute stress-experience (Scharf et al., 2011; Touma et al., 2011). A further down-regulation of baseline levels may be related to a potent GR-mediated negative feedback tone from the hippocampus and from an ultrashort local feedback loop within the PVN (Fries et al., 2015).

5. Conclusion

Taken together, our results show that in mice that are genetically predisposed for increased stress reactivity, early-life adversity leads to endophenotypes associated with affective disorders, leaving more stress-resilient individuals relatively unaffected in their later life. We suggest that this G × E interaction is initiated through an increased HPA axis activation during the stress-hypo-responsive period in HR animals. Abnormally elevated corticosterone levels overcome the suppression of the HPA axis and set off a programming cascade that lastingly modifies neuroendocrine functions and gene expression profiles, probably through epigenetic modifications in key regulators. The down-stream effects are stress hyper-reactivity and excess glucocorticoid exposure, with negative repercussions affecting brain function and behavior. In conclusion, the presented findings make the three SR mouse lines a highly valuable animal model to explore how genetic predispositions and environmental stress factors interact to increase, or to buffer, the risk for developing endophenotypes associated with affective disorders and therefore contribute to our understanding of the pathomechanisms underlying psychiatric diseases.

Conflict of interest

The authors have no commercial interest and report no conflict of interest with regard to the submitted manuscript and the experiments conducted.

Contributors

Silja McIlwrick and Chadi Touma designed the study. Silja McIlwrick performed all the experiments and prepared the manuscript. Chadi Touma edited the manuscript and supervised the study. Alon Chen gave advice regarding the format of the manuscript and data presentation. Alexandra Rechenberg assisted with the sample preparation for qPCR. Mariana Matthes assisted with the USV experiment. Jessica Burgstaller assisted with the analysis of the maternal behavior and USV recordings. Thomas Schwarzbauer assisted with the recording of maternal behavior. All authors have approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2016.04.023.

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SUPPLEMENTRAY MATERIAL

Genetic predisposition for high stress reactivity amplifies effects of early-life adversity

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1. Methods (supplementary)

1.1. The Stress Reactivity mouse model

The Stress Reactivity (SR) mouse model is a recently established genetic animal for affective disorders. A selective breeding approach was used to generate three mouse lines, which differ in their HPA axis responsiveness to stressors, thus creating a high reactivity (HR), intermediate reactivity (IR) and low reactivity (LR) mouse line. A detailed description of the breeding and selection procedure is published in Touma et al., 2008. Briefly, a founder generation, consisting of 100 male and 100 female outbred CD-1 mice, was subjected to the stress reactivity test (SRT, see manuscript section 2.5.3.). Based on the CORT increase (response minus initial value) measured in this test, high reactivity breeding pairs, as well as intermediate, and low reactivity breeding pairs were selected as founders to produce the F1 generation of the three mouse lines (HR, IR and LR, respectively). Through routine SRT testing of every new generation of animals at the age of 7-8 weeks, new breeding pairs are selected and three stable mouse lines have thus been created, differing significantly in their HPA axis reactivity. Extensive endophenotyping of the SR mouse model lines has henceforth revealed several phenotypic similarities between HR animals and the melancholic subtype of depression and LR animals and the atypical depression subtype, including altered activity, bodyweight, sleep architecture, stress-coping behavior and cognition (Fenzl et al., 2011;

Heinzmann et al., 2014,; Knapman et al., 2010 a,b 2011, 2012; Touma et. al, 2008, 2009). The mice used to generate the experimental animals for the present study were taken from breeding generations XXIII-XXVIII of the SR mouse model."

1.2. Candidate gene expression in adult mice

Three days after the last behavioral test, the mice (14-16 week old males, cohort I) were decapitated under basal conditions after a brief isoflurane anesthesia. Brains were dissected, snap-frozen in methylbutane, wrapped in aluminum foil and stored at -80 °C until further processing. The pituitary was removed from the scull, frozen on dry ice in a labeled tube, and stored at -80 °C until further processing. As previously described (Heinzmann et al., 2014), the frozen brains were sliced on a cryostat into 200 µm thick coronal sections and tissue punches of the paraventricular nucleus of the hypothalamus (PVN) (0.8- to 1.4 mm from *Bregma*), dorsal (-1.2 to -2,0 mm from Bregma) and ventral (-3.0 to -3,80 mm from Bregma) hippocampus and the basolateral amygdala (BLA) (-1.0 to -1,8 mm from Bregma) were acquired by micropuncture (needle ⌀ 0.8 mm). Total RNA was isolated from the collected brain tissue punches and pituitaries using RNA spin column (RNeasy Micro Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. Approximately 200 ng of the extracted RNA was reverse transcribed into cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Gene transcripts were analyzed in 384 well-plates using a qPCR kit (QuantiFast SYBR Green, Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. All samples were analyzed in duplicates using the Roche Lightcycler® 480 instrument (Roche Diagnostics, Mannheim, Germany). TATA-binding protein (*Tbp*) and Hypoxanthine-Guanine Phosphoribosyltransferase (*Hprt*) were used as housekeeping genes. A list of all measured candidate genes with the applied oligonucleotide primers is provided in supplementary table 1. Relative gene expression was calculated using the 2-ΔΔCT algorithm (Livak and Schmittgen, 2001). Crossing points were normalized to the mean of the two housekeeping genes and to the relative expression mean of the IR STD group.

1.3. Ultrasonic vocalization test

On P3 and P7, one randomly chosen male pup (cohort III) was removed from each litter, weighed and gently placed onto a glass dish (20 cm diameter) in a noise protected chamber. Since a decrease in body temperature can affect a pups' ultrasonic vocalization (USV) frequency (Allin and Banks, 1971; Blumberg et al., 1992a), we measured the body surface temperature of each pup immediately before and after the USV test, using a non-invasive infrared thermometer (Visual IR Thermometer VT02, FLUKE, Eindhoven, Netherlands). In addition, to limit the effect of thermal stress due to cooling during the USV test, a styrofoam board and a heating pad (HK 35, Beurer, Neu-Ulm, Germany) were positioned underneath the dish to maintain a temperature of 27 ± 2 °C. At the beginning of the test, the pup was positioned in the center of the dish on its four paws. USVs were recorded for a 5 min period using a sensitive microphone, mounted 12.5 cm above the center of the glass dish and analyzed using specialized software (microphone and software: Avisoft-SASLab Pro, Glienicke, Germany). After the test on P3, the pup's back was marked (Animal marker, #50441, Stoeling Co, Wood Dale, U.S.A) before returning it to the cage to exclude the possibility of testing the same animal again on P7.

1.4. Candidate gene expression in pups

In the morning of P9, one male pup per litter (cohort IV) was removed from the nest and immediately decapitated within less than 2 min from disturbing the nest. Brains were collected, snap-frozen in methylbutane, wrapped in aluminum foil and stored at -80 °C until further processing, as described above in section 1.2 of the supplementary material. Total RNA from tissue punches of the PVN was isolated, reverse transcribed the cDNA, and gene expression was analyzed using qPCR.

1.5. Effects of the ELS paradigm on dams and nesting environment

1.5.1. Bodyweight of dams

Dams were weighed once per week during the regular changing of cages on P2, P9, P17 and P25.

1.5.2. Nest temperature

During the early postnatal period, mouse pups are not able to regulate their body temperature and are therefore dependent on external sources of warmth and insulation (Sokoloff, 2001). The reduced amount of nesting material in the ELS housing condition is likely to result in a difference in nest temperature between ELS and STD nests. To quantify this thermal stressor, the nest temperature was measured on P2, 3, 5, 7, 9, 13 and 17 (cohort III). A non-invasive method using an infrared thermometer (Visual IR Thermometer VT02, FLUKE, Eindhoven, Netherlands) was employed to minimize the disturbance of the animals. Briefly, the dam was gently removed from the cage and the thermometer was centred approximately 10 cm above the pups in the nest, guided by the color-coded screen (see Suppl. Fig. 5.D). In STD cages with a full dome nest, the nest was briefly uncovered to allow an accurate measurement. The temperature in the warmest place of the nest, usually in the centre of the assembled pups, was noted down, and the dam was immediately returned to her pups (time between removal and return of the dam to the cage was below 30 seconds).

1.5.3. Nest score

As a further measure of the early-life environment, the nest quality was evaluated on P5 (cohort III), using the scale established by Deacon (2006). Nest scores ranging from 1 to 5 were assigned based on the following criteria: (1) no nest and pups scattered, (2) no intact nest but pups assembled, (3) basic nest and pups assembled, (4) incomplete dome and all pups in nest, (5) full dome and all pups in nest.

1.5.4. Maternal behavior

The limited nesting and bedding material paradigm has been reported to lead to erratic maternal behavior in rats and mice (Brunson et al., 2005; Rice et al., 2008), which in turn creates a stressful environment for the pups. To verify this finding in the SR mouse lines and to detect potential differences in maternal behavior between HR, IR and LR dams, we mounted light-sensitive video cameras above the ELS and STD cages (cohort II) and recorded the dams' behavior for 24 hrs on P3 and P7. During the dark phase dim red light was used to illuminate the cages. On both days, three one-hour time periods of the video

recordings (light phase: 9-10 a.m. and 3-4 p.m.; dark phase: 9-10 p.m.) were analyzed by a trained observer to determine the number of exits from the nest area and the time the dams spent on the nest.

2. Results (supplementary)

2.1. Effects of the ELS paradigm on dams and nesting environment

2.1.1. Bodyweight of dams

The ELS paradigm did not cause any significant changes in maternal bodyweight, i.e. within each mouse line, dams in the ELS and the STD condition did not statistically differ in bodyweight throughout the first four postnatal weeks (Suppl. Fig. 5.A). However, the dam's bodyweight on P2 showed a main effect of mouse line ($F_{2,44}$=10.500, p<0.001, Suppl. Fig. 5.B), and a repeated-measures ANOVA over all time points between P2 and P25 showed a trend for a main effect of line ($F_{2,43}$=2.604, p=0.086). From P2 until P25, all dams initially gained, and then lost some bodyweight, but this was independent of line and condition.

2.1.2. Nest temperature

The nest temperature measurements taken between P2 to P17 were analyzed using a repeated-measures ANOVA and revealed a main effect of condition ($F_{1,32}$=15.098, p<0.001, Suppl. Fig. 5.C). Post-hoc tests confirmed that the temperature in ELS nests was significantly lower than in STD nests during the ELS paradigm (P3: p<0.001), P5: p<0.001), P7: p=0.005, and P9: p=0.002), but not before (P2) or after (P13, P17), and there was no difference in nest temperature between the three mouse lines.

2.1.3. Nest score

The nest quality assessment on P5 showed that ELS nests were of a significantly lower quality than STD nests ($F_{1,42}$=70.226, p<0.001, post-hoc tests: all p<0.001), reflected in lower nest scores (Suppl. Fig. 5.E). However, there was no difference in nest quality between the three mouse lines.

2.1.4. Maternal behavior during the ELS paradigm

The video recordings of the maternal behavior on P3 revealed that dams in the ELS condition exited the nest area significantly more often than STD-housed dams ($F_{1,42}$=34.634, p<0.001) and post-hoc tests confirmed that this effect was significant in all three mouse lines (HR: p=0.004, IR: p<0.001, LR: p=0.042, Suppl. Fig. 6.A). However, the more frequent nest exits did not lead to a reduction in the overall time spent on the nest. On the contrary, ELS dams spent significantly more time on their nest than STD dams of all three lines ($F_{1,42}$=47.582, p<0.001, post-hoc tests: all $p\leq$0.001, Suppl. Fig. 6.B). On P7, there remained a strong statistical trend for a main effect of condition on the number of exits from the nest area ($F_{1,41}$=4.016, p=0.053, Suppl. Fig. 6.C), but there was no longer any difference in the time that ELS and STD dams spent on their nest (Suppl. Fig. 6.D).

Comparing the maternal behavior of the HR, IR and LR mouse lines, we detected no significant differences in any of the measured parameters of maternal behavior both on P3 and on P7, confirming pervious findings showing no difference in maternal behavior between the three lines of the SR mouse model (Touma et al., 2008).

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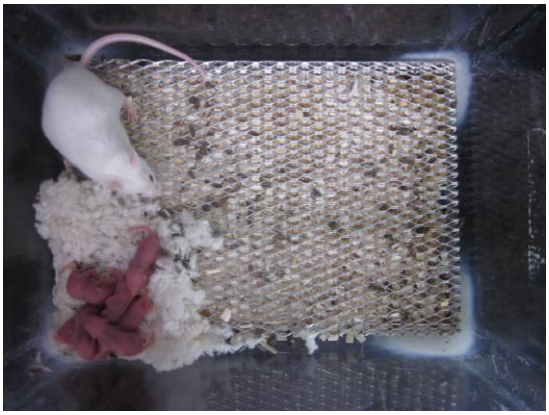
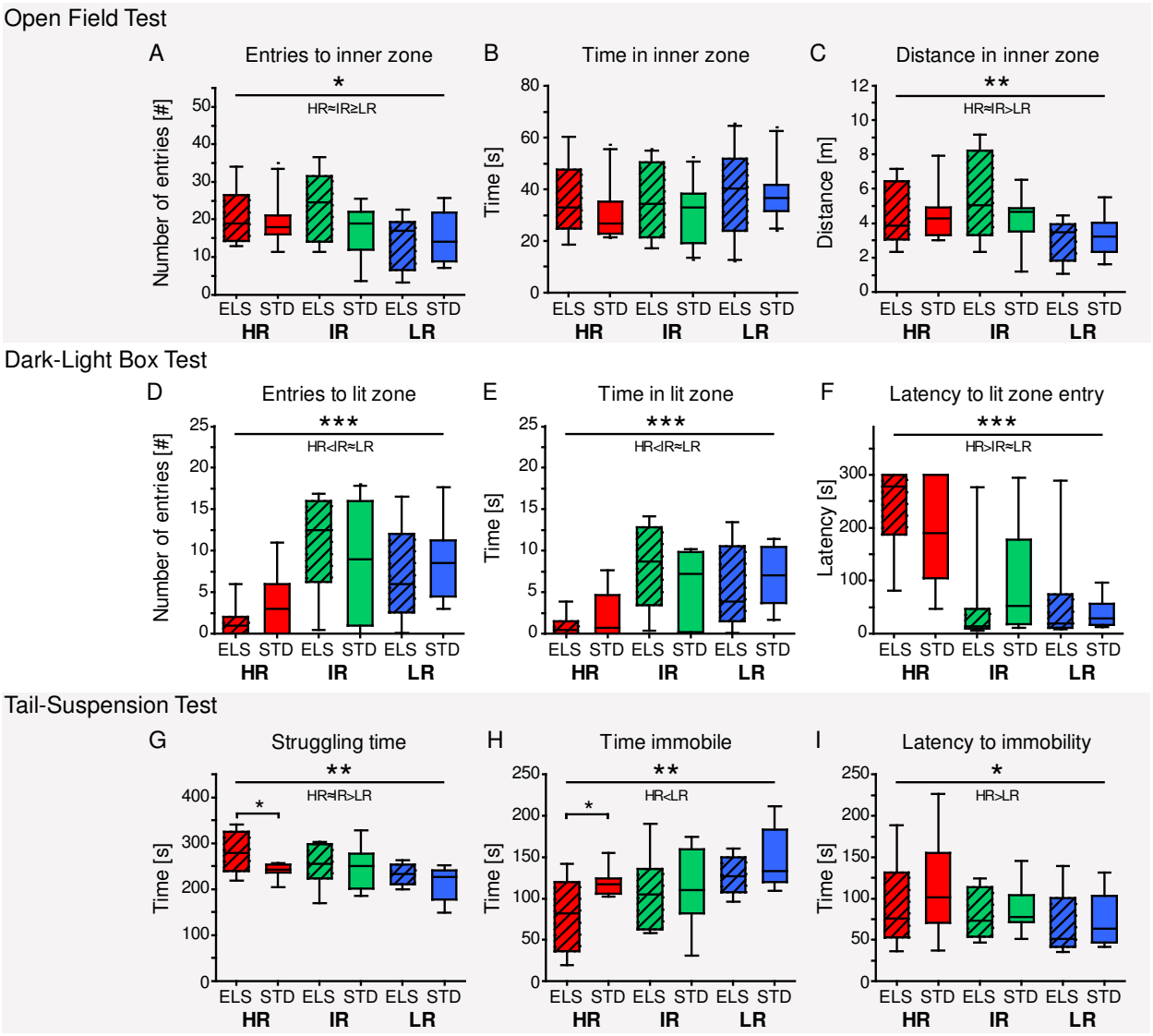
A Early-life stress (ELS) housing



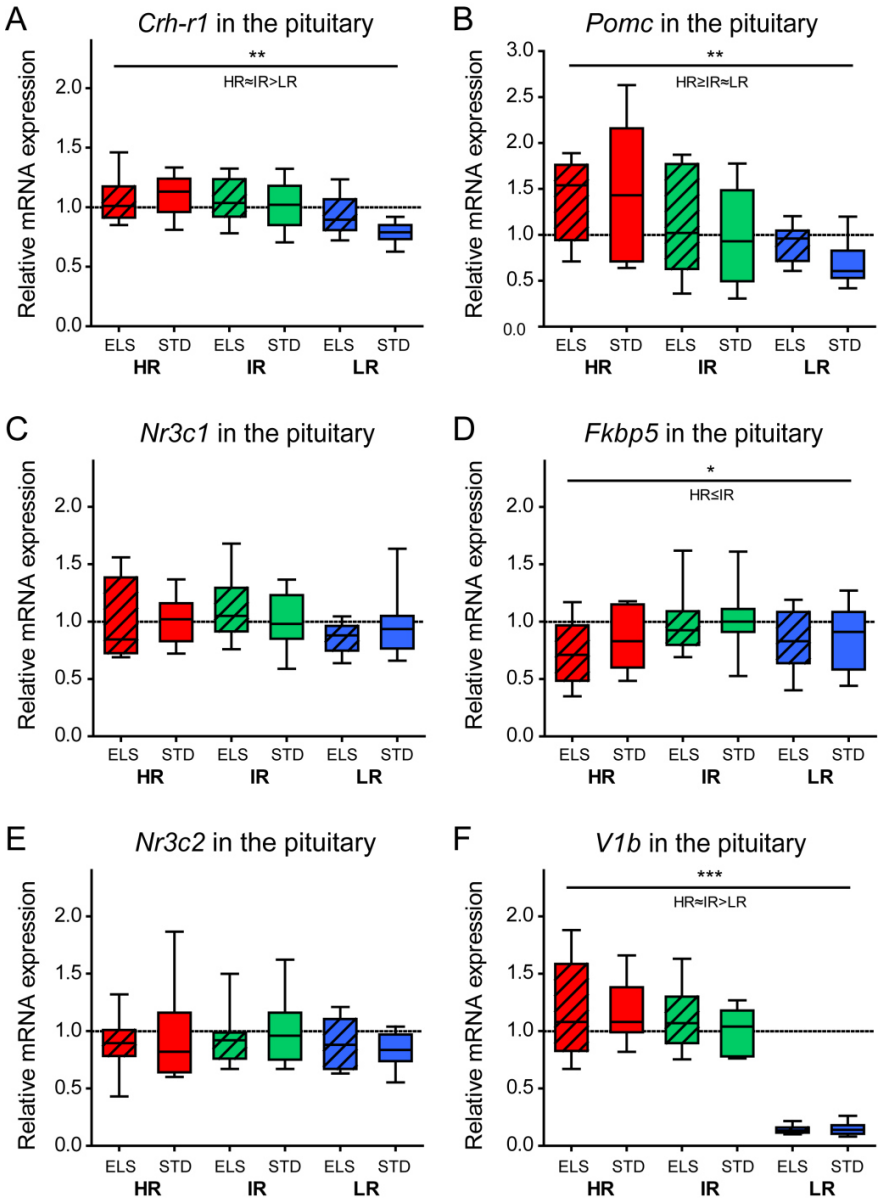
B Standard (STD) housing

Supplementary Figure 1. Representative pictures of cages from the early-life stress and standard housing conditions. (A) Early-life stress (ELS) cages were equipped with ~20 g of sawdust bedding covered by an aluminum grid and half a nestlet (~5 g) as nesting material. **(B)** Standard (STD) cages were equipped with normal amounts of sawdust bedding (~100 g) and two nestlets (~20 g).



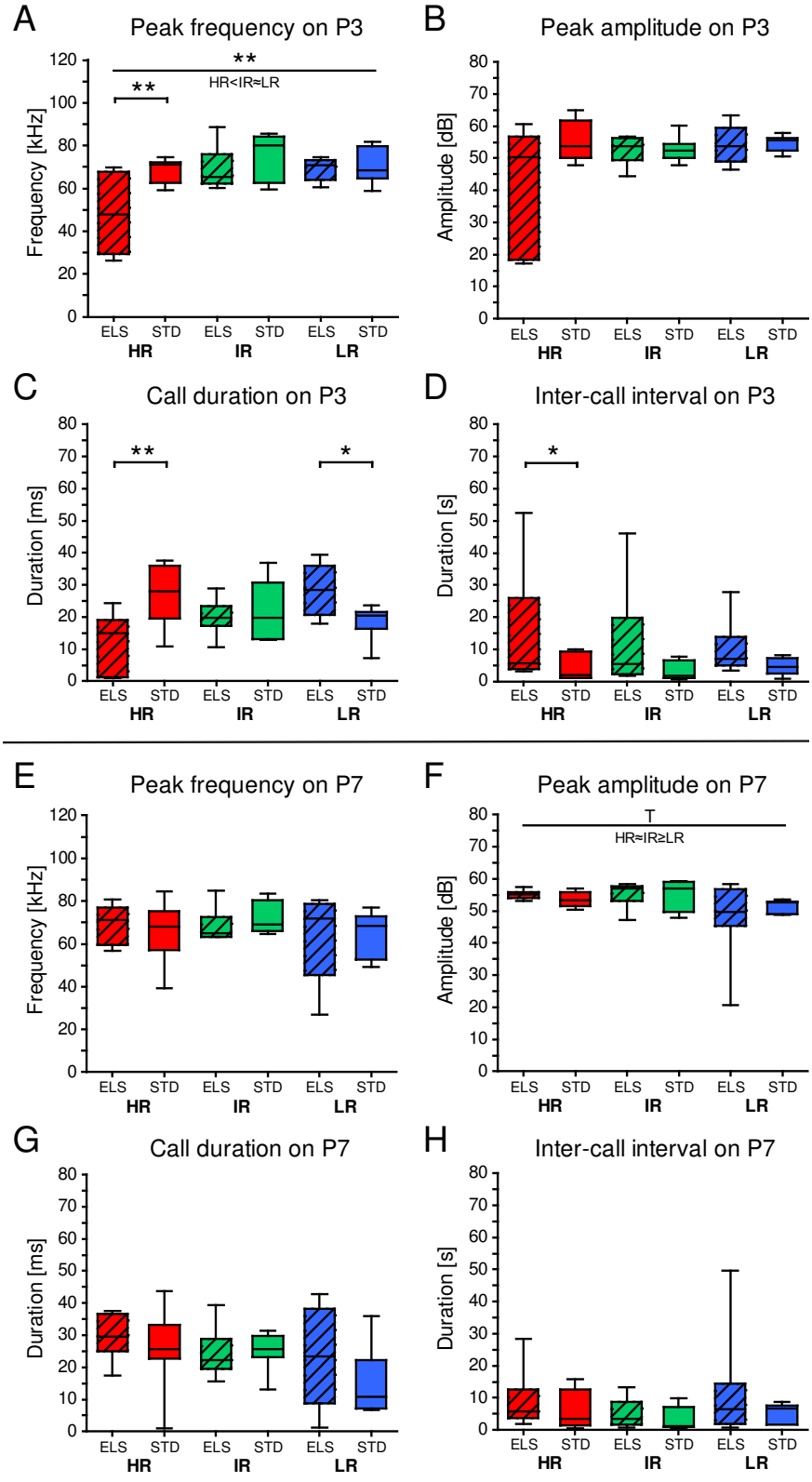
Supplementary Figure 2. Behavior testing of adult animals. Results of the open field test, dark-light box test, and tail-suspension test of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions, are presented as box plots, showing the median (horizontal line in the boxes), 25-75% (boxes) and 10-90 % (whiskers), *N*=8-11 per group. **(A)** The number of entries to the inner zone of the open field arena showed a main effect of line ($F_{2,54}$=3.480, p=0.038, post-hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.098, IR vs LR: p=0.081). **(B)** The time in the inner zone did not differ between lines or conditions. **(C)** The distance traveled in the inner zone showed a main effect of line ($F_{2,54}$=6.046, p=0.004, post-hoc tests: HR vs IR: p=1.0, HR vs LR: p<0.054, IR vs LR: p=0.005). **(D)** The number of entries to the lit zone of the dark-light test showed a main effect of mouse line ($F_{2,52}$=10.562, p<0.001, post-hoc tests: HR vs IR: p<0.001, HR vs LR: p=0.032, IR vs LR: p=0.226). **(E)** The time in the lit zone showed a main effect of mouse line ($F_{2,52}$=9.475, p<0.001, post-hoc tests: HR vs IR: p<0.001, HR vs LR: p=0.010, IR vs LR: p=1.0). **(F)** The latency to the first lit zone entry showed a main effect of mouse line ($F_{2,52}$=18.404, p<0.001, post-hoc tests: HR vs IR: p<0.001, HR vs LR: p<0.001, IR vs LR: p=1.0). **(G)** The time spent struggling during the tail suspension test showed a main effect of

mouse line ($F_{2,54}$=5.987, p=0.004, post-hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.004, IR vs LR: p=0.041) and a trend for an interaction of line and condition ($F_{2,54}$=3.740, p=0.058, post-hoc tests: HR ELS vs STD: p=0.029, IR ELS vs STD: p=0.998, LR ELS vs STD: p=0.289) **(H)** The time spent immobile during the tail-suspension test showed a main effect of mouse line $F_{2,54}$=6.104, p=0.004, post-hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.003, IR vs LR: p=0.039) and a trend for an interaction of line and condition ($F_{2,54}$=3.676, p=0.061, post-hoc tests: HR ELS vs STD: p=0.030, IR ELS vs STD: p=0.998, LR ELS vs STD: p=0.292). **(I)** The latency to the first immobile episode showed a main effect of mouse line ($F_{2,54}$=3.398, p=0.041, post-hoc tests: HR vs IR: p=0.484, HR vs LR: p=0.040, IR vs LR: p=0.778). Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line (≈ p≥0.1, ≤/≥, p<0.1, </> p<0.05). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T p<0.1, * p<0.05, ** p<0.01, *** p<0.001).



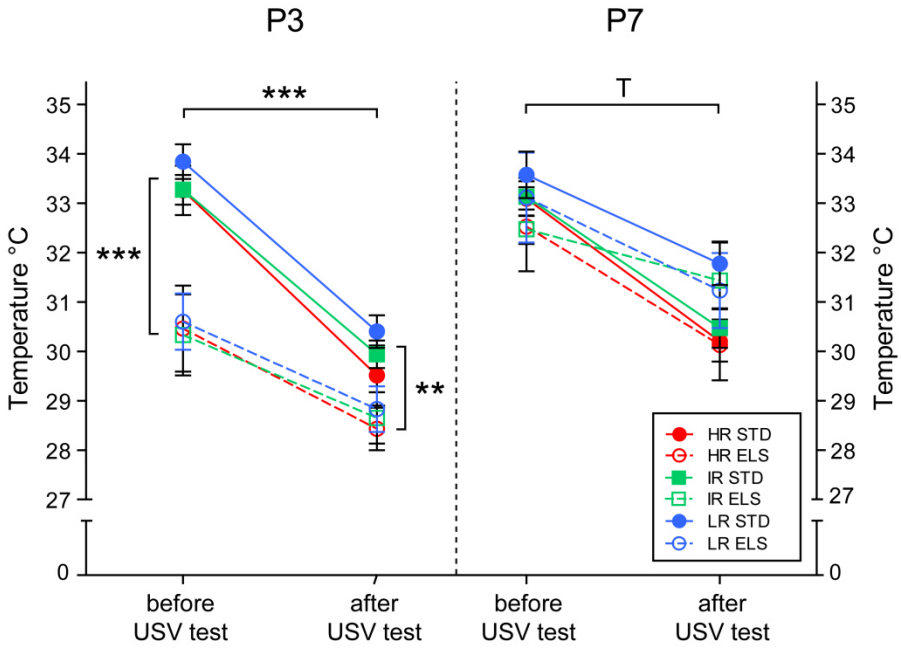
Supplementary Figure 3. Candidate gene expression in the pituitary of adult animals. Relative gene expression in the pituitary of adult high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions, is presented as box plots, showing the median (horizontal line in the boxes), 25-75% (boxes) and 10-90 % (whiskers), *N*=8-11 per group. **(A)** The expression of *Crh-r1* showed a significant difference between the three lines ($F_{2,54}$=7.875, p=0.001, post-hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.001, IR vs LR: p=0.011), but no effect of condition. **(B)** The expression of *Pomc* showed a main effect of line ($F_{2,53}$=7.694, p=0.001, post-hoc tests: HR vs IR: p=0.059, HR vs LR: p=0.001, IR vs LR: p=0.353). **(C)** The expression of *Nr3c1* showed no effect of line or condition. **(D)** The expression of *Fkbp5* showed a significant effect of line ($F_{2,54}$=3.243, p=0.047, post-hoc tests: HR vs IR: p=0.056, HR vs LR: p=1.0, IR vs LR: p=0.320), but no effect of condition. **(E)** The expression of *Nr3c2* showed no effect of line or condition. **(F)** The expression of *V1b* showed a significant effect of line ($F_{2,53}$=105.617, p<0.001, post-hoc tests: HR vs IR: p=0.356, HR vs LR and IR vs LR: p<0.001). Main effects of line are represented by a horizontal line above the graphs. The respective post-

hoc test statistics are indicated underneath the line (≈ p≥0.1, ≤/≥, p<0.1, </> p<0.05). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T p<0.1, * p<0.05, ** p<0.01, *** p<0.001).

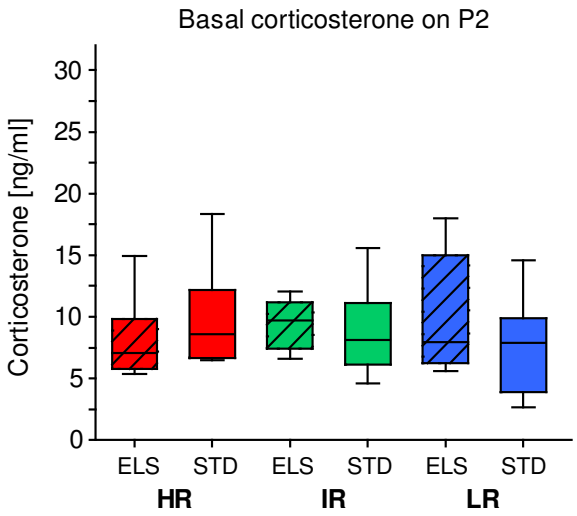


Supplementary Figure 4. Parameters assessed in the ultrasonic vocalization test in pups on P3 and P7. The frequency, amplitude, duration and inter-call interval of ultrasonic vocalization (USV) calls

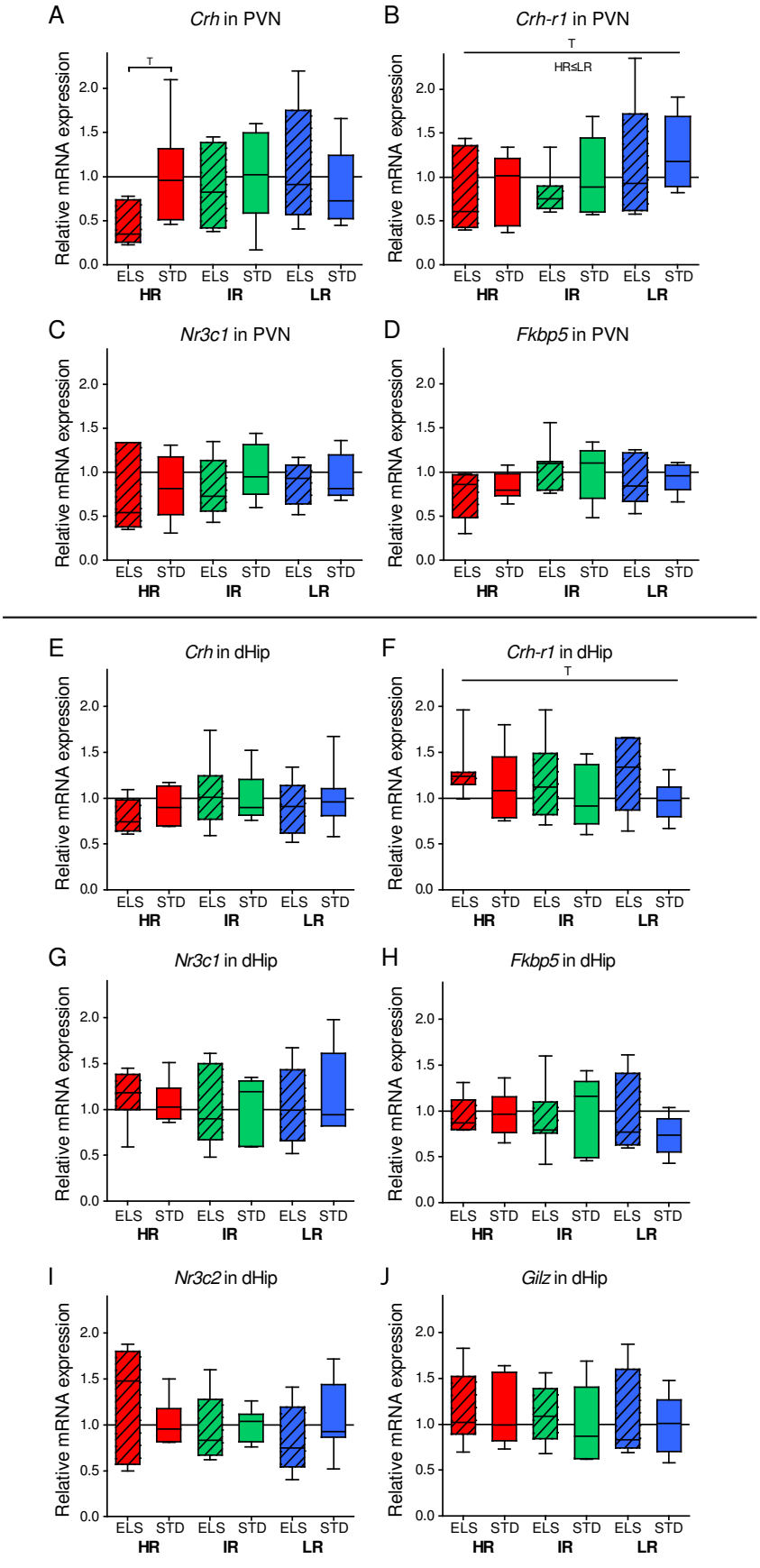
recorded in high (HR), intermediate (IR), and low (LR) reactivity mouse pups raised in early-life stress (ELS) or standard (STD) housing conditions, measured on postnatal day (P) 3 and P7, are presented as box plots, showing the median (horizontal line in the boxes), 25-75% (boxes) and 10-90 % (whiskers), *N*=6-8 per group. **(A)** The peak frequency of USV calls on P3 showed a main effect of line ($F_{2,36}$=6.125, p=0.005, post-hoc tests: HR vs IR: p=0.007, HR vs LR: p=0.024, LR ELS vs STD: p=1.0), a main effect of condition ($F_{1,36}$=6.981, p=0.012) and a strong trend for an interaction ($F_{2,36}$=3.020, p=0.061, post-hoc tests: HR ELS vs STD: p=0.001, IR ELS vs STD: p=0.405, LR ELS vs STD: p=0.816) **(B)** The peak amplitude of USV calls on P3 showed no significant differences between the lines or conditions. **(C)** The duration of calls on P3 showed a significant interaction of line and condition ($F_{2,36}$=8.314, p=0.001, post-hoc tests: HR ELS vs STS: p=0.002, IR ELS vs STD: p=0.669, LR ELS vs STD: p=0.021). **(D)** The inter-call intervals on P3 were significantly longer in ELS-housed pups compared to STD-housed pups ($F_{1,36}$=7.779, p=0.008, post-hoc tests: HR ELS vs STS: p=0.027, IR ELS vs STD: p=0.143, LR ELS vs STD: p=0.316), but there was no main effect of line. **(E)** The peak frequency of USV calls on P7 showed no significant effect of line or condition. **(F)** The peak amplitude of USV calls on P7 showed a strong trend for a main effect of line ($F_{2,35}$=3.237, p=0.051, post-hoc tests: HR vs IR: p=1.0 and HR vs LR: p=0.145, IR vs LR: p=0.072). **(H)** The duration of calls on P7, and **(G)** the inter-call intervals on P7 both showed no significant effect of line or condition. Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line (≈ p≥0.1, ≤/≥, p<0.1, </> p<0.05). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T p<0.1, * p<0.05, ** p<0.01, *** p<0.001).



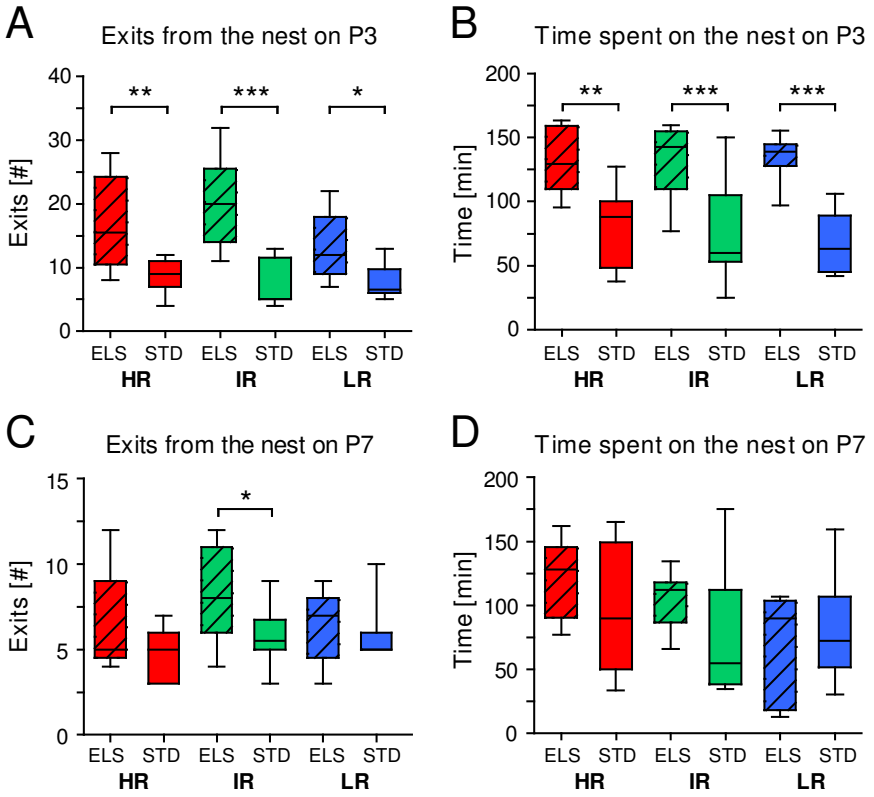
Supplementary Figure 5. Body surface temperature before and after the USV test. Body surface temperature of high (HR), intermediate (IR), and low (LR) reactivity mouse pups raised in early-life stress (ELS) or standard (STD) housing conditions, measured on postnatal day (P) 3 and 7 before and after a 5-min USV test is presented as line plots, showing means and SEM, N=6-10 per group. On P3 (left panel), there was a main effect of condition on body surface temperature both before ($F_{1,36}$=37.456, p<0.001, post hoc tests: HR and IR: p=0.002, LR: p<0.001) and after the USV test ($F_{1,36}$=12.811, p=0.001, post hoc tests: HR: p=0.096, IR: p=0.058, LR: p=0.015). In addition, there was a main effect of condition on temperature loss during the test ($F_{1,36}$=22.934, p<0.001, post hoc tests: HR and IR: p=0.007, LR: p=0.013). On P7 (right panel), the pups' body surface temperature before and after the test showed no significant difference between groups, but there was a trend for increased cooling during the test in the STD- compared to the ELS-housed animals ($F_{1,35}$=3.282, p=0.079, post hoc tests: HR and LR p≥0.1, IR: p=0.020). Statistical significance of post-hoc tests for main effects of condition are presented in the panels (T p<0.1, * p<0.05, ** p<0.01, *** p<0.001).



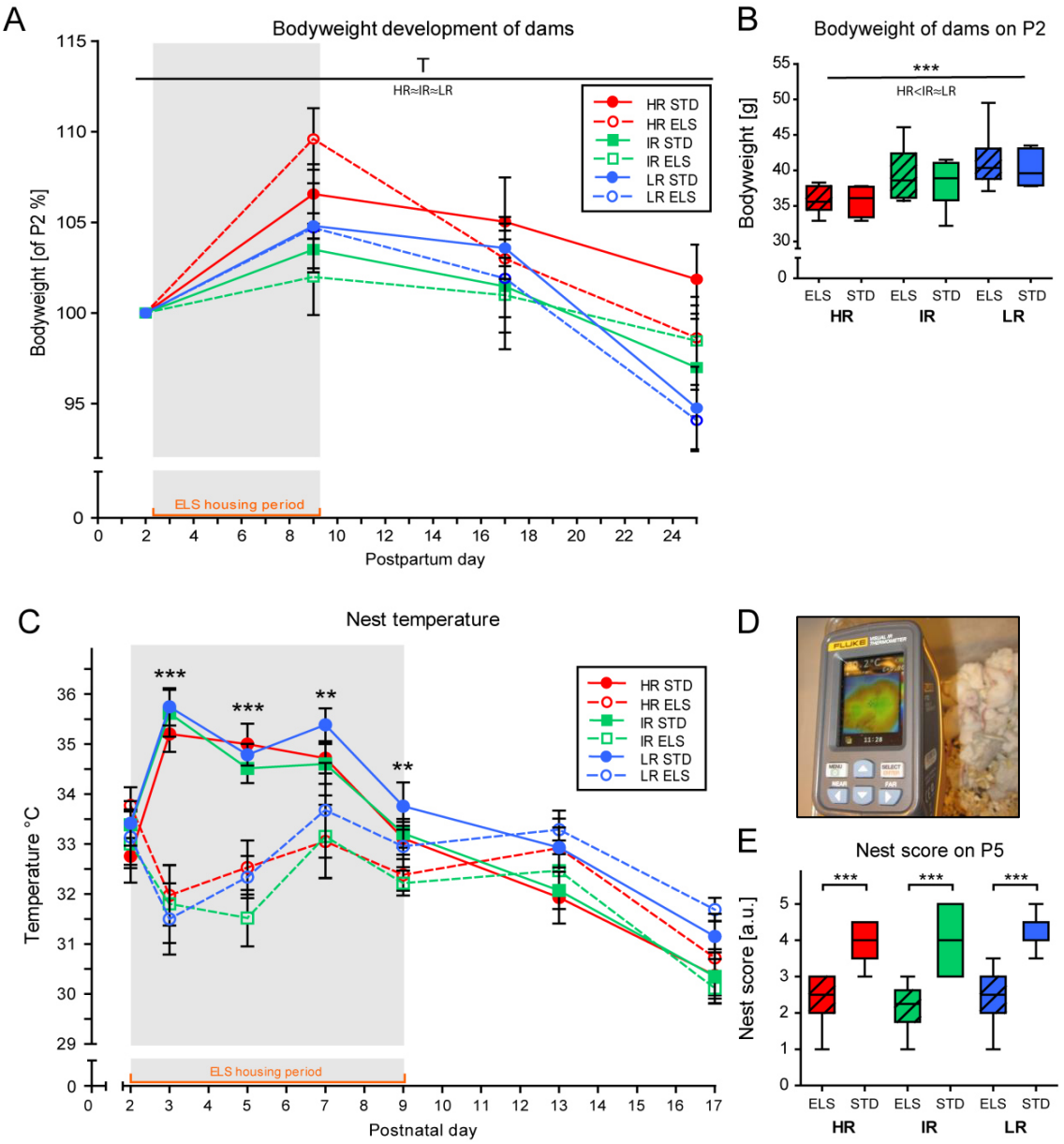
Supplementary Figure 6. Basal corticosterone levels before the early-life stress period. Basal plasma corticosterone levels of high (HR), intermediate (IR), and low (LR) reactivity mouse pups raised in early-life stress (ELS) or standard (STD) housing conditions, measured on postnatal day (P) 2, i.e. before the start of the ELS paradigm, are presented as box plots, showing the median (horizontal line in the boxes), 25-75% (boxes) and 10-90 % (whiskers), *N*=5-6 per group. Corticosterone levels on P2 did not differ significantly between the three mouse lines, nor between animals assigned to the STD and ELS housing condition.


A
Crh in PVN
B
Crh-r1 in PVN
T
HR≤LR
C
Nr3c1 in PVN
D
Fkbp5 in PVN
E
Crh in dHip
F
Crh-r1 in dHip
G
Nr3c1 in dHip
H
Fkbp5 in dHip
I
Nr3c2 in dHip
J
Gilz in dHip
Relative mRNA expression
0.0
0.5
1.0
1.5
2.0
ELS STD ELS STD ELS STD
HR IR LR

Supplementary Figure 7. Candidate gene expression in the pups. Relative gene expression in the paraventricular nucleus of the hypothalamus (PVN) **(A-D)** and the dorsal hippocampus (dHip) **(E-J)** of high (HR), intermediate (IR), and low (LR) reactivity mouse pups raised in early-life stress (ELS) or standard (STD) housing conditions, measured on postnatal day (P) 9, i.e. at the end of the ELS paradigm, is presented as box plots, showing the median (horizontal line in the boxes), 25-75% (boxes) and 10-90 % (whiskers), *N*=6-8 per group. **(A)** The expression of *Crh* showed a strong trend for an interaction of line and condition ($F_{2,36}$=2.598, p=0.088, post-hoc tests: HR ELS vs STD: p=0.051, IR ELS vs STD: p=0.691, LR ELS vs STD: p=0.249). **(B)** The expression of *Crh-r1* showed a trend for a main effect of line ($F_{2,36}$=2.859, p=0.071, post-hoc tests: HR vs IR: p=1.00, HR vs LR: p=0.088, IR vs LR: p=0.214). **(C)** The expression of *Nr3c1* showed no significant effect of line or condition. **(D)** The expression of *Fkbp5* showed no significant effect of line or condition. **(E)** The expression of *Crh* showed no significant difference between lines or between conditions. **(F)** The expression of *Crh-r1* showed a trend for a main effect of condition ($F_{1,34}$=3.706, p=0.063, post-hoc tests: all p>0.1). **(G)** The expression of *Nr3c1* showed no significant effect of line or condition. **(H)** The expression of *Fkbp5* showed no significant effect of line or condition. **(I)** The expression of *Nr3c2* showed no significant effect of line or condition. **(J)** The expression of *Gilz* showed no significant effect of line or condition. Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line (≈ p≥0.1, ≤/≥, p<0.1, </> p<0.05). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T p<0.1, * p<0.05, ** p<0.01, *** p<0.001).



Supplementary Figure 8. Maternal behavior. The number of nest exits and the time spent in the nest by high (HR), intermediate (IR), and low (LR) reactivity dams in early-life stress (ELS) or standard (STD) housing conditions, measured on postpartum (P) day 3 and 7, are presented as box plots, showing the median (horizontal line in the boxes), 25-75% (boxes) and 10-90 % (whiskers), *N*=7-10 per group. **(A)** The number of exits from the nest area on P3 showed a main effect of condition ($F_{1,42}$=34.634, p<0.001, post-hoc tests: HR ELS vs STD: p=0.004, IR ELS vs STD: p<0.001, LR ELS vs STD: p=0.042). **(B)** The time the dams spent on the nest on P3 showed a main effect of condition ($F_{1,42}$=47.582, p<0.001, post-hoc tests: all p≤0.001). **(C)** The number of exits from the nest area on P7 showed a trend for a main effect of condition ($F_{1,41}$=4.016, p=0.053, HR ELS vs STD: p=0.305, IR ELS vs STD: p=0.033, LR ELS vs STD: p=0.689. **(D)** The time the dams spent on the nest on P7 showed no significant effect of line or condition. Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line (≈ p≥0.1, ≤/≥, p<0.1, </> p<0.05). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T p<0.1, * p<0.05, ** p<0.01, *** p<0.001).



Supplementary Figure 9. Effects of early-life stress housing on dams and nesting environment. Bodyweight of dams and nest quality in cages of high (HR), intermediate (IR), and low (LR) reactivity dams in early-life stress (ELS) or standard (STD) housing conditions, measured from postpartum day (P) 2 until P25 (nest temperature measurements until P17) are presented as line plots, showing means and SEM, and as box plots, showing the median (horizontal line in the boxes), 25-75% (boxes) and 10-90 % (whiskers), *N*=7-10 per group. **(A)** The change in bodyweight between P2 and P25 (weaning) was analyzed in a repeated-measures ANOVA. The results show a trend for a main effect of line over all time points ($F_{2,43}$=2.604, p=0.086, post-hoc tests: HR vs IR: p=0.173, HR vs LR: p=0.147, IR vs LR: p=1.0. **(B)** The bodyweight of the dams on P2 showed a main effect of line ($F_{2,44}$=10.500, p<0.001, post-hoc tests: HR vs IR: p=0.011, HR vs LR: p<0.001, IR vs LR: p=0.288). **(C)** The nest temperature measurements,

analyzed using repeated-measures ANOVA, showed a main effect of condition ($F_{1,32}$=15.098, p<0.001, post-hoc tests: HR ELS vs STD: p=0.002, IR and LR ELS vs STD: p<0.001). Specifically, the temperature was significantly lower in the ELS condition compared to the STD condition nests on P3 (p<0.001), P5 (p<0.001), P7 (p=0.005), and P9 (p=0.002), i.e. only during the ELS period. **(D)** Photograph of the infra-red thermometer (Visual IR Thermometer VT02, FLUKE, Eindhoven, Netherlands) used to measure nest and pup surface temperature. **(E)** The nest scores, assigned on P5, showed a main effect of condition ($F_{1,36}$=70.226, p<0.001, post-hoc tests: all p<0.001), i.e. nest quality was reduced in all three mouse lines in the ELS housing condition. Main effects of line are represented by a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line (≈ p≥0.1, ≤/≥, p<0.1, </> p<0.05). Statistical significance of post-hoc tests for main effects of condition and the interaction are presented above the appropriate boxes (T p<0.1, * p<0.05, ** p<0.01, *** p<0.001).

Supplementary Table 1. List of investigated candidate genes. Candidate genes measured by quantitative real-time polymerase chain reaction (qPCR), including full designation, oligonucleotide primer sequence, melting temperature (T_m) and the amplicon length in base pairs (bp).

Candidate gene	Designation	Direction	Sequence	T_m	Amplicon length [bp]
Avp	Arginine Vasopressin	forward	TCGCCAGGATGCTCAACAC	67.6	174
		reverse	TTGGTCCGAAGCAGCTC	67.7	
Crh	Corticotropin releasing hormone	forward	GCATCCTGAGAGAAGTCCCTCTG	67.5	135
		reverse	GCAGGACGACAGAGCCA	64.2	
Crh-r1	Corticotropin releasing hormone receptor 1	forward	GGTCCTGCTGATCAACTTTA	59.2	152
		reverse	ACATGTAGGTGATGCCCA	59.9	
Fkbp4	FK506-binding protein 4	forward	CAACGCCACACTTGTATTTGA	63.5	143
		reverse	CTTCCACCATAGCACCATCAT	63.7	
Fkbp5	FK506-binding protein 5	forward	AGAATCAAACGGAAAGGCGAG	66.3	103
		reverse	CTCGGCAATCAAATGTCCTTC	65.6	
Gilz	Glucocorticoid-induced leucine zipper	forward	GTGGCCCTAGACAACAAGATT	61.8	122
		reverse	GAGTTCTTCTCAAGCAGCTCA	61.4	
Hprt	Hypoxanthine guanine phosphoribosyl transferase	forward	GTTGGATACAGGCCAGACTTTGT	65.1	225
		reverse	CCACAGGACTAGAACACCTGCTA	64.3	
Nr3c1	Glucocorticoid receptor	forward	CAAGGGTCTGGAGAGGACAA	64.2	220
		reverse	TACAGCTTCCACACGTCAGC	64.1	
Nr3c2	Mineralocorticoid receptor	forward	GTGTGTGGAGATGAGGC	57.2	155
		reverse	GGACAGTTCTTTCTCCGAAT	59.6	
Pomc	Proopiomelanocortin	forward	GAAGATGCCGAGATTCTGCT	63.4	222
		reverse	TTTTCAGTCAGGGGCTGTTC	64.1	
Tbp	TATA box binding protein	forward	CCCCCTTGTACCCTTCACC	65.4	285
		reverse	TGGATTGTTCTTCACTCTTGG	65.3	
V1b	Arginine vasopressin receptor 1b	forward	CCTTTCTTCAGTGTCCAGATG	61.2	141
		reverse	GTTGAAGCCCATATAGATCCA	60.3	

Supplementary Table 2. Relative mRNA expression of candidate genes. Candidate gene expression as assessed by qPCR in selected brain nuclei of adult high (HR), intermediate (IR) and low (LR) reactivity mice raised in either early-life stress (ELS) or standard (STD) housing conditions (*N*=8-11 per group) is given (mean, SEM) relative to two housekeeping genes (HPRT and TBP) and normalized to the IR STD group. Significant results of the statistical analysis (univariate ANOVA, p<0.05) are indicated (bold), including Bonferroni-corrected post-hoc tests. **(A)** Relative expression of candidate genes in the paraventricular nucleus of the hypothalamus (PVN). **(B)** Relative expression of candidate genes in the dorsal hippocampus. **(C)** Relative expression of candidate genes in the ventral hippocampus. **(D)** Relative expression of candidate genes in the basolateral amygdala (BLA). **(E)** Relative expression of candidate genes in the pituitary.

A

mRNA expression in the PVN

Gene		HR		IR		LR		ANOVA				
		ELS	STD	ELS	STD	ELS	STD	Main effect: Line	Post-hoc tests	Main effect: Condition	Interaction effect	Post-hoc tests
Nr3c1	Mean	0.93	1.10	0.77	1.00	1.02	1.02	F(2,54)=0.755, p=0.475		F(1,54)=1.696, p=0.198	F(2,54)=1.319, p=0.276	
	SEM	0.13	0.12	0.09	0.09	0.12	0.16					
Nr3c2	Mean	0.95	0.94	1.01	1.00	0.81	0.95	F(2,54)=0.965, p=0.388		F(1,54)=0.466, p=0.498	F(2,54)=0.440, p=0.647	
	SEM	0.15	0.07	0.09	0.08	0.08	0.04					
Crh	Mean	**0.62**	**1.25**	**0.81**	**1.00**	**1.01**	**0.82**	F(2,54)=0.187, p=0.830		F(1,54)=2.453, p=0.123	F(2,53)=3.592, p=0.034	HR ELS vs STD: p=0.010
	SEM	0.08	0.15	0.10	0.13	0.13	0.09					
Crh-r1	Mean	**1.36**	**0.96**	**1.02**	**1.00**	**0.92**	**0.96**	F(2,54)=2.419, p=0.020	HR vs LR: p=0.026	F(1,54)=4.349, p=0.042	F(2,54)=4.977, p=0.010	HR ELS vs STD: p=0.001
	SEM	0.12	0.05	0.06	0.05	0.07	0.05					
Fkbp4	Mean	1.00	0.98	0.94	1.00	0.86	0.97	F(2,54)=0.687, p=0.508		F(1,54)=0.555, p=0.460	F(2,53)=1.150, p=0.325	
	SEM	0.10	0.04	0.06	0.07	0.04	0.05					
Fkbp5	Mean	**0.72**	**0.96**	**0.74**	**1.00**	**0.84**	**0.76**	F(2,54)=0.380, p=0.686		F(1,54)=5.094, p=0.028	F(2,54)=2.762, p=0.072	HR ELS vs STD: p=0.011
	SEM	0.03	0.10	0.11	0.09	0.13	0.12					IR ELS vs STD: p=0.074
AVP	Mean	0.77	0.96	0.74	1.00	0.75	0.62	F(2,54)=0.725, p=0.489		F(1,54)=1.249, p=0.269	F(2,54)=0.394, p=0.676	
	SEM	0.23	0.21	0.08	0.24	0.08	0.15					

B

mRNA expression in the dorsal hippocampus

Gene		HR		IR		LR		ANOVA				
		ELS	STD	ELS	STD	ELS	STD	Main effect: Line	Post-hoc tests	Main effect: Condition	Interaction effect	Post-hoc tests
Nr3c1	Mean	**0.91**	**0.83**	**0.95**	**1.00**	**0.70**	**0.79**	F(2,53)=6.141, p=0.004	IR vs LR: p=0.003	F(1,54)=0.176, p=0.677	F(2,54)=0.944, p=0.395	
	SEM	0.09	0.05	0.07	0.08	0.06	0.04					
Nr3c2	Mean	**0.93**	**0.82**	**1.05**	**1.00**	**1.23**	**1.11**	F(2,53)=3.430, p=0.040	HR vs LR: p=0.027	F(1,54)=0.997, p=0.323	F(2,54)=0.053, p=0.948	
	SEM	0.12	0.08	0.12	0.10	0.13	0.11					
Crh	Mean	**1.38**	**0.88**	**1.10**	**1.00**	**0.92**	**1.12**	F(2,54)=0.486, p=0.618		F(1,54)=2.076, p=0.155	F(2,54)=4.662, p=0.014	HR ELS vs STD: p=0.004
	SEM	0.16	0.07	0.10	0.08	0.15	0.14					
Crh-r1	Mean	0.81	0.91	0.94	1.00	0.88	0.98	F(2,54)=0.871, p=0.424		F(1,54)=1.714, p=0.196	F(2,54)=0.033, p=0.967	
	SEM	0.06	0.09	0.06	0.09	0.06	0.09					
Fkbp4	Mean	0.97	0.86	0.99	1.00	0.94	1.15	F(2,54)=1.366, p=0.264		F(1,54)=0.264, p=0.610	F(2,54)=2.003, p=0.145	
	SEM	0.07	0.06	0.08	0.10	0.09	0.07					
Fkbp5	Mean	**0.89**	**0.86**	**1.09**	**1.00**	**0.66**	**0.73**	F(2,53)=8.531, p=0.001	IR vs LR: p<0.001	F(1,54)=0.103, p=0.749	F(2,54)=0.444, p=0.644	
	SEM	0.09	0.06	0.09	0.09	0.05	0.10					
Gilz	Mean	**0.99**	**1.03**	**0.98**	**1.00**	**0.82**	**0.87**	F(2,54)=3.692, p=0.031	HR vs LR: p=0.045	F(1,54)=0.440, p=0.510	F(2,54)=0.023, p=0.977	
	SEM	0.05	0.07	0.05	0.08	0.04	0.08		IR vs LR: p=0.087			
V1b	Mean	0.85	1.19	1.31	1.00	1.08	0.79	F(2,54)=0.593, p=0.557		F(1,54)=0.260, p=0.613	F(2,54)=1.589, p=0.215	
	SEM	0.13	0.18	0.19	0.28	0.24	0.13					

C

mRNA expression in the ventral hippocampus

Gene		HR		IR		LR		ANOVA				
		ELS	STD	ELS	STD	ELS	STD	Main effect: Line	Post-hoc tests	Main effect: Condition	Interaction effect	Post-hoc tests
Nr3c1	Mean	**0.40**	**0.44**	**0.61**	**1.00**	**0.82**	**0.77**	F(2,52)=5.241, p=0.008	HR vs IR p=0.018	F(1,52)=1.312, p=0.257	F(2,52)=1.529, p=0.226	
	SEM	0.10	0.09	0.15	0.16	0.14	0.14		HR vs LR p=0.022			
Nr3c2	Mean	**0.55**	**0.73**	**0.85**	**1.00**	**1.03**	**0.81**	F(2,53)=3.955, p=0.025	HR vs IR p=0.066	F(1,53)=0.169, p=0.683	F(2,53)=1.908, p=0.158	
	SEM	0.07	0.09	0.10	0.15	0.13	0.11		HR vs LR p=0.074			
Crh	Mean	**0.84**	**0.82**	**1.06**	**1.00**	**1.01**	**1.15**	F(2,53)=3.783, p=0.029	HR vs LR p=0.029	F(1,53)=0.132, p=0.718	F(2,53)=0.620, p=0.542	
	SEM	0.08	0.06	0.14	0.10	0.07	0.10					
Crh-r1	Mean	0.87	0.81	0.78	1.00	1.04	0.95	F(2,53)=0.322, p=0.726		F(1,53)=0.091, p=0.765	F(2,53)=1.651, p=0.202	
	SEM	0.08	0.06	0.08	0.08	0.15	0.16					
Fkbp4	Mean	1.06	1.09	1.03	1.00	1.11	1.08	F(2,53)=0.294, p=0.747		F(1,53)=0.131, p=0.719	F(2,53)=0.008, p=0.992	
	SEM	0.20	0.11	0.17	0.09	0.13	0.17					
Fkbp5	Mean	0.72	0.80	0.81	1.00	0.77	0.73	F(2,53)=2.151; p=0.126		F(1,53)=1.171, p=0.284	F(2,53)=0.899, p=0.413	
	SEM	0.08	0.08	0.09	0.09	0.08	0.09					
Gilz	Mean	0.61	0.72	0.73	1.00	0.77	0.79	F(2,53)=1.246; p=0.297		F(1,53)=1.667, p=0.203	F(2,53)=0.072, p=0.626	
	SEM	0.10	0.12	0.11	0.15	0.13	0.14					
V1b	Mean	1.07	1.16	1.03	1.00	1.39	1.35	F(2,53)=0.249; p=0.780		F(1,53)=0.047, p=0.829	F(2,53)=0.095, p=0.909	
	SEM	0.24	0.18	0.09	0.24	0.31	0.29					

D

mRNA expression in the BLA

Gene		HR		IR		LR		ANOVA				
		ELS	STD	ELS	STD	ELS	STD	Main effect: Line	Post-hoc tests	Main effect: Condition	Interaction effect	Post-hoc tests
Nr3c1	Mean	1.11	1.27	1.11	1.00	1.03	1.12	F(2,53)=0.281; p=0.756		F(1,53)=0.838, p=0.364	F(2,53)=0.036, p=0.965	
	SEM	0.17	0.15	0.11	0.09	0.11	0.11					
Nr3c2	Mean	1.33	1.33	1.18	1.00	1.12	1.03	F(2,53)=1.022; p=0.367		F(1,53)=0.066, p=0.799	F(2,53)=0.286, p=0.752	
	SEM	0.22	0.13	0.14	0.10	0.13	0.14					
Crh	Mean	**1.26**	**1.28**	**1.07**	**1.00**	**0.79**	**0.51**	F(2,53)=10.367,p<0.001	HR vs LR p<0.001	F(1,53)=3.163, p=0.081	F(2,53)=1.741, p=0.190	LR ELS vs STD: p=0.014
	SEM	0.12	0.20	0.20	0.13	0.07	0.10		IR vs LR p=0.007			
Crh-r1	Mean	0.77	0.97	0.74	1.00	1.03	0.85	F(2,52)=0.162; p=0.851		F(1,52)=0.601, p=0.442	F(2,52)=1.403, p=0.255	
	SEM	0.08	0.07	0.07	0.25	0.13	0.13					
Fkbp4	Mean	1.33	1.22	1.21	1.00	1.28	0.98	F(2,52)=0.517; p=0.851		F(1,52)=2.119, p=0.152	F(2,53)=0.145, p=0.865	
	SEM	0.22	0.14	0.20	0.16	0.19	0.16					
Fkbp5	Mean	0.98	1.19	0.79	1.00	0.92	0.94	F(2,53)=0.987; p=0.379		F(1,53)=1.662, p=0.203	F(2,52)=0.286, p=0.753	
	SEM	0.09	0.14	0.12	0.18	0.12	0.14					

E

mRNA expression in the Pituitary

Gene		HR		IR		LR		ANOVA				
		ELS	STD	ELS	STD	ELS	STD	Main effect: Line	Post-hoc tests	Main effect: Condition	Interaction effect	Post-hoc tests
Nr3c1	Mean	1.02	1.01	1.12	1.00	0.86	0.97	F(2,53)=1.615, p=0.209		F(1,53)=0.11, p=0.916	F(2,53)=1.008, p=0.372	
	SEM	0.12	0.06	0.10	0.07	0.04	0.09					
Nr3c2	Mean	0.89	1.01	0.93	1.00	0.90	0.83	F(2,53)=0.693, p=0.505		F(1,53)=0.281, p=0.598	F(2,53)=0.538, p=0.587	
	SEM	0.09	0.13	0.08	0.09	0.07	0.05					
Crh-r1	Mean	**1.06**	**1.09**	**1.06**	**1.00**	**0.94**	**0.79**	F(2,54)=7.875, p=0.001	HR vs LR: p=0.001	F(1,54)=1.855, p=0.179	F(2,54)=1,.236, p=0.299	
	SEM	0.07	0.05	0.06	0.06	0.05	0.03		IR vs LR p=0.011			
Fkbp5	Mean	**0.72**	**0.82**	**0.99**	**1.00**	**0.84**	**0.89**	F(2,54)=3.243, p=0.047	HR vs IR; p=0.056	F(1,54)=0.387, p=0.537	F(2,54)=0.152, p=0.859	
	SEM	0.10	0.08	0.09	0.09	0.08	0.11					
Pomc	Mean	**1.24**	**1.34**	**1.11**	**1.00**	**0.96**	**0.74**	F(2,53)=7.694, p=0.001	HR vs IR: p=0.059	F(1,53)=0.330, p=0.568	F(2,53)=0.482, p=0.620	
	SEM	0.15	0.22	0.17	0.16	0.06	0.09		HR vs LR p=0.001			
V1b	Mean	**1.17**	**1.08**	**1.10**	**1.00**	**0.14**	**0.15**	F(2,53)=105.6, p<0.001	HR vs LR: p<0.001	F(1,53)=0.301, p=0.586	F(2,53)=0.301, p=0.742	
	SEM	0.15	0.12	0.09	0.06	0.01	0.02		IR vs LR p<0.001			

CHAPTER 4:

Late-onset consequences of early-life stress are moderated by differences in genetic stress reactivity

4.1 Declaration of author contributions

All listed authors contributed to this manuscript: Silja McIlwrick, Tobias Pohl, Alon Chen, and Chadi Touma.

The study was designed by Silja McIlwrick and Chadi Touma. Silja McIlwrick performed the experiments and prepared the manuscript. Tobias Pohl assisted with behavioral testing in the late adulthood animal cohort. Chadi Touma edited the manuscript and supervised the study. Alon Chen gave advice regarding the format of the manuscript and data presentation. All authors approved to all modifications and approval of the final version of the manuscript.

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Late-onset consequences of early-life stress are moderated by differences in genetic stress reactivity

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Abstract

Early-life stress (ELS) has been associated with lasting cognitive impairments and with an increased risk for affective disorders. A dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis, the body's main stress response system, forms a central pathway leading from adverse experience to adverse behavioral outcomes. It remains unclear to what extent a genetic predisposition for HPA axis sensitivity or resilience influences the relationship between ELS and cognitive impairments, and which neuroendocrine and molecular mechanisms may be involved. To investigate this, we exposed animals of the stress reactivity mouse model, consisting of three independent lines selectively bred for high (HR), intermediate (IR), or low (LR) HPA axis reactivity to a stressor, to ELS and assessed their cognitive performance, neuroendocrine function, and hippocampal gene expression in early and in late adulthood. Our results show that HR animals that were exposed to ELS exhibited an HPA axis hyper-reactivity in early and late adulthood, associated with cognitive impairments in hippocampus-dependent tasks, as well as molecular changes in genes involved in the regulation of the HPA axis (*Crh*) and in neurotropic action (*Bdnf*). In contrast, LR animals showed intact cognitive function across adulthood, with no change in stress reactivity. Intriguingly, LR animals that were exposed to ELS even showed some indications of enhanced cognitive performance in late adulthood, which may be related to some late-onset changes observed in the expression of *Crh* and *Crhr1* in the dorsal hippocampus. Collectively, our findings demonstrate that the lasting consequences of ELS at the level of cognition differ as a function of genetic predispositions and suggest that an innate tendency for low stress reactivity may be protective against late-onset cognitive impairments after ELS.

1. Introduction

Many affective disorders have their roots in early-life, during the perinatal phase of development when important networks in the central nervous system (CNS) are being shaped (Heim and Nemeroff, 2002, Provencal and Binder, 2015). Offering a window of opportunity to prepare the organism for its future environment, the CNS is particularly sensitive to environmental cues during these periods, so that key signaling pathways and neuronal ensembles in the brain can be lastingly programmed in their response to relevant stimuli (Bale et al., 2010, Barker et al., 2013). While this may be an adaptive process in some cases (e.g. the attenuation of the enzyme 11β-HSD-2 can be beneficial to regulate sodium retention in nutrient poor environments (Yehuda and Seckl, 2011)), the lasting effects of early-life stress (ELS) exposure on the hypothalamic-pituitary-adrenal (HPA) axis, one of the body's main stress response system, can be detrimental for future health and coping (Meaney, 2001, Pryce and Feldon, 2003). Several studies both in humans and animal models have shown that ELS can precipitate a dysregulation of the HPA axis in later life (Heim et al., 2000b, Korosi and Baram, 2010, McIlwrick et al., 2016, Shea et al., 2005), evidenced by alterations in the corticotrophin-releasing hormone (CRH) system, exaggerated release of glucocorticoid hormones from the adrenal cortex in response to stressors, and by impaired negative feedback via glucocorticoid and mineralocorticoid receptors (GR and MR) in the brain. A lasting dysregulation of HPA axis function increases the risk for affective disorders, such as major depressive disorders (MDD) and anxiety disorders (Heim and Binder, 2012, Holsboer, 1999, Holsboer, 2000, Sanchez et al., 2001, Yehuda and Seckl, 2011).

Limbic brain areas, such as the hippocampal formation, are particularly sensitive to the signaling of stress hormones, as they abundantly express GRs and MRs, as well as CRH receptor 1 (CRH-R1). High levels of glucocorticoids (cortisol in humans, corticosterone (CORT) in murine rodents) can activate signaling pathways which exert neurotoxic effects when excessively or chronically activated (Conrad, 2008, Raber, 1998). Importantly, the hippocampus develops perinatally, so that ELS can directly impact on its development. Manifestations of the exitotoxicity of stress, or of the exposure to excessive levels of glucocorticoids, include reduced survival of newborn cells (Gould et al., 1991, Naninck et al., 2015, Sapolsky, 1985), downregulation of brain derived neurotrophic factor (BDNF) (Daskalakis et al., 2015), and reduced synaptic plasticity (Anacker et al., 2011, Liston and Gan, 2011), as well as changes of dendritic morphology and dendritic atrophy of pyramidal neurons (Alfarez et al., 2009, Brunson et al., 2005, Magarinos et al., 1996, McKittrick et al., 2000). Imaging studies in MDD patients

have extended these findings by demonstrating that reduced hippocampal volume is associated with ELS (Kronmuller et al., 2008, Teicher et al., 2012, Woon and Hedges, 2008). Furthermore, ELS has been causally linked to impaired hippocampus-dependent memory (Brunson et al., 2005, Gould et al., 2012, Nelson et al., 2007), probably via excessive glucocorticoid-induced activation of hippocampal CRH-R1 and subsequent changes in the CRH system (Ivy et al., 2010, Wang et al., 2013). In concert, the accumulated evidence demonstrates that ELS can alter HPA axis function and adversely affect the hippocampus, thus impairing cognitive performance and increasing the risk for affective disorders, such as MDD.

Genetic factors also play an important role in the etiology of MDD (Kendler, 2001, Lohoff, 2010) and are likely moderators of individual trajectories towards resilience or vulnerability. Evidence suggests that individuals with a genetic predisposition for dysregulated stress reactivity may be at greater risk for affective disorders (Holsboer, 1999, Pariante and Lightman, 2008). Interestingly, within the group of patients diagnosed with MDD, two subtypes can be distinguished based on the profile of their HPA axis function (Antonijevic, 2006, Gold and Chrousos, 1999, Gold and Chrousos, 2002). On one extreme are patients with stress hyper-reactivity (psychotic or melancholic depression subtype), displaying symptoms such as restlessness, hyperactivity, a shift in their diurnal neurohormone rhythms, impaired sleep architecture with increased REM sleep, weight loss, and cognitive impairments. On the other extreme are patients with a markedly reduced HPA axis reactivity, i.e. stress hypo-reactivity (atypical depression subtype), showing symptoms of lethargy, hypersomnia, weight gain, and a heightened sensitivity for social rejection, but no signs of cognitive impairments. This stratification of MDD patients by HPA axis function suggests that different genetic predispositions may be underlying the divergent endophenotypes (Heinzmann et al., 2014). Using the stress reactivity (SR) mouse model, our group has recently shown that a genetic predisposition for extremes in stress reactivity (high or low) interacts with ELS to shape short-term, as well as lasting consequences at the level of stress-coping behavior, neuroendocrine function, and gene expression (McIlwrick et al., 2016). The SR mouse model is a genetic animal model, which recapitulates several of the key endophenotypes of the two MDD subtypes described above (Heinzmann et al., 2014, Touma et al., 2008), including associated changes in bodyweight (Touma et al., 2009), sleep architecture (Fenzl et al., 2011, Touma et al., 2009), stress hormone profiles (Touma et al., 2008, Touma et al., 2009, Heinzmann et al., 2014), and cognitive performance (Knapman et al., 2010a, Knapman et al., 2010b, Knapman et al., 2012).

The SR mouse model thus offers an ideal starting point to investigate the interaction of genetic predisposition and environmental adversity.

In the present study we asked how a genetic predisposition for increased or decreased HPA axis reactivity interacts with ELS, laying a particular focus on cognitive function, the role of neurotrophic factors, and the expression of stress-related genes in the hippocampus. To this end, we exposed animals of the three SR mouse lines to a well-established paradigm of ELS, based on limited nesting and bedding material (Rice et al., 2008), and assessed cognitive performance, stress reactivity, and the expression of selected candidate genes (*Bdnf*, *Ntrk2* (TrkB), *Crh*, *Crh-r1*, *Nr3c1* (GR), *Nrc32* (MR), *Fkbp5*) in the dorsal and ventral hippocampus of these animals at two time points during early and late adulthood, as evidence points towards cumulative effects of glucocorticoid exposure over time.

2. Materials and methods

All presented work is in accordance with the accepted standards of humane care and use of experimental animals and was approved by the appropriate local authority.

2.1. The stress reactivity mouse model

The SR mouse model consists of three independent mouse lines selectively bred for either high (HR) or low (LR) HPA axis reactivity in response to a psychological stressor. The IR mouse line, bred for intermediate stress reactivity, serves as a reference line. Briefly, to generate this animal model, a founder generation of 100 outbred CD-1 male and female mice was tested in the stress reactivity test (SRT), which measures the animal's CORT release in response to a psychological stressor (15 min restraint, a detailed description of the SRT is provided in section 2.9.). Based on the test results, breeding pairs were selected to generate the HR, IR, and LR mouse lines. Through repeated testing and re-selection of every new generation at the age of 7-8 weeks, three inbred mouse lines were established. The SR mouse model has been extensively phenotyped and several parallels regarding symptoms associated with MDD have been highlighted. In the HR line, these include a reduced bodyweight, increased locomotor activity, hyperactive stress-coping behavior, altered sleep architecture with increased rapid eye movement (REM) sleep, and impaired cognition, akin to endophenotypes of melancholic/psychotic depression. On the other hand, LR animals show an increased bodyweight, reduced locomotor activity, passive stress-coping behavior, and intact sleep and

cognitive function, in line with endophenotypes of the atypical depression subtype (Fenzl et al., 2011, Heinzmann et al., 2014, Knapman et al., 2010a, Knapman et al., 2010b, Knapman et al., 2012, Pillai et al., 2012, Touma et al., 2009, Touma et al., 2008).

2.2. The early-life stress paradigm

To induce ELS, we used the limited nesting and bedding material paradigm (Rice et al., 2008), which creates a stressful early-life situation for the dam and her pups, without having to physically remove the dam from the litter. This ELS paradigm has been described as more ecologically valid than maternal separation (Molet et al., 2014b) and its lasting effect on the offspring has been replicated in rats and mice in several studies (Avishai-Eliner et al., 2001b, Gunn et al., 2013, Machado et al., 2013, McIlwrick et al., 2016, Naninck et al., 2015). Briefly, 16 dams of each SR mouse line were randomly assigned to either the ELS or the standard (STD) housing condition. On P2, after regulating the litters (see 2.4.1.), dams in the ELS condition were placed, together with their pups, into a macrolone cage type II, the floor of which was covered by an aluminum grid (mesh dimensions 0.4 x 0.9 cm, catalog no. 57398; McNichols Co., Tampa, U.S.A). Twenty grams of wood chip bedding and half a nestlet (~5 g) were provided for nest building. Dams in the STD condition were moved, together with their pups, into standard macrolone cage type II with ample wood chip bedding (~100 g) and 2 nestlets (~20 g) for nest building. The animals were then left undisturbed until P9, when all litters were moved to standard cages, where they stayed with their mother until weaning (P25). In a previous study using this ELS paradigm in the SR mouse model, we conducted a detailed analysis of the maternal behavior (McIlwrick et al., 2016). Briefly, the dams of all three mouse lines show similar changes in their maternal behavior (number of exits and time spent on the nest) when rearing their pups in the ELS condition. Emerging differences between the pups can thus not be attributed to differences in maternal care between the three mouse lines.

2.3. Experimental design

To investigate the gene × environment interaction of a genetic predisposition for extremes in stress reactivity with ELS, we used a three-by-two experimental design (i.e. the three SR mouse lines, HR, IR, and LR, and two conditions, ELS and STD), thus resulting in a total of six experimental groups. This two-factorial design was employed both when testing animals during early and late adulthood. In total, the data presented in this manuscript was collected from three sequential cohorts of experimental animals, generated from breeding generations XXIII, XXVI, and XXVII of the SR mouse model. Animals from experimental cohorts I and II were tested

starting at 16 ± 1 weeks of age (early adulthood), while animals from cohort III were tested starting at 26 ± 1 weeks of age (late adulthood). There was some natural stratification in the birth dates of the litters of each breeding cohort (~14 days), so that the mean age of the animals in each cohort was used to determine the date to start testing.

2.4. Animals

For this study, male animals from the three SR mouse lines (HR, IR, and LR) were used. All mice were bred in-house, and housed in sibling-pairs until two weeks before the behavioral testing, when they were single housed to avoid influences of dominance hierarchy. Mice were housed in macrolone cages under standard laboratory conditions, with standard chow and water ad libitum, a 12 h light/dark cycle (lights on at 8 a.m.) and constant humidity (55 ± 10 %). Cages were changed once per week, but never on the day of or immediately before testing.

2.4.1. Breeding of experimental animals

For breeding, 16 females and 8 males of each of the three SR mouse lines were housed in triplets for 14 days to allow mating. Thereafter, the females were moved to fresh single cages with ample nesting material. Cages were checked daily at 5 p.m. for the delivery of litters. The day a litter was discovered was defined as postnatal day 0 (P0). On P2, litters were culled to seven pups (including at least 5 males), to maximize the similarity in the early-life situation between litters. Litters with less than 5 pups and litters with only same-sex pups were not included in the experiment. On P25, pups were weaned and pair-housed with same-sex siblings until adulthood.

2.5. Bodyweight

During and after the ELS paradigm, the animals' bodyweight was closely monitored, as research has shown that it can be lastingly affected by stress manipulations during early-life (Maniam et al., 2014). Pups' weight was assessed on P2, P9, P17 and P25, when the cages were changed, and in adulthood bodyweight was measured two weeks before the behavioral testing.

2.6. Behavioral and cognitive testing

All behavioral tests were performed between 8 a.m. and 1 p.m., when the animals' CORT levels are in the circadian trough (Ishida et al., 2005, Touma et al., 2009). Between the different behavioral tests, the animals were allowed at least 48 h of rest in order to avoid carry-over effects influencing behavioral readouts in subsequent tests (McIlwain et al., 2001). During the

tests, the animals' behavior was video-recorded and tracked using automated tracking software (ANY-maze, Stoeling GmbH), with the exception of the water cross-maze test, which was scored in real-time by a trained observer. At the beginning and between different animals, the testing apparatus was cleaned with soapy water and 10% ethanol solution and dried to remove any odor cues.

2.6.1. Open field test

We used the Open field test (OFT) to assess the animals' exploratory and anxiety-related behavior. In this classic behavioral test (for review see Bailey and Crawley, 2009) the animal is placed into the center of a circular arena (⌀ 60 cm), which is dimly lit (15 Lux), and is allowed to explore freely for 5 min. Behavioral measures of exploration include the total distance travelled by the animal in the arena, as well as the time and distance it travelled in the more aversive central zone (⌀ 30 cm). For animal cohort I, tested during early adulthood, the OFT was the first in a battery of behavioral tests. In cohort III, tested during late adulthood, the OFT was incorporated into the habituation phase for the object recognition test (see section 2.6.4.). The entire habituation phase in the OF arena lasted for 20 min. To compare the results with those from the early adulthood cohort, we extracted the data from the first 5 min and analyzed these separately.

2.6.2. Water cross-maze test

The water cross-maze (WCM) is a test for hippocampus-dependent learning and memory. It was described in detail by Kleinknecht et al. (2012). Briefly, the apparatus consists of a cross-shaped maze, made of transparent acrylic glass (maze dimensions: arm length: 50 cm, arm width: 10 cm, wall height: 30 cm), which is filled with water (~23 °C, 11 cm deep). The maze was placed on a wooden platform (30 cm above the floor) in an evenly and dimly lit room (~14 Lux), containing some environmental spatial cues (shelves, ceiling pipes). We used a place learning protocol as described previously (Kleinknecht et al., 2012) to assess the animals' spatial memory performance. Briefly, the cross-maze was converted to a T-maze by blocking the arm opposite to the start arm with an acrylic glass shield. Each animal was gently placed into the water facing the back wall of the start arm and had to swim to a location where a small platform (acrylic glass, 8 x 8 cm) was submerged under the water surface at the end of the goal arm. Once the mouse had climbed onto the hidden platform, it was removed from the water, dried and returned to its home cage (which was placed partly under an infrared lamp for voluntary heating). If an animal failed to locate the platform within 30 s, it was manually guided

to the platform and 31 s was entered as latency for this trial. In all other cases, the experimenter remained motionless behind the start arm until the animal had reached the platform, so as not to provide any cue for the platform location. Each animal completed six trials per day on eight consecutive days, with an inter-trial interval (ITI) of ~10 min. As required by the place learning protocol, the start arm varied in a pseudo-random order, while the hidden platform always remained in the same position. Thus, the animals had to make use of distal spatial cues in order to minimize the time to reach their target. This place learning strategy involves building a cognitive map of the environment and is dependent on intact hippocampal function (Gutierrez-Guzman et al., 2011, Morris et al., 1982, O'Keefe et al., 1975). To assess the animals' performance, three main variables were quantified in each trial: (1) accuracy (scored as 0 if the animals entered any other arm before entering the goal arm, scored as 1 if the animal directly entered the goal arm), (2) latency (the time from entering the water to climbing onto the hidden platform), (3) number of wrong platform visits (scored as 0 if the animal did not enter the outer third of any arm apart from the goal arm, scored as 1 (or more) if the animal swam into the outer third of any non-goal arm). For the analysis, the scores on all six daily trials were averaged per animal on every training day. The WCM test was used to assess spatial learning only in animals during early adulthood (cohort I).

2.6.3. Y-maze test

The Y-maze is a frequently used behavioral test to assess hippocampus-dependent spatial memory in rodents (Dellu et al., 2000). The test is based on the innate tendency of rodents to explore unfamiliar areas. The apparatus consists of a Y-shaped maze (3 arms joining in a central area, arm length: 30 cm, arm width: 11 cm, wall height: 15 cm) made of dark grey plastic, evenly illuminated with 15 Lux. The walls of each of the three arms were marked with a white symbol (a triangle, a bar, or a plus), so that they can be clearly distinguished. The test consisted of an acquisition phase (10 min), followed by an ITI of 60 min, and a retrieval phase (5 min). During the acquisition phase, the plus-arm was blocked by a removable wall. The animal was placed into the central area, facing the corner joining the two open arms together, and was allowed to freely explore the maze. In the ITI, the animal was returned to its home cage. For the retrieval phase, the wall blocking the plus-arm was removed; the mouse was again placed into the center area and allowed to explore the entire maze. To derive a measure of cognitive performance, a discrimination ratio was calculated using the following formula: (distance in the novel arm - the mean exploration distance in the two familiar arms) / total distance in all three arms. The discrimination ratio provides a measure of whether an animal distinguished between the novel

and the familiar arms (i.e. if the ratio is larger than zero). It is also possible to calculate a discrimination ratio based on the time the animal spent in each of the arms (we provide both measures here), but this time-based discrimination ratio may sometimes be less sensitive to detect subtle differences in task performance, as animals can spend a lot time sitting in one arm, without actually exploring it. We used the Y-maze test to assess spatial memory in mice during both early and late adulthood (cohorts II and III).

2.6.4. Object recognition test

The Object Recognition Test (ORT) is one of the most frequently used tests for non-spatial memory in rodents (Akkerman et al., 2012). The performance in the ORT is dependent on hippocampal function (Clark et al., 2000), as well as on perirhinal and entorhinal cortex activity (Aggleton et al., 2010, Buckmaster et al., 2004). Similar to the Y-maze test, the ORT relies on the animals' natural preference for novelty. We followed the testing protocol described by Leger et al. (2013). Briefly, 24 h before the familiarization phase, each animal was placed into the open field arena to freely explore for 20 min to allow habituation and to reduce the stressfulness of the subsequent testing phases. On the next day, the animal was returned to the arena, where two identical objects (constructed from LEGO blocks, Lego Group, Bilund, Denmark) had been placed, and was allowed to explore for 10 min (familiarization phase). During the ITI (60 min) the animal was returned to its home cage. In the test phase, one familiar and one novel object (also built from LEGO blocks, but with a different shape and color) were placed at the identical locations to where the objects had been in the familiarization phase, and the animal was again allowed to explore for 10 min. The objects we used were same as those previously employed by Knapmann et al. (2010a) and have been pretested to make sure they were equally "interesting" to the animals. To assess the animals' memory performance, a discrimination ratio (Akkerman et al., 2012) was calculated (formula: (time exploring novel object - time exploring familiar object) / time exploring both objects). Exploration was defined as the animal's head being within a 3 cm circumference from the object's center. Any animal that failed to explore any of the objects for less than 5 s during the familiarization was excluded from the analysis. The ORT was performed in animals during late adulthood (cohort III).

2.7. Stress reactivity and CORT measurement

As a measure of HPA axis responsiveness we used the SRT, as previously described (Touma et al., 2008). Briefly, each mouse was removed from its home cage and an initial blood sample was obtained through a small incision from the ventral tail vessel (to ensure the reference sample

was very close to baseline levels, the time between initial handling of the cage until completing the blood sampling was less than 2 min). The mouse was then placed into a small restrainer (50 ml plastic tube, with holes for ventilation and an aperture in the cap for the tail) for 15 min, whereafter it was decapitated (after a very brief isoflurane anesthesia), and a "reaction sample" was collected from the trunk blood. We employed the SRT to measure the animals' stress reactivity during both early and late adulthood (cohorts II and III). All blood samples were kept on ice until they were centrifuged (4 °C) and plasma was removed for measurement of CORT using radioimmunoassay, according to the manufacturer's protocol (DRG Instruments GmbH, Marburg, Germany), with slight modifications (Touma et al., 2008). All samples were measured in duplicates and the intra- and inter-assay coefficients of variation were both below 10 %.

2.8. Gene expression

To detect lasting consequences of ELS exposure at the level of gene regulation in the three SR mouse lines, we analyzed the relative expression of selected candidate genes using quantitative polymerase chain reaction (qPCR). Early and late adulthood samples were collected from cohorts I and III to explore whether the effects of ELS changed over time. Briefly, after the SRT on the last day of testing, the animals were decapitated, the brain was removed, snap frozen in iced methylbutane, and stored at -80 °C until further processing. The brains were sectioned into 200 µm thick coronal slices and mounted onto glass slides. Tissue punches of the dorsal (-1.2 to -2,0 mm from Bregma) and ventral (-3.0 to -3,80 mm from Bregma) hippocampus (dHip and vHip) were collected via micropuncture (for further details see Heinzmann et al., 2014). Total mRNA was extracted using RNeasy columns (RNeasy Micro Kit, Qiagen, Hilden, Germany) and 200 ng of the extracted mRNA was reverse transcribed to cDNA using high-capacity transcription kits (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA). The expression of candidate genes was measured using qPCR kits (QuantiFast SYBR Green, Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. All samples were measured in duplicates (Standard deviation (SD) < 1.0) on 384 well-plates with three genes per plate, including standard curves. A list of all measured candidate genes with the applied oligonucleotide primers is provided in Table 1. The relative fold expression of each gene was calculated using the ΔΔCT method (Livak and Schmittgen, 2001) by normalizing to two housekeeping genes (TATA-binding protein (*Tbp*) and Hypoxanthine-Guanine Phosphoribosyltransferase (*Hprt*) and again normalizing to the mean of IR STD group.

2.9. Statistical analysis

All statistical data analysis was conducted in PASW 18, using two-way analysis of variance (ANOVA), with the independent factors "line" and "condition". To detect the difference between early and late adulthood data, "age" was added as an independent variable. When data points were collected repeatedly from the same animal in one test, a three-way repeated-measures ANOVA, with "line" and "condition" as between-subjects factors, and "sampling time" as a within-subjects variable, was employed. Where appropriate, post-hoc tests were conducted and corrected using the Bonferroni method. Statistical significance was accepted for $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), while $p \leq 0.1$ (T) was considered a trend.

Some data from cohort I animals was presented in a previous publication (McIlwrick et al., 2016) (OFT, expression of *Crh* and *Crhr1*). This data is included here as we aimed to provide an early adulthood comparison time point for the late adulthood data in all assessed read-outs, without increasing the number of animals sacrificed for this research.

3. Results

3.1. ELS influences bodyweight development

Figure 1A illustrates the development of the animals' bodyweight throughout the entire experimental time span. Before the start of the ELS paradigm, on P2, there were no significant differences in bodyweight between pups of the three mouse lines, or between pups assigned to the ELS or STD condition (Fig. 1B). After one week of ELS or STD housing, on P9, the analysis revealed a significant main effect of condition ($F_{1,90}$=71.557, p<0.001, post-hoc tests: all p<0.001), showing that pups that had been exposed to ELS had gained significantly less weight than STD-housed pups (Fig. 1C). In addition, there was a small difference in bodyweight between the mouse lines ($F_{2,90}$=2.846, p=0.063, post-hoc tests: HR vs LR: p=0.089, all other between-lines comparisons: p>0.1). On P17, the main effect of housing condition remained significant ($F_{1,87}$=20.396, p<0.001, post-hoc tests for ELS vs STD: HR: p=0.004, IR: p=0.012, LR: p=0.023) and a comparison between the three lines revealed that LR pups weighed more than HR pups ($F_{2,87}$=3.339, p=0.040, post-hoc tests: HR vs LR: p=0.036, all other: p>0.1) (Fig. 1D). At weaning on P25, only the main effect of condition was significant ($F_{1,87}$=18.181, p<0.001, post-hoc tests: HR: p=0.032, IR: p=0.007, LR: p=0.016) (Fig. 1E). During early adulthood, there was a clear differences in bodyweight between animals of the three lines, increasing from HR to IR to

LR ($F_{2,127}$=169.490, p<0.001, post-hoc tests: all p<0.001), as well as a main effect of ELS exposure ($F_{1,127}$=17.543, p<0.001), and an interaction of line and condition ($F_{2,127}$=5.317, p=0.007). Further analysis specified that the ELS effect was significant in the HR (p<0.001) and the LR mouse line (p=0.026) (Fig. 1F). During late adulthood, HR mice still weighed significantly less than animals of the other two lines ($F_{2,65}$=54.141, p<0.001, post-hoc tests: HR vs IR and LR: p<0.001), but IR and LR mice no longer differed (IR vs LR: p=1.0). In addition, the effect of ELS housing was still significant ($F_{1,65}$=5.331, p=0.024), and post-hoc tests showed a statistical trend in the LR line (p=0.096) (Fig. 1G).

3.2. Results of the behavioral and cognitive testing

3.2.1. Anxiety-related behavior was not affected by ELS

When animals were tested during early adulthood, the total distance travelled in the OF arena revealed a main effect of mouse line ($F_{2,54}$=23.371, p<0.001). Specifically, LR mice traveled shorter distances than HR and IR mice (post-hoc tests: HR vs IR: p=0.409, HR vs LR: p<0.001, IR vs LR: p<0.001) (Fig. 2A), confirming previous findings in the SR mouse model (Heinzmann et al., 2014, Touma et al., 2008). ELS exposure had no effect on the animals' locomotor activity in the OFT at this time point. When animals were tested during late adulthood, there was again a significant effect of mouse line on the total distance traveled ($F_{2,54}$=7.462, p=0.001, post-hoc tests: HR vs IR: p=0.084, HR vs LR: p=0.001, IR vs LR: p>0.1) (Fig. 2B). In addition, the analysis revealed a main effect of condition ($F_{1,54}$=4.130, p=0.047), and post-hoc tests showed that ELS-exposed mice in the HR line tended to move around less than STD-housed HR mice (ELS vs STD: HR: p=0.019, IR and LR: p>0.1).

At both time points (early and late adulthood), there was no indication of an effect of ELS on anxiety-related behavior (time in the inner zone: early adulthood: $F_{2,54}$=0.635, p=0.429, late adulthood: $F_{2,54}$=0.093, p=0.762) (Fig. 2C, D). Animals tested in late adulthood showed trend for a main effect of mouse line regarding the time spent in the inner zone of the OF ($F_{2,54}$=3.002, p=0.058, post-hoc tests: HR vs IR: p=0.055, HR vs LR and IR vs LR: p>0.1), indicating that HR mice spent slightly more time in the more aversive inner zone than IR animals. This may, however, be related to the increased locomotor activity of HR mice in general. In line with this, at both time points, the ratio of the path length the animals traveled in the inner and outer zone showed no significant differences between the lines (early: $F_{2,54}$=1.642, p=0.203; late:

$F_{2,54}$=0.576, p=0.565) or between conditions (early: $F_{1,54}$=0.006, p=0.940; late: $F_{1,54}$=2.320, p=0.134) (Fig. 2 E, F).

3.2.2. ELS hampers spatial learning in HR animals

To assess spatial learning and memory, we first evaluated each animal's performance during the eight days of training in the WCM test. A repeated-measures ANOVA confirmed that, overall, the animals in all six experimental groups improved their task performance over time, showing an increased accuracy ($F_{7,378}$=62.794, p<0.001) (Fig. 3A), a decreased latency to reach the hidden platform ($F_{7,378}$=85.502, p<0.001) (Fig. 3B), and a decreasing number of wrong platform visits ($F_{7,378}$=60.502, p<0.001) (Fig. 3C). We next analyzed the data for between-group effects and found a trend for a main effect of condition on accuracy ($F_{1,54}$=2.882, p=0.095) (Fig. 3A), a significant effect of condition on latency ($F_{1,54}$=4.123, p=0.047) (Fig. 3B), as well as a trend for a main effect of condition on the number of wrong platform visits ($F_{1,54}$=3.376, p=0.072) (Fig. 3C). Post-hoc tests for the accuracy measures revealed that on training days two, three, and five HR ELS mice performed worse than HR STD mice (p=0.040, p=0.032, and p=0.094, respectively), leading, overall, to a statistical trend for decreased accuracy in HR ELS compared to HR STD mice ($F_{1,54}$=3.085, p=0.085). IR ELS animals also had lower accuracy scores than IR STD animals on training day eight (p=0.048), but overall, the performance of IR ELS mice was not significantly different from IR STD animals. LR mice showed no significant effects of ELS on accuracy in this task. Post-hoc tests for the latency to reach the platform showed that both HR and IR ELS mice had some deficits on different testing days (HR, day three: p=0.032; IR, days seven and eight: p=0.060 and p=0.040), but over the course of the entire eight days of testing, the difference in latency scores between ELS and STD mice was not significant in any of the three lines. Post-hoc analysis of the number of wrong platform visits, overall, revealed that, although there were no significant differences on any particular training day, there was a trend for more platform errors in HR ELS compared to HR STD mice ($F_{1,54}$=2.891, p=0.095), but no difference between ELS and STD mice in the other two mouse lines. Together this data indicates some ELS-induced deficiencies in the acquisition of hippocampus-dependent place learning and spatial navigation mainly in HR mice, and some slight effects also in IR animals.

3.2.3. HR and LR mice show divergent effects of ELS on spatial memory

The animals' spatial memory performance was assessed using the Y-maze test. In early adulthood, the animals' distance-based discrimination ratio revealed that LR animals differentiated between novel and familiar and more extensively explored the novel arm,

compared to the familiar arms (one sample t-test against test value zero: LR ELS: t_9=4.346, p=0.001, LR STD: t_9=3.487, p=0.004) (Fig. 4A). Similarly, HR and IR mice that had been raised in STD conditions also made this distinction by travelling longer distances in the novel than in the familiar arms (HR STD: t_9=3.255, p=0.005, IR STD: t_9=2.648, p=0.014). However, HR and IR mice that had been exposed to ELS showed no preference for the novel arm (HR ELS: t_9=-1.302, p=0.113, IR ELS: t_9=-0.412, p=0.345). A comparison between all six experimental groups revealed a main effect of line ($F_{2,54}$=7.785, p=0.001, post-hoc tests: HR vs IR: p>0.1, HR vs LR: p=0.002, IR vs LR: p=0.006), a main effect of condition ($F_{1,54}$=6.828, p=0.012), and an interaction of line and condition ($F_{1,54}$=3.297, p=0.045, post-hoc tests: HR: p=0.009, IR: p=0.020 and LR: p>0.1).

In line with the results seen in young adult animals, the analysis of the Y-maze test in late adulthood revealed that, in terms of exploration distance, only HR ELS did not discriminate between the novel and the familiar arms (one sample t-test against test value zero: HR ELS: t_9=1.134, p=0.142, HR STD: t_8=3.768, p=0.003, IR ELS: t_9=3.256, p=0.005, IR STD: t_8=4.362, p=0.001, LR ELS: t_9=6.119, p<0.001, LR STD: t_9=4.614, p=0.001) (Fig. 4B). In this cohort, one HR STD and one IR STD animal had to be excluded from the analysis due to difficulties in video tracking. The ANOVA comparing the performance of all six groups showed a main effect of mouse line ($F_{2,52}$=4.410, p=0.017, post-hoc tests: HR vs IR, and IR vs LR: p>0.1, HR vs LR: p=0.012), as well as a significant interaction of line and condition ($F_{2,52}$=8.978, p<0.001). Post-hoc tests specified that HR ELS animals performed significantly worse than HR STD mice (p=0.009), while LR ELS mice actually outperformed LR STD animals (p=0.005).

Both at the early and the late adulthood time point, there was a main effect of mouse line on the total exploration distance in the Y-maze (early adulthood: $F_{2,54}$=40.514, p<0.001, post-hoc test: all p≤0.001; late adulthood: $F_{2,52}$=13.059, p<0.001, post-hoc test: HR vs IR and vs LR: p≤0.001, IR vs LR: p=1.0), showing that HR mice were more active than the other two lines (Fig. 4C, D). Importantly, there was no difference in the total distance traveled by ELS and STD animals at both time points (early adulthood: $F_{1,54}$=0.114, p=0.737; late adulthood: $F_{1,52}$=0.103, p=0.750).

As a further measure of spatial memory performance, we analyzed the time the animals spent in the different areas of the maze during the test. In early adulthood, only LR mice discriminated between the novel and the familiar arms of the Y-maze in terms of the time they spent exploring the different arms (one sample t-test against test value zero: LR ELS: t_9=2.361, p=0.022, LR STD:

t_9=2.104, p=0.033) (Fig. 4E). IR STD and HR STD animals showed a statistical trend (IR STD: t_9=1.754, p=0.056, HR STD: t_9=1.739, p=0.058), but IR ELS and HR ELS animals failed to show this discrimination or spent more time exploring the familiar arms (IR ELS: t_9=-1.493, p=0.085, HR ELS: t_9=-0.258, p=0.401). When comparing the groups in a univariate ANOVA, a main effect of mouse line was confirmed ($F_{2,54}$=3.315, p=0.044, post-hoc tests: HR vs IR and vs LR: p>0.1, IR vs LR: p=0.058), but condition did not affect this read-out of spatial memory performance.

Animals tested during late adulthood showed a similar pattern of results. Specifically, LR mice showed a significant preference for the novel arm (LR ELS: t_9=3.258, p=0.005, LR STD: t_9=2.394, p=0.020) (Fig. 4F). In the HR and IR mouse lines, those animals that had been raised in STD conditions made a distinction between novel and familiar arms (HR STD: t_8=1.905, p=0.045, IR STD: t_8=1.715, p=0.060), while ELS-exposed animals did not (HR ELS: t_9=0.392, p=0.352, IR ELS: t_9=-0.939, p=0.186). The ANOVA showed a main effect of mouse line ($F_{2,54}$=4.039, p=0.023, post-hoc tests: HR vs IR, and vs LR: p>0.1, IR vs LR: p=0.022), but no effect of condition.

To detect an effect of aging on the animals' spatial memory performance in the Y-maze test, "age" was included as independent factor in a univariate ANOVA. The results showed a main effect of age on the animals' performance in the distance-based discrimination ratio ($F_{1,106}$=5.016, p=0.027), but there was no significant difference between early and late adulthood performance in the time-based discrimination measure.

3.2.4. ELS impairs object recognition in HR animals

To detect whether the animals were able to distinguish between the previously encountered and the novel object, a discrimination ratio was calculated for each animal, as described in the methods section. One HR STD and one LR STD animal had to be excluded from the analysis, because they did not reach the criterion of exploring both objects for at least 5 s during the familiarization phase. Using one-sample t-tests (against test value zero), the analysis showed that both HR ELS and IR ELS animals did not spend more time exploring the novel object (HR ELS mice actually showed a trend for favoring the familiar object) (one-sided t-tests: HR ELS: t_9=-1.499, p=0.084, IR ELS: t_9=0.118, p=0.454) (Fig 5A). HR STD mice showed a trend for positive object discrimination (HR STD: t_8=1.617, p=0.073), and animals from the IR STD group, as well as LR ELS and LR STD all spent significantly more time investigating the novel object (IR STD: t_9=2.943, p=0.008, LR ELS: t_9=2.834, p=0.010, LR STD: t_8=3.835, p=0.003), thus showing they remembered the previously encountered familiar object. Comparisons between the

experimental groups using a univariate ANOVA revealed a significant main effect of line ($F_{2,52}$=6.003, p=0.005), showing that LR animals performed significantly better in this task than HR (p=0.006) and IR mice (p=0.025). In addition, the analysis showed a main effect of condition ($F_{1,52}$=6.925, p=0.011) and post-hoc tests specified that this effect was only significant in the HR mouse line (p=0.011), i.e. overall, HR ELS mice performed significantly worse that HR STD animals.

During the testing phase, LR animals spent overall less time exploring both objects than IR and HR mice ($F_{2,52}$=6.663, p=0.003, post-hoc tests HR vs IR: p=0.1, HR vs LR: p=0.039, IR vs LR: p=0.003), but there was no difference between ELS and STD-housed animals (Fig. 5B). A similar pattern was seen when examining the animals' exploration of each object separately (Fig. 5C, D).

3.3. HR animals show increased stress reactivity after ELS exposure

The absolute levels of plasma CORT concentration before (initial) and after (response) 15 min of restraint were analyzed using a repeated-measures ANOVA. In early adulthood, the data showed a strong effect of time point (initial vs response), confirming a significant rise in plasma CORT levels in response to the stressor in animals of all three lines (within-subjects effect: $F_{1,54}$=1459.996, p<0.001) (Fig. 6A). The initial CORT levels (taken within 2 min after the first disturbance of the animals' cage) showed that HR and IR mice had higher baseline CORT levels than LR mice ($F_{2,54}$=6.253, p=0.004, post-hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.007, IR vs LR: p=0.015), but there was no difference between conditions ($F_{1,54}$=0.148, p=0.702). In the response levels measured after restraint, the main effect of mouse line was exacerbated, with evident differences in CORT concentrations between all three lines ($F_{2,54}$=214.164, p<0.001, post-hoc tests: all p<0.001). In addition, the analysis revealed a main effect of condition ($F_{1,54}$=5.675, p=0.021), as well as an interaction of line and condition ($F_{2,54}$=4.232, p=0.020), and post-hoc comparisons showed that HR ELS mice had significantly higher plasma CORT levels in response to the stressor than HR STD mice (p<0.001), while no other mouse line showed this effect of ELS.

As there had been differences between the groups at baseline, we also analyzed the CORT increase (reaction CORT minus initial CORT level) and this measure confirmed the main effect of mouse line ($F_{2,54}$=223.319, p<0.001, post-hoc tests: all p<0.001) (Fig. 6B). In addition, the increase in plasma CORT concentration also revealed a main effect of condition ($F_{1,54}$=6.022,

p=0.017), as well as an interaction of line and condition ($F_{2,54}$=6.243, p=0.016), showing that HR ELS mice had a significantly higher increase in CORT levels than HR STD mice (p<0.001), while IR and LR mice did not show this effect of ELS exposure on stress reactivity (p=0.776 and p=0.949, respectively).

The SRT carried out in mice during late adulthood matched the earlier results (Fig. 6C). Again, there was a strong effect of time point (initial vs response) ($F_{1,53}$=1957.027, p<0.001) and the initial CORT values showed a strong trend for a main effect of line ($F_{2,53}$=3.023, p=0.057, post-hoc tests: HR vs IR: p=0.063, HR vs LR: p=0.309 IR vs LR: p=1.0), but no effect of condition ($F_{1,53}$=0.381, p=0.540). The difference between the three lines became highly significant after 15 min of restraint ($F_{2,53}$=140.272, p<0.001, post-hoc tests: all p<0.001), when, in addition, there was also an interaction of line and condition ($F_{2,53}$=3.468, p=0.038). Specifically, ELS-exposed HR mice showed a significantly stronger CORT response than STD-raised HR mice (p=0.048), while IR and LR mice seemed more resilient to this early-life programming of stress reactivity. The analysis of the increase in plasma CORT levels confirmed a main effect of mouse line ($F_{2,53}$=166.189, p<0.001, post-hoc tests: all p<0.001), and the interaction of line and condition ($F_{2,53}$=4.544, p=0.015, post-hoc tests: HR: p=0.026, IR: p=0.092, LR: p=0.344) (Fig. 6D).

3.4. ELS is associated with long-term changes in hippocampal gene expression

3.4.1. Expression of Bdnf and Ntrk2 in the hippocampus

The analysis of gene expression in the dHip of animals sacrificed during early adulthood showed that *Bndf* was downregulated in HR mice that had been exposed to ELS compared to STD-raised HR mice (p=0.031) (Fig. 7A). In addition, there was a main effect of line ($F_{1,54}$=4,091, p=0.022), showing that, overall, HR mice had a lower *Bdnf* expression levels than LR animals (p=0.038). When the expression levels of *Bdnf* were measured in animals sacrificed during late adulthood, the data showed a statistical trend in the same direction ($F_{2,52}$=2.651, p=0.080, post-hoc tests: HR vs LR: p=0.080) (Fig. 7B). Interestingly, the difference between HR ELS and STD mice was not present in the older animals; rather, at this later time point, the *Bdnf* expression levels in HR STD animals resembled those measured in HR ELS mice. The BDNF receptor coding gene *Ntrk2* showed no changes in expression between lines or conditions at either of the two time points in the dHip (Fig. 7C, D).

Mirroring the pattern seen in the dHip, the levels of *Bdnf* in the vHip during early adulthood were significantly downregulated in HR ELS compared to HR STD mice (p>0.001) (Fig. 7E). In

addition, the data showed a strong statistical trend for a main effect of line ($F_{2,52}$=2.993, p=0.059), a main effect of condition ($F_{1,52}$=4.513, p=0.038), and an interaction of line and condition ($F_{2,52}$=5.677, p=0.006). As seen in the dHip, the difference in *Bdnf* levels between HR ELS and STD mice was no longer significant when gene expression in the vHip was measured in late adulthood. Again, this seemed to be mainly due to a downward shift in the expression levels in HR STD animals (Fig. 7F). There was a clear main effect of line ($F_{2,54}$=5.218, p=0.008), and, overall, HR animals had lower *Bdnf* levels than LR mice (p=0.007), while there was no effect of condition. As in the dHip, the expression of *Ntrk2* in the vHip showed no effect of line or condition both in early and late adulthood (Fig. 7G, H).

3.4.2. Expression of Crh and Crhr1 in the hippocampus

The expression of *Crh* in the dHip was affected by an interaction of line and condition ($F_{2,48}$=4.358, p=0.018). Post-hoc analyses revealed that HR ELS mice had higher *Crh* levels than HR STD animals (p=0.008) (Fig. 8A). When measured in late adulthood, the same interaction was detected ($F_{2,52}$=5.247, p=0.009) and further analysis revealed that, as before, HR ELS mice had significantly higher *Crh* levels than HR STD mice (p=0.047) and that the opposite was true in LR animals (p=0.015), i.e. LR ELS animals displayed reduced *Crh* expression compared to LR STD mice (Fig. 8B). The expression of the CRH-R1 gene did not differ significantly between lines and conditions in early adulthood (Fig. 8C). However, in late adulthood, the data showed an interaction of line and condition ($F_{2,50}$=3.487, p=0.038), with post-hoc tests specifying that *Crhr1* levels were significantly reduced in LR ELS compare to LR STD mice (p=0.001) (Fig. 8D).

In the vHip, there was a main effect of line on the expression of *Crh* in early adulthood ($F_{1,53}$=3.782, p=0.029, post-hoc tests: HR vs LR: p=0.029) (Fig. 8E), but this effect was not observed in the later adulthood samples (Fig. 8F). The expression of *Crhr1* in the vHip showed no significant changes associated with line or condition in early or late adulthood (Fig. 8G,H).

4. Discussion

Here, we confirm previous findings showing that ELS produces late-onset and long-lasting effects on cognitive function (Brunson et al., 2005, Gould et al., 2012, Mehta and Schmauss, 2011). Our results further reveal that the nature of these effects differs between individuals, contingent with their innate stress reactivity (high versus low). The genetic predisposition is therefore centrally involved in shaping the cognitive phenotype after ELS, as well as in

mediating the consequences at the level of neuroendocrine regulation and gene expression. Below, we discuss and integrate the key findings of the presented experiments in the light of past and current research regarding the complex interaction of genes and environment.

4.1. Bodyweight and behavior

Exposure to the ELS paradigm caused a substantial delay in bodyweight development in pups of all three SR mouse lines, evidenced by reduced bodyweight gain from P2 to P9 in litters raised in ELS conditions (Fig. 1C). This main effect of ELS confirms previous findings in the SR mouse model, as well as in other rodent models (Avishai-Eliner et al., 2001b, Bath et al., 2016, Gilles et al., 1996, McIlwrick et al., 2016, Naninck et al., 2015, Rice et al., 2008). The difference in bodyweight remained significant all throughout development into early and late adulthood in the HR and the LR lines, similar to observations by others (Bath et al., 2016). Animals in the IR ELS group matched their STD housed control group by early adulthood (Fig. 1D-G). However, in a previous study in our animal model (McIlwrick et al., 2016), and as reported by others (Rice et al., 2008), no weight differences had been observed between ELS and STD mice by early adulthood. The lasting effects of ELS on bodyweight will thus need to be investigated in further studies to allow a better understanding of the circumstances under which long-term changes in bodyweight occur and how ELS influences the underlying metabolic processes.

In line with previous studies using the limited nesting material paradigm or maternal separation (Brunson et al., 2005, Millstein and Holmes, 2007, Rice et al., 2008), ELS did not affect anxiety-related behavior in the adult offspring (Fig. 2C-F). In general, the OFT results showed that HR mice were more active than LR mice, a phenotype which was present at both measurement time points (Fig. 2A, B) and confirms earlier studies (Heinzmann et al., 2014, Touma et al., 2008). While there was an indication for reduced locomotor activity in aged HR ELS mice (Fig. 2B), this finding was not confirmed in a slightly different testing set-up (Fig. 4D), pointing towards a context-specific, rather than a general effect of ELS.

4.2. Cognitive function

Cognitive function was clearly influenced by ELS in the SR mouse model. However, the three lines were not equally vulnerable to the deleterious effects of early-life adversity. HR ELS animals showed the most pronounced phenotype in terms of cognitive impairments, emerging in early adulthood [reduced spatial learning performance (Fig. 3) and impaired place memory (Fig. 4A)] and lasting into older age [impaired place memory (Fig. 4B) and impaired object

memory (Fig. 5A)]. IR ELS animals showed a similar effect in early adulthood [partially reduced spatial learning (Fig. 3) and impaired place memory (Fig. 4A)], but appeared to recover with increasing age [intact place memory (Fig. 4B) and no significant difference in object memory (Fig. 5A)]. In contrast, LR ELS animals showed no indication of cognitive deficits in early adulthood [normal spatial learning (Fig. 3) and good place memory (Fig. 4A)], and there was even some evidence for improved cognitive function in aged ELS-exposed LR mice compared to STD animals (Fig. 4B). These results highlight that the consequences of ELS on cognitive function depend very much on the genetic predisposition of the individual. Previous studies in the SR mouse model have provided evidence for deficits in cognitive function in HR animals (Knapman et al., 2010a, Knapman et al., 2010b) and have linked this to reduced hippocampal activity and neuronal integrity (Knapman et al., 2012). The proposed mechanism underlying this phenotype is a cumulative neurotoxic effect of glucocorticoids, as lifetime exposure to elevated stress hormones can give rise to progressive deficits in learning and memory (Hibberd et al., 2000, Lupien et al., 2009, Sapolsky et al., 2000). Our new data complements these findings by showing that the cognitive deficits observed in HR mice become exacerbated by ELS exposure. In a set of studies, Brunson et al. demonstrated that ELS can set off a cascade of structural and functional changes in different subfields of the hippocampus, including aberrant mossy fiber expansion, impaired long-term potentiation (LTP), and dendritic atrophy, which contribute to several cognitive impairments emerging with increased age (Brunson et al., 2005). We suggest that these same mechanisms may be acting in HR mice and that they may become increasingly detrimental through the additional sensitization of the HPA axis set in motion by the exposure to ELS.

4.3. Stress reactivity

To verify the central role of HPA axis programming in the effects of ELS, the animals' stress reactivity was tested at two time points. Both in early and in late adulthood, the results showed that HR ELS mice had an increased stress reactivity compared to STD-raised HR mice (Fig. 6). This ELS-induced augmentation in CORT release supports the hypothesis that the cognitive deficits displayed by HR ELS animals are due to excessive, cumulative glucocorticoid exposure and its adverse downstream consequences in stress sensitive regions of the brain, such as the hippocampus. Earlier work from our group demonstrated that directly after a week-long period of ELS exposure HR ELS pups had elevated basal CORT levels, which normalized by the age of weaning (McIlwrick et al., 2016). In the present study, we found no differences in basal CORT levels between adult HR ELS and STD mice, but differences in stress reactivity. This indicates

that a disruption of the HPA axis suppression during the stress-hyporesponsive period (SHRP) led to changes in the neuroendocrine programming of stress reactivity in these animals. During the SHRP, lasting from P2 –P12 in mice, moderate stressors fail to elicit a measurable physiological stress response in pups, due to a desensitization at all levels of the HPA axis (Sapolsky and Meaney, 1986). The suppression of the pups' stress reactivity is tightly controlled by maternal care and can only be disrupted by severe stressors, such as removal of the dam (Schmidt et al., 2003, Levine, 2002). The SHRP coincides with a critical period of postnatal brain development, and it is evolutionary purpose most likely to minimize the damaging effects of glucocorticoids on the developing brain (Sapolsky and Meaney, 1986). In HR mice, the fragmented maternal care induced by the ELS paradigm was apparently sufficient to disrupt the suppression of the stress response system, leading to elevated basal CORT levels during the SHRP. As the GR-mediated negative feedback loop is not yet functioning in pups at this young age (Meaney et al., 1985), the CORT levels remained elevated, with the potential to interfere with neuroendocrine receptor expression and programming throughout the brain. Thus, once the negative feedback loop became instantiated, basal CORT levels of HR ELS pups dropped to normal levels, while neuroendocrine programming mechanisms led to enhanced stress reactivity in adult HR ELS animals.

In contrast, adult LR mice showed no differences in their stress response associated with ELS rearing conditions. However, we noted that the baseline CORT levels of LR ELS mice increased significantly from early to late adulthood (pairwise comparisons LR ELS early vs late adulthood: $p=0.031$), and overall, LR mice displayed a rise in their stress response CORT levels in late adulthood (pairwise comparisons LR early vs late adulthood: $p=0.051$). To date, only some acute and no lasting effects of ELS exposure have been reported in LR animals (McIlwrick et al., 2016). Our new data now suggests that there are indeed some lasting consequences, but that these only appear with a late adulthood onset. Strikingly, the ELS-induced effects in LR mice, while not being very pronounced, seem to be rather favorable in nature: aged LR ELS animals showed signs of improved cognitive function and had slightly raised baseline CORT levels compared to LR STD mice. Since the effects of stress hormones have an inverted U-shaped relationship to cognition (de Kloet et al., 1999, Mateo, 2008, Sapolsky, 2015) and LR mice usually have a very low baseline HPA axis tone (Touma et al., 2008, Touma et al., 2009), a small increase in baseline activation may convey some beneficial aspects for attention and behavioral reactivity, by increasing the relative occupancy of MRs compared to GRs in the hippocampus

(de Kloet et al., 1999, Ferguson and Sapolsky, 2007, Herbert et al., 2006) and thus promote cognitive function.

4.4. Gene expression

BDNF is an important mediator of neural growth, maturation, and survival, as well as synaptic plasticity (Daskalakis et al., 2015, Huang and Reichardt, 2001), and thereby plays a central role in the underlying processes of learning and memory (Cunha et al., 2010). Moreover, ELS has been shown to impact on BDNF expression in the hippocampus (Liu et al., 2000), suggesting a link to cognitive deficits that have been described (Daskalakis et al., 2015). In our animals, we found that, during early adulthood, *Bdnf* levels were downregulated in the dorsal and ventral hippocampus of HR ELS mice compared to HR STD animals (Fig. 7A, E). This change in gene expression may reflect a downstream effect of exaggerated glucocorticoid stimulation, as the BDNF- and glucocorticoid-signaling pathways are closely interlinked and show bi-directional cross talk (Daskalakis et al., 2015, Jeanneteau and Chao, 2013). In late adulthood, the difference in *Bdnf* levels between HR ELS and STD mice was reduced, due to a downward shift in the expression levels of HR STD animals (Fig 7B, F). Overall, HR mice had lower *Bdnf* levels than LR mice, confirming previous findings at the level of proteins in the hippocampus (Knapman et al., 2010b). Taken together, these results indicate that a reduced availability of neurotrophins, such as BDNF, may contribute to the cognitive impairments observed in HR mice, and HR ELS in particular. Since in early adulthood, BDNF mRNA expression was changed as a function of ELS experience, it is likely to reflect a dynamic signature of downstream glucocorticoid signaling, rather than genomic differences between the mouse lines. Glucocorticoid signaling can affect the regulation of transcription factors, the epigenome, and mircoRNAs, implicating a wide array of potential programming pathways (de Kloet et al., 2009, McGowan et al., 2009, Suri and Vaidya, 2013).

Furthermore, changes in the CRH system have repeatedly been implicated in the adverse effects of ELS (Avishai-Eliner et al., 2001a, Rice et al., 2008, Fuge et al., 2014, Ivy et al., 2010, Korosi et al., 2010, Liao et al., 2014, McIlwrick et al., 2016, Wang et al., 2013). In this study, we detected an upregulation of *Crh* in the dorsal hippocampus of ELS-exposed HR mice compared to HR STD animals, which was stable over time from early to late adulthood (Fig. 8A, B), while the expression level of the *Crhr1* remained unchanged (Fig. 8C, D, G, H). An increased tone of CRH activity in the hippocampus has been implicated in dendritic remodeling (Chen et al., 2012, Chen et al., 2008) and could constitute a further pathway contributing to the observed deficits

in hippocampus-dependent cognitive tasks. In LR mice no ELS-associated changes in *Crh* and *Crhr1* expression were observed in the dorsal hippocampus during early adulthood (Fig. 8A, C), but a down regulation of both genes became evident in late adulthood (Fig. 8B, D), coinciding with somewhat enhanced cognitive performance of LR ELS mice (Fig. 4B). In conclusion, our data suggests that ELS exposure sets in motion a range of neuroendocrine and molecular alterations, the effects of which emerge gradually in adulthood and strongly depend on the animal's genetic predisposition for high or low stress reactivity.

4.5. Shortcomings and future directions

The selection of appropriate behavioral tests is critical to reliably measure small effects of experimental manipulations in behaving animals. To assess cognitive function in early and late adulthood we used the Y-maze test at both time points, but supplemented this with different behavioral tasks (i.e. the WCM was used only in early adulthood; the object recognition test was used only in late adulthood), which creates some asymmetry in the data. The reason why we did not repeat the WCM in the late adulthood animal cohort was that this test presupposes the animals' ability to navigate using visual cues and albino mice are poorly equipped for vision-based tasks (Brown and Wong, 2007). Hence, animals of the SR mouse model in general preformed relatively poorly compared to e.g. wild type C57Bl6/N (Kleinknecht et al., 2012). In addition, the WCM is a quite stressful test, due to the need for the animals to swim, which may impact on the animals' performance. Therefore, we decided to use a less stressful and less vision-dependent test in the late adulthood cohort of animals. Since the Y-maze test was identical at both time points and the results concurred well with the results of both other cognitive tasks, we believe that our conclusions regarding changes in cognitive function from early to late adulthood are nonetheless valid and justifiable.

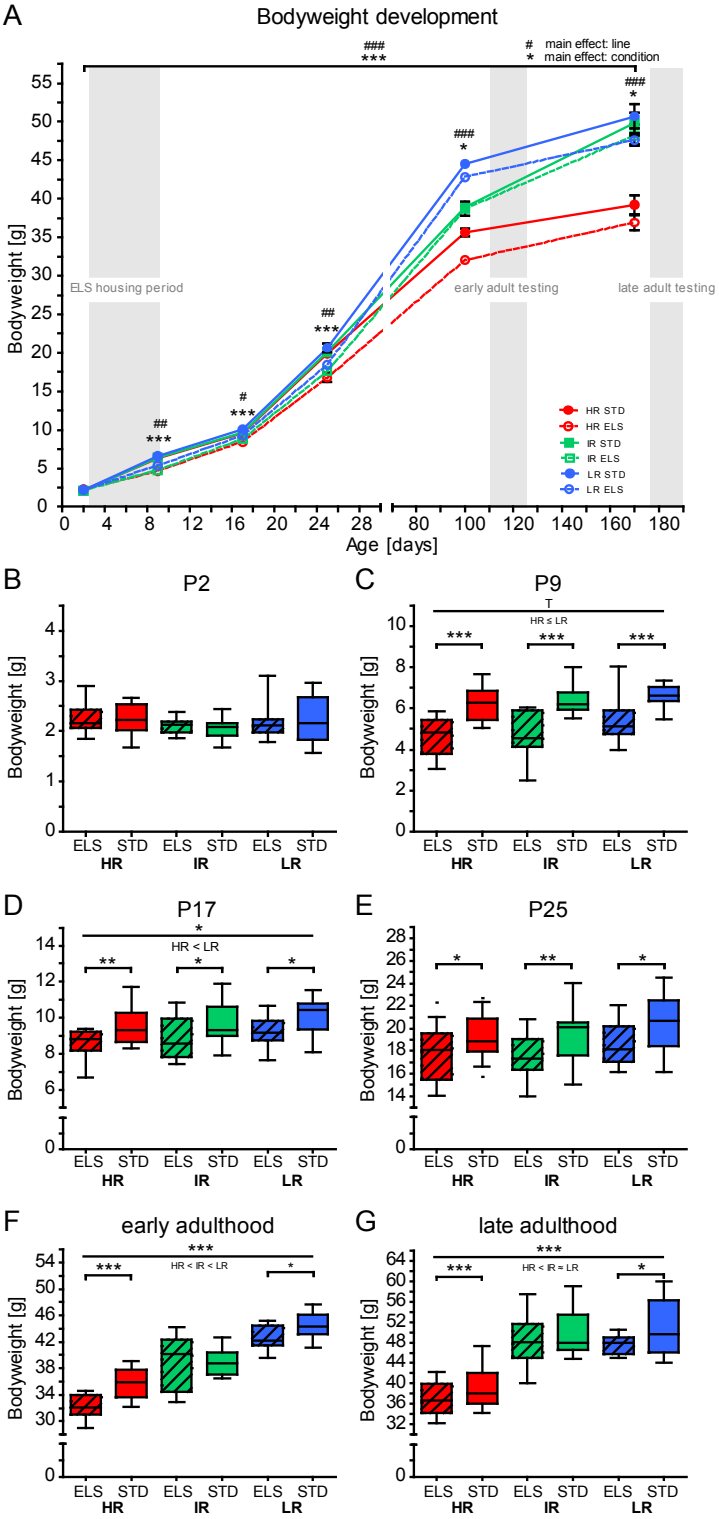
In the presented work, we investigated the effects of ELS in male mice, only. However, the clinical reality shows that women are at a 2-fold increased risk for affective and stress-related disorders (Castle, 2007, Gater et al., 1998). Several factors may play a role in this enhanced vulnerability, including differences in neuroendocrine regulation and interaction of reproductive hormones (Bale, 2006, Seeman, 1997, Young et al., 2001), which may be exacerbated through ELS experiences. As the findings from male animals are not necessarily directly translatable to females and therefore only provide limited information regarding large parts of the patient population, future studies should include female subjects in the investigation.

In recent years, several gene variants that contribute to individual risk or resilience for affective disorders have been identified, including *Nr3c1* (Wust et al., 2009), *Nr3c2* (DeRijk et al., 2006), *Fkbp5* (Ising et al., 2008), *Crhr1* (Clarke and Schumann, 2009), *Crhbp* (Wang et al., 2007), *Gabra6* (Uhart et al., 2004), and *Slc6a4* (Way and Taylor, 2010). It would contribute to our understanding of the gene × environment interaction described here to have a better knowledge about the genomic sequence of the three SR mouse lines, in order to seek confirmation for some of the known risk polymorphisms and to detect new potential candidates. Moreover, an analysis of the methylation status of candidate genes may add valuable information about epigenetic changes induced by ELS in the three SR mouse lines.

4.6. Conclusion

In summary, we present evidence showing that the lasting effects of ELS can differ greatly between individuals and that one key determinant of the long-term outcome is the individual's genetic predisposition for high or low stress reactivity. The SR mouse model provided us with an ideal tool to investigate the role of innate differences in neuroendocrine function in this gene × environment interaction. Using this animal model we could show that, while HR mice display cognitive deficits already in early adulthood, accompanied by a hyper-reactive HPA axis and lasting changes in the regulation of *Crh* and *Bdnf*, LR mice appear to be largely protected against these adverse effects of ELS. Taken together, our findings contribute to improve our understanding of factors influencing vulnerability and resilience to early-life adversity and to stress-related psychopathology. Future studies using the SR mouse model could yield valuable insights into the molecular mechanism underlying resilience and vulnerability and epigenetic programming is a promising candidate, likely to be involved in these processes. A better understanding of how ELS interacts with genetic predisposition to program individuals for increased stress sensitivity and risk for affective disorders could guide the design of future treatment options by reversing or otherwise targeting these pathological processes.

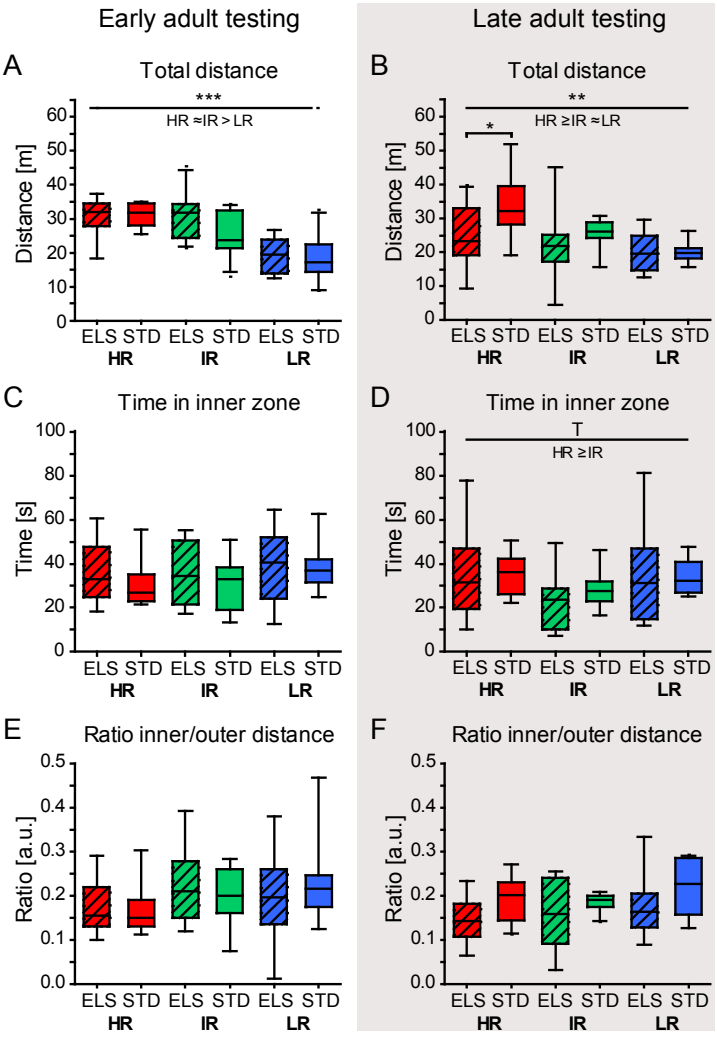
Figure 1



Bodyweight development of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions was analyzed by repeated-measures or univariate ANOVA. Data are presented as line plots showing means and standard error of the mean (SEM) (error bars) and as boxplots showing the median (horizontal line in the box), 25-75% (boxes) and 10-90 % (whiskers). **(A)** The animals' bodyweight shows different developmental trajectories. A repeated-measures ANOVA over all time points revealed a main effect of line ($F_{2,35}$=87.404, p<0.001, post hoc tests: P2: p=0.208, P9: p=0.002, P17: p=0.020, P25: p=0.005, P100: p<0.001, P170: p<0.001) and a main effect of condition ($F_{1,35}$=45.631, p<0.001, post hoc tests: P2: p=0.872, P9: p<0.001, P17: p<0.001, P25: p<0.001, P100: p=0.031, P170: p=0.018). Panels b-g show each time point in more detail: **(B)** On P2, there were no significant differences in bodyweight between the lines ($F_{2,93}$=2.367, p=0.099, post hoc tests: all p>0.1) or conditions ($F_{1,93}$=0.63, p=0.803), N=14-18. **(C)** On P9, there was a statistical trend main effect of line on bodyweight ($F_{2,90}$=2.846, p=0.063, post hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.086, IR vs LR: p=0.279), and a main effect of condition $F_{1,90}$=71.557, p<0.001, post hoc tests: all p<0.001), N=14-17. **(D)** On P17, there was a main effect of line on bodyweight ($F_{2,87}$=3.339, p=0.040, post-hoc tests: HR vs IR: p=1.0, IR vs LR: p=0.295, HR vs LR: p=0.036), and a main effect of condition

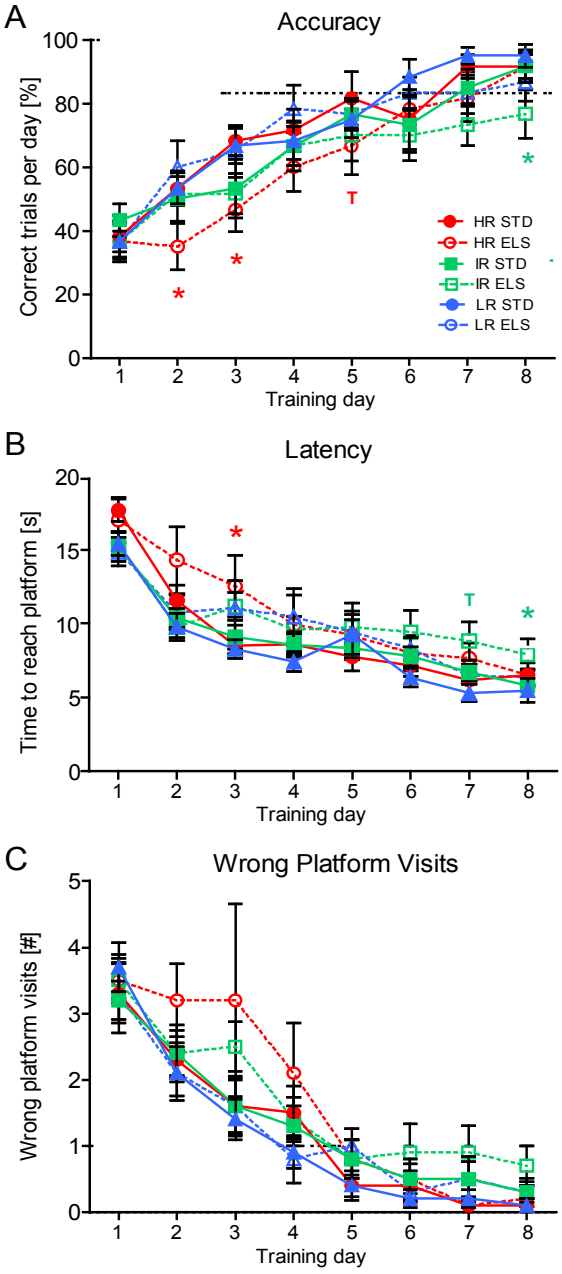
($F_{1,87}$=20.396, p<0.001, post-hoc tests: HR ELS vs STD: p=0.004, IR ELS vs STD: p=0.012, LR ELS vs STD: p=0.023), N=14-16. **(E)** On P25, the main effect of line was not significant ($F_{2,87}$=2.336, p=0.103), but there was a main effect of condition ($F_{1,87}$=18.181, p<0.001, post-hoc tests: HR ELS vs STD: p=0.032, IR ELS vs STD: p=0.007, LR ELS vs STD: p=0.016), N=14-17. **(F)** During early adulthood, there was a main effect of line ($F_{2,127}$=169.490, p<0.001, post-hoc tests: all: p<0.001), a main effect of condition ($F_{1,127}$=17.543, p<0.001), and an interaction of line and condition ($F_{2,127}$=5.317, p=0.007, post-hoc tests: HR ELS vs STD: p<0.001, IR ELS vs STD: p=0.811, LR ELS vs STD: p=0.026), N=22-23. **(G)** During late adulthood, there was a main effect of line ($F_{2,65}$=54.141, p<0.001, post-hoc tests: HR vs IR and HR vs LR: p<0.001, IR vs LR: p=1.0) and a main effect of condition ($F_{1,65}$=5.331, p=0.024, post-hoc tests: HR ELS vs STD: p=0.181, IR ELS vs STD: p=0.349, LR ELS vs STD: p=0.096), N=10-13. Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Main effects of line are represented above a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line with: </>, p≤0.05; ≤/≥, p≤0.1; ≈, p>0.1. Post-hoc statistics for main effects of condition and the interaction are presented above the appropriate boxes.

Figure 2



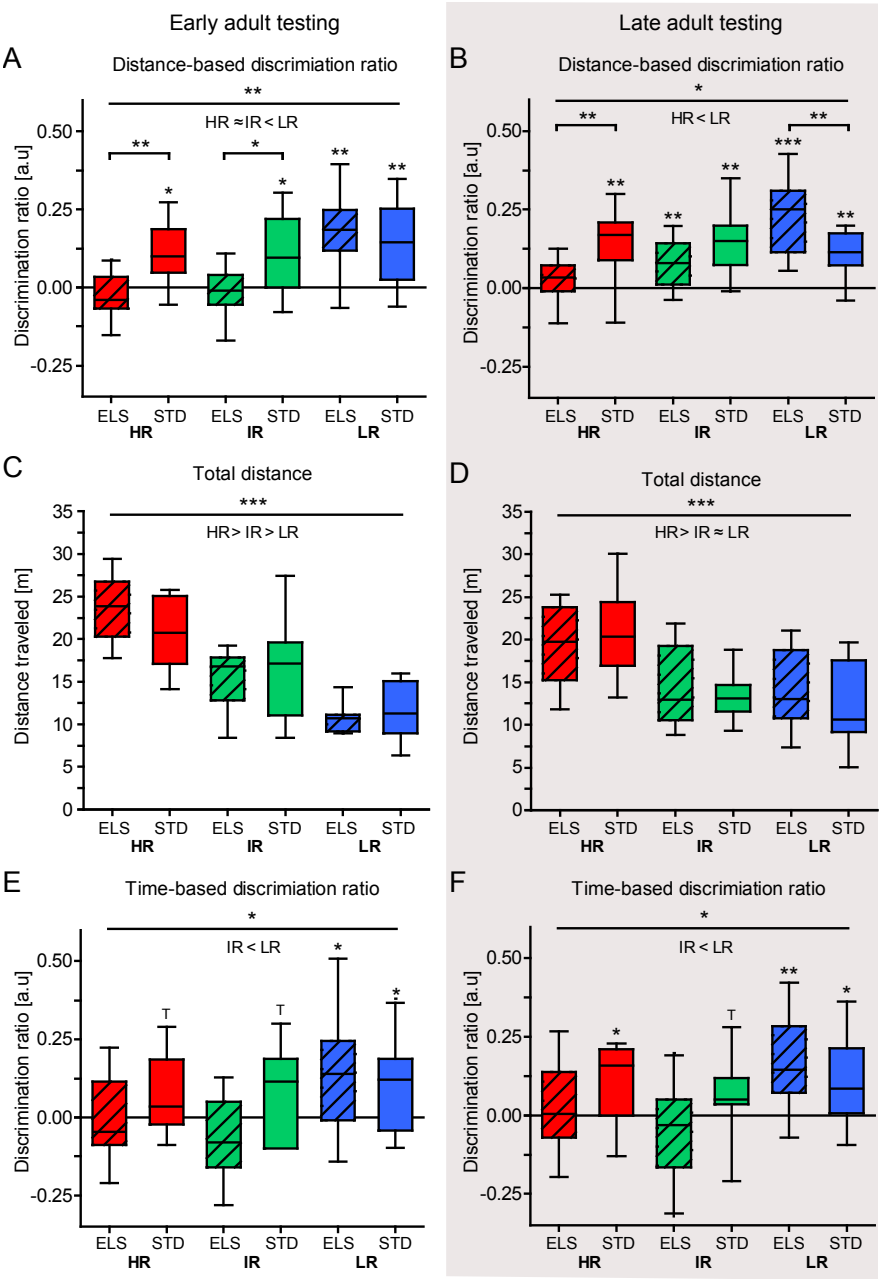
Open field test (OFT) in early and late adulthood. The behaviour of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions was analyzed by univariate ANOVA, N=10 per group. Data are presented as boxplots showing the median (horizontal line in the box), 25-75% (boxes) and 10-90 % (whiskers). **(A)** The total distance travelled in the OFT in early adulthood differed significantly between the three mouse lines ($F_{2,54}$=23.371, p<0.001, post hoc tests: HR vs IR: p=0.409, HR vs LR: p<0.001, IR vs LR: p<0.001), but not between conditions ($F_{1,54}$=1.219, p=0.274). **(B)** The total distance travelled during the OFT in late adulthood showed a main effect of line ($F_{2,54}$=7.462, p=0.001, post hoc tests: HR vs IR: p=0.084, HR vs LR: p=0.001, IR vs LR: p=0.357), as well as a main effect of condition ($F_{1,54}$=4.130, p=0.047, HR: p=0.019, IR: p=0.299 and LR: p=0.954). **(C)** In early adulthood, the time the animals spent in the inner zone of the OF was not affected by line ($F_{2,54}$=0.980, p=0.382) or condition ($F_{1,54}$=0.635, p=0.429). **(D)** In late adulthood, the time in the inner zone showed a trend for a main effect of line ($F_{2,54}$=3.002, p=0.058, post hoc tests: HR vs IR: p=0.055, HR vs LR: p=0.985, IR vs LR: p=0.459), butno effect of condition ($F_{1,54}$=0.093, p=0.762). **(E)**, **(F)** Both in early and late adulthood, the ratio of the inner to outer path length showed no significant effect of line (early: $F_{2,54}$=1.642, p=0.203; late: $F_{2,54}$=0.576, p=0.565) or condition (early: $F_{1,54}$=0.006, p=0.940; late: $F_{1,54}$=2.320, p=0.134). Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Main effects of line are represented above a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line with: </>, p≤0.05; ≤/≥, p≤0.1; ≈, p>0.1. Post-hoc statistics for main effects of condition and the interaction are presented above the appropriate boxes.

Figure 3



Water Cross-Maze test. Test performance of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions was analyzed by univariate ANOVA, N=10 per group. Data was analyzed using repeated-measures ANOVA and is presented as line plots showing means and standard error of the mean (SEM) (error bars). **(A)** The accuracy of the animals task performance showed a within-subjects main effect of training day ($F_{7,378}$=62.794, p<0.001), and a trend for a between-subjects main effect of condition ($F_{1,54}$=2.882, p=0.095), but no effect of line ($F_{2,54}$=1.753, p=0.183). Specifically, HR ELS mice were less accurate than HR STD mice on testing days two, three, and five (p=0.040, p=0.032, p=0.094) and IR ELS mice were less accurate than IR STD mice on day eight (p=0.048). Overall pairwise comparisons showed a trend for poorer accuracy in the HR ELS compared to HR STD animals ($F_{1,54}$=3.085, p=0.085), but no difference in the IR and LR lines($F_{1,54}$=0.986, p=0.325, and $F_{1,54}$=0.036, p=0.849). **(B)** The latency to reach the hidden platform showed a within-subjects main effect of training day ($F_{7,378}$=85.502, p<0.001), and a between-subjects main effect of condition ($F_{1,54}$=4.123, p=0.047), but no effect of line ($F_{2,54}$=0.635, p=0.534). Specifically, HR ELS mice had a higher latency than HR STD mice on testing day three (p=0.032) and IR ELS had a higher latency than IR STD mice on days seven and eight (p=0.060 and p=0.040). Overall pairwise comparisons were not significant (HR: $F_{1,54}$=1.562, p=0.217, IR: $F_{1,54}$=1.218, p=0.275, LR: $F_{1,54}$=1.353, p=0.250). **(C)** The number of wrong platform visits showed a within-subjects main effect of training day ($F_{7,378}$=60.502, p<0.001), and a trend for a between-subjects main effect of condition ($F_{1,54}$=3.376, p=0.072), but no effect of line ($F_{2,54}$=1.348, p=0.268). No particular day showed significant differences between conditions, but overall pairwise comparisons revealed a trend for an increased number of wrong platform visits in the HR ELS compared to HR STD mice ($F_{1,54}$=2.891, p=0.095), but not in IR and LR animals IR: $F_{1,54}$=1.188, p=0.281, LR: $F_{1,54}$=0.154, p=0.696). Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Post-hoc statistics for main effects of condition and the interaction are presented above the appropriate data points of the line plot in the corresponding color.

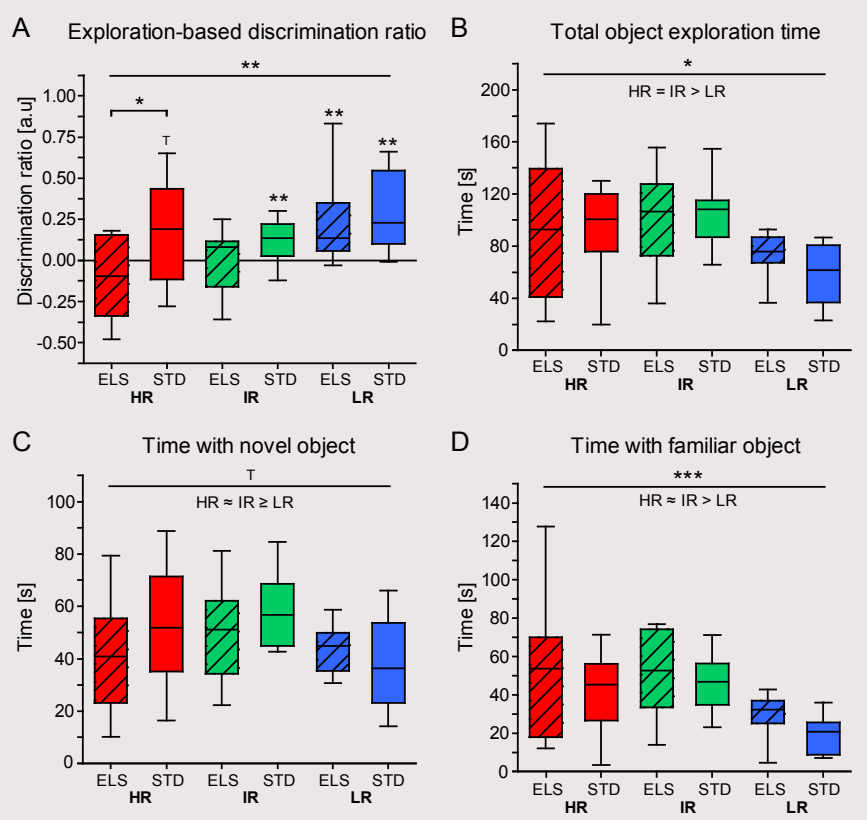
Figure 4



Y-maze test in early and late adulthood. Test performance of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions was analyzed by one-sample t-tests and univariate ANOVA, N=9-10 per group. Data are presented as boxplots showing the median (horizontal line in the box), 25-75% (boxes) and 10-90 % (whiskers). **(A)** In early adulthood, the discrimination ratio (based on distance) showed that LR animals, as well as HR STD and IR STD animals, discriminated between novel and familiar arms by traveling further distances in the novel arm, but HR ELS and IR ELS mice did not (HR ELS: t_9=-1.302, p=0.113, HR STD: t_9=3.255, p=0.005, IR ELS: t_9=-0.412, p=0.345, IR STD: t_9=2.648, p=0.014, LR ELS: t_9=4.346, p=0.001, LR STD: t_9=3.487, p=0.004). The ANOVA revealed a main effect of line ($F_{2,54}$=7.785, p=0.001, post hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.002, IR vs LR: p=0.006), a main effect of condition ($F_{1,54}$=6.828, p=0.012), and an interaction of line and condition ($F_{1,54}$=3.297, p=0.045, post hoc tests: HR: p=0.009, IR: p=0.020 and LR: p=0.564). **(B)** In late adulthood, the discrimination ratio (based on distance) showed that only HR ELS mice did not discriminate between novel and familiar arms (HR ELS: t_9=1.134, p=0.142, HR STD: t_8=3.768, p=0.003, IR ELS: t_9=3.256, p=0.005, IR STD: t_8=4.362, p=0.001, LR ELS: t_9=6.119,

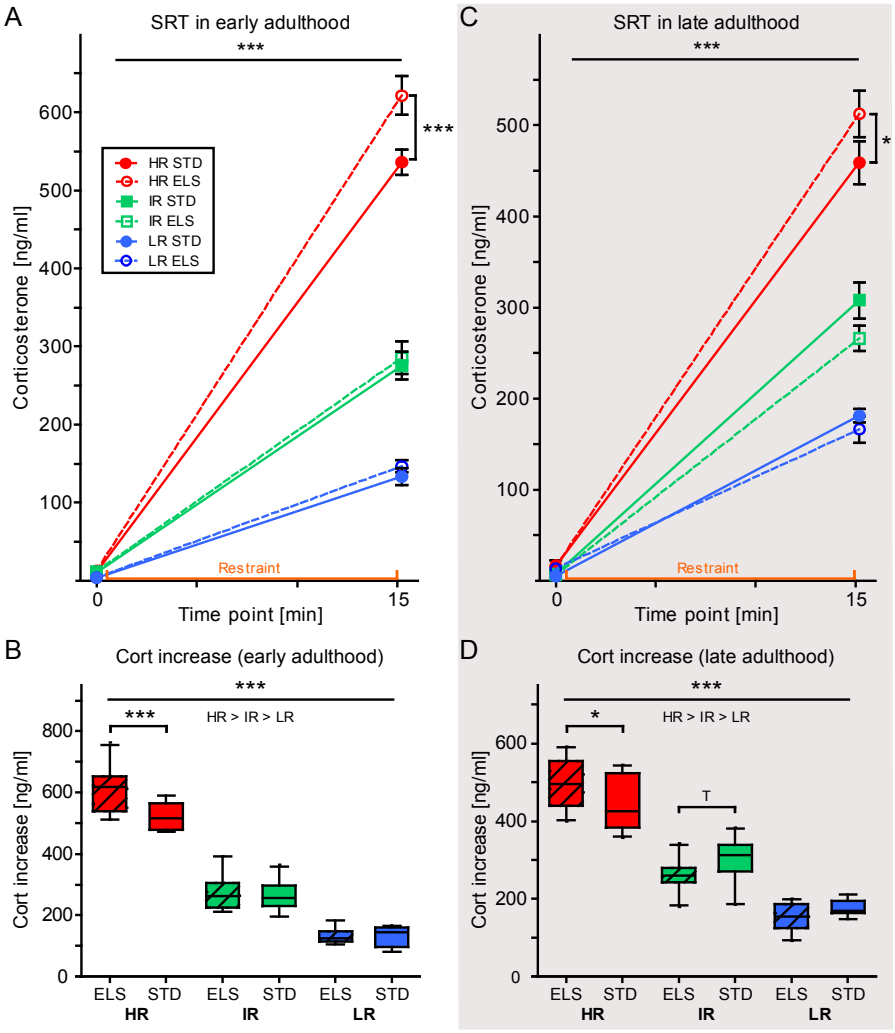
p<0.001, LR STD: t_9=4.614, p=0.001). The ANOVA showed a main effect of mouse line ($F_{2,52}$=4.410, p=0.017, post hoc tests: HR vs IR: p=0.940, HR vs LR: p=0.012, IR vs LR: p=0.157), and a significant interaction ($F_{2,52}$=8.978, p>0.001, post hoc tests: HR: p=0.009, LR: p=0.128, LR: p=0.005). **(c)** The total distance travel by the animals in early adulthood in the Y-maze showed a main effect of line ($F_{2,54}$=40.514, p<0.001, post hoc test: all p≤0.001), while condition had no effect ($F_{1,54}$=0.114, p=0.707). **(D)** The total distance travel by the animals in late adulthood in the Y-maze showed a main effect of line ($F_{2,52}$=13.059, p<0.001, post hoc test: HR vs IR and vs LR: p≤0.001, IR vs LR: p=1.0), while condition had no effect ($F_{1,52}$=0.737, p=0.484). **(E)** In early adulthood, the discrimination ratio (based on time) showed that only LR mice discriminated between the novel and familiar arms by spending significantly more time in the novel arm (HR ELS: t_9=-0.258, p=0.401, HR STD: t_9=1.739, p=0.058, IR ELS: t_9=-1.493, p=0.085, IR STD: t_9=1.754, p=0.056, LR ELS: t_9=2.361, p=0.022, LR STD: t_9=2.104, p=0.033). The ANOVA revealed a main effect of line ($F_{2,54}$=3.315, p=0.044, post hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.154, IR vs LR: p=0.058), but no effect of condition ($F_{1,54}$=2.404, p=0.127) and no interaction ($F_{2,54}$=2.037, p=0.140). **(F)** In late adulthood, the discrimination ratio (based on time) showed that LR mice (from both conditions), IR STD mice, and HR STD mice discriminated between novel and familiar arms by spending significantly more time in the novel arm, but not IR and HR ELS animals (HR ELS: t_9=0.392, p=0.352, HR STD: t_9=1.905, p=0.045, IR ELS: t_9=-0.939, p=0.186, IR STD: t_9=1.715, p=0.060, LR ELS: t_9=3.258, p=0.005, LR STD: t_9=2.394, p=0.020). The ANOVA revealed only a significant main effect of line ($F_{2,54}$=4.039, p=0.023, post hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.196, IR vs LR: p=0.022; main effect of condition: $F_{1,54}$=1.118, p=0.295, interaction: $F_{2,54}$=1.884, p=0.162). Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Main effects of line are represented above a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line with: </>, p≤0.05; ≤/≥, p≤0.1; ≈, p>0.1. Post-hoc statistics for main effects of condition and the interaction are presented above the appropriate boxes.

Figure 5



Object recognition test in late adulthood. Test performance of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions was analyzed using one-sample t-tests and univariate ANOVA, *N*=10 per group. Data are presented as boxplots showing the median (horizontal line in the box), 25-75% (boxes) and 10-90 % (whiskers). **(A)** The discrimination ratio, based on the exploration time animals spent with both objects, showed that IR STD, LR ELS and LR STD mice preferentially explored the novel object, while HR STD mice showed a trend. IR ELS and HR ELS mice did not show a preference for the novel object or even showed a trend for preferring the familiar object (HR ELS: t_9=-1.499, p=0.084, HR STD: t_8=1.617, p=0.073, IR ELS: t_9=0.118, p=0.454, IR STD: t_9=2.943, p=0.008, LR ELS: t_9=2.834, p=0.010, LR STD: t_8=3.835, p=0.003). The ANOVA showed a main effect of mouse line ($F_{2,52}$=6.003, p=0.005, post-hoc tests: HR vs IR: p=0.1, HR vs LR: p=0.006, IR vs LR: p=0.025) and a main effect of condition ($F_{1,52}$=6.925, p=0.011, post-hoc tests: HR ELS vs STD: p=0.011, IR p=0.279, LR ELS vs STD: p=0.412). **(B)** The total exploration time animals spent with both objects showed a main effect of line ($F_{2,52}$=6.663, p=0.003, post-hoc tests HR vs IR: p=1.0, HR vs LR: p=0.039, IR vs LR: p=0.003), but no effect of condition. **(C)** The time animals spent with the novel object showed a trend for a main effect of line ($F_{2,52}$=2.532, p=0.089, post-hoc tests HR vs IR: p=0.526, HR vs LR: p=1.0, IR vs LR: p=0.094), but no effect of condition. **(D)** HR and IR mice spent more time exploring the familiar objects compared to LR animals ($F_{2,52}$=7.884, p=0.001, post-hoc tests HR vs IR: p=0.1, HR vs LR: p=0.005, IR vs LR: p=0.002), and there was a trend for a main effect of condition ($F_{1,52}$=3.191, p=0.080, post hoc tests: HR ELS vs STD: p=0.156, IR p=0.543, LR ELS vs STD: p=0.307). Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Main effects of line are represented above a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line with: </>, p≤0.05; ≤/≥, p≤0.1; ≈, p>0.1. Post-hoc statistics for main effects of condition and the interaction are presented above the appropriate boxes.

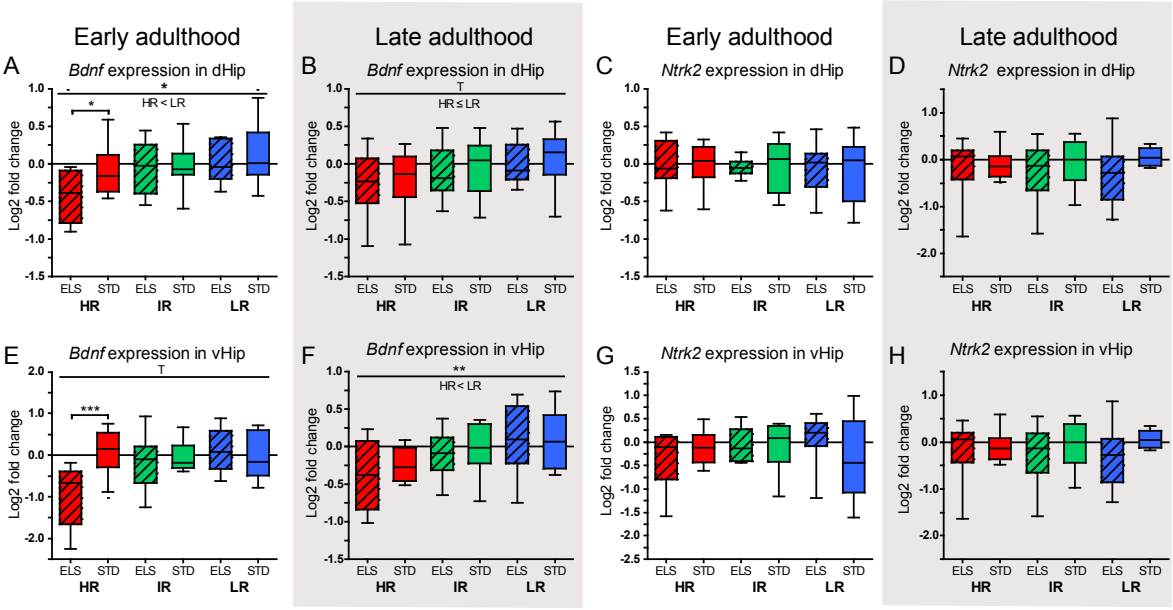
Figure 6



Stress reactivity. Corticosterone concentrations measured in the plasma of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions, collected during the SRT was analyzed using repeated-measured and univariate ANOVA. Data is presented as line plots showing means and standard error of the mean (SEM) (error bars) and as boxplots showing the median (horizontal line in the box), 25-75% (boxes) and 10-90 % (whiskers). **(A)** In early adulthood, there was a significant effect of time point (initial vs response) on the plasma corticosterone concentration (within-subjects effect: $F_{1,54}$=1459.996, p<0.001). The initial corticosterone concentration showed a main effect of line ($F_{2,54}$=6.253, p=0.004, post hoc tests: HR vs IR: p=1.0, HR vs LR: p=0.007, IR vs LR: p=0.015), but no effect of condition ($F_{1,54}$=0.148, p=0.702). The response levels of corticosterone showed a main effect of mouse line ($F_{2,54}$=214.164, p<0.001, post hoc tests: all p<0.001), as well as a main effect of condition ($F_{1,54}$=5.675, p=0.021), and an interaction of line and condition ($F_{2,54}$=4.232, p=0.020, post hoc tests, ELS vs STD: HR: p<0.001, IR: p=0.742, LR: p=0.857). **(B)** The increase in plasma corticosterone levels in response to 15 restraint showed a main effect of line ($F_{2,54}$=223.319, p<0.001, post hoc tests: all p<0.001), a main effect of condition ($F_{1,54}$=6.022, p=0.017), as well as an interaction of line and condition ($F_{2,54}$=6.243, p=0.016, post hoc tests ELS vs STD: HR: p<0.001, IR: p=0.776, LR: p=0.949). **(C)** In late adulthood, there was a significant effect of time point (initial vs response) on the plasma corticosterone concentration (within-subjects effect: $F_{1,53}$=1957.027, p<0.001). The initial corticosterone concentration showed a trend for a main effect of mouse line ($F_{2,53}$=3.023, p=0.057, post hoc tests: HR vs IR: p=0.063, HR vs LR: p=0.278, IR vs LR: p=1.0), but no effect

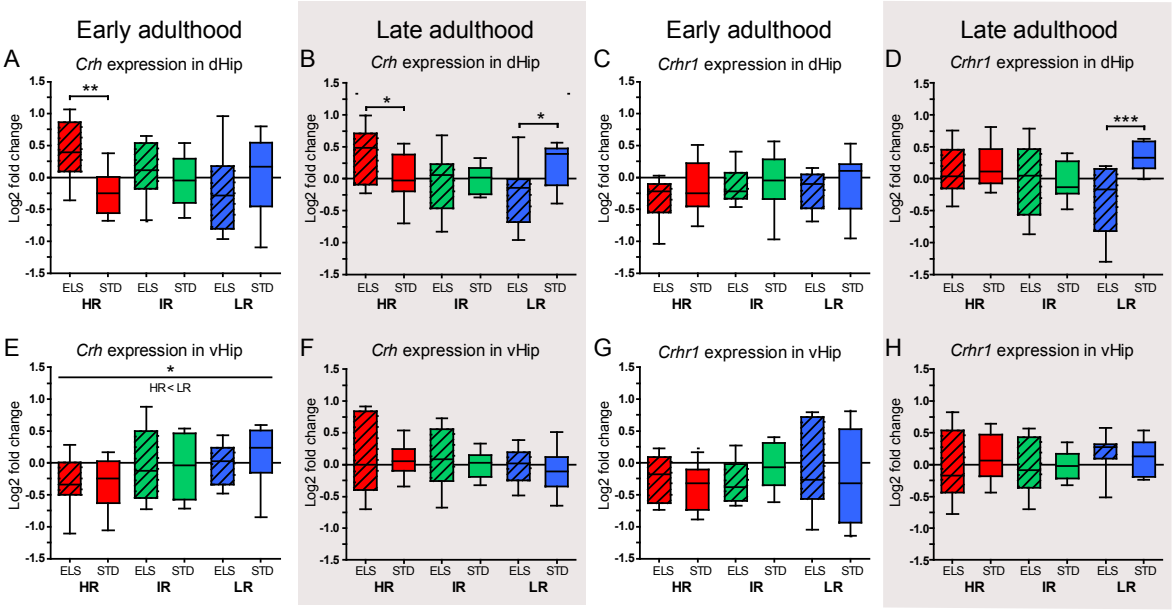
of condition ($F_{1,53}$=0.381, p=0.540). The response levels of corticosterone showed a main effect of mouse line ($F_{2,54}$=140.272, p<0.001, post hoc tests: all p<0.001), and interaction of line and condition ($F_{2,53}$=3.468, p=0.038, post hoc tests, ELS vs STD: HR: p=0.048, IR: p=0.118, LR: p=0.580). **(D)** The increase in plasma corticosterone levels in response to 15 restraint showed a main effect of line ($F_{2,53}$=166189, p<0.001, post hoc tests: all p<0.001), and an interaction of line and condition ($F_{2,53}$=4.544, p=0.015, post hoc tests, ELS vs STD: HR: p=0.026, IR: p=0.0.092, LR: p=0.344). Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Main effects of line are represented above a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line with: </>, p≤0.05; ≤/≥, p≤0.1; ≈, p>0.1. Post-hoc statistics for main effects of condition and the interaction are presented above or next to the appropriate boxes.

Figure 7



Relative expression levels of *Bdnf* and *Ntrk2*. The relative expression levels of the genes coding for the neurotrophin BDNF (*Bdnf*) and its receptor TRKB (*Ntrk2*) were measured in selected brain regions of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions, at an early and a late time point in adulthood. The data was normalized to the IR STD animals and analyzed using two-way ANOVAs, and is presented as boxplots showing the median (horizontal line in the box), 25-75% (boxes) and 10-90 % (whiskers). **(A)** In early adulthood, the expression of *Bndf* in the dHip showed a main effect of line ($F_{1,54}$=4,091, p=0.022, post hoc tests: HR vs IR: p=0.301, HR vs LR: p=0.038 IR vs LR: p=1.0), as well as a trend for a main effect of condition ($F_{1,54}$=3.177, p=0.080, post hoc tests ELS vs STD: HR: p=0.031, IR: p=0.972, LR: p=0.442). **(B)** In late adulthood, the expression of *Bndf* in the dHip showed a trend for a main effect of line ($F_{2,52}$=2.651, p=0.080, post hoc tests: HR vs IR: p=0.615, HR vs LR: p=0.080, IR vs LR: p=0.931). **(C)**, **(D)** In early adulthood and in late adulthood, there was no effect of line of condition on the expression of *Ntrk2* in the dHip. **(E)** In early adulthood, the expression of *Bndf* in the vHip showed a trend for main effect of line ($F_{2,52}$=2.993, p=0.059, post hoc test: all p>0.1), a main effect of condition ($F_{1,52}$=4.513, p=0.038), and an interaction of line and condition ($F_{2,52}$=5.677, p=0.006, post hoc tests ELS vs STD: HR: p>0.001, IR: p=0.649, LR: p=0.492). **(F)** In late adulthood, the expression of *Bndf* in the vHip showed a main effect of line ($F_{2,54}$=5.218, p=0.008, post hoc tests: HR vs IR: p=0.174, HR vs LR: p=0.007, IR vs LR: p=0.628). **(G)**, **(H)** In early adulthood and in late adulthood, there was no effect of line of condition on the expression of *Ntrk2* in the vHip. Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Main effects of line are represented above a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line with: </>, p≤0.05; ≤/≥, p≤0.1; ≈, p>0.1. Post-hoc statistics for main effects of condition and the interaction are presented above the appropriate boxes.

Figure 8



Relative expression levels of *Crh* and *Crhr1*. The relative expression levels of the genes coding for the neuropeptide CRH (*Crh*) and its receptor CRH-R1 (*Crhr1*) were measured in selected brain regions of high (HR), intermediate (IR), and low (LR) reactivity mice, raised in early-life stress (ELS) or standard (STD) housing conditions, at an early and a late time point in adulthood. The data was normalized to the IR STD animals and analyzed using two-way ANOVAs, and is presented as boxplots showing the median (horizontal line in the box), 25-75% (boxes) and 10-90 % (whiskers). **(A)** In early adulthood, the expression of *Crh* in the dHip showed an interaction of line and condition ($F_{2,48}$=4.358, p=0.018, post hoc tests ELS vs STD: HR: p=0.008, IR: p=0.556, LR: p=0.170). **(B)** In late adulthood, the expression of *Crh* in the dHip showed an interaction of line and condition ($F_{2,52}$=5.247, p=0.009, post hoc tests ELS vs STD: HR: p=0.047, IR: p=0.743, LR: p=0.015). **(C)** In early adulthood, the expression of *Crhr1* in the dHip showed no effect of line of condition. **(D)** In late adulthood, the expression of *Crhr1* in the dHip showed a main effect of condition ($F_{1,50}$=4.798, p=0.033) and an interaction of line and condition ($F_{2,50}$=3.487, p=0.038, post hoc tests ELS vs STD: HR: p=0.661, IR: p=0.949, LR: p=0.001). **(E)** In early adulthood, the expression of *Crh* in the vHip showed a main effect of line ($F_{1,53}$=3.782, p=0.029, post hoc tests: HR vs IR: p=0.162, HR vs LR: p=0.029, IR vs LR: p=1.0). **(F)** In late adulthood, the expression of *Crh* in the vHip showed no effect of line or condition. **(G)**, **(H)** In early and late adulthood, the expression of *Crhr1* in the vHip showed no effect of line or condition. Symbols: ***, p≤0.001; **, p≤0.01; *, p≤0.05; T, p≤0.1. Main effects of line are represented above a horizontal line above the graphs. The respective post-hoc test statistics are indicated underneath the line with: </>, p≤0.05; ≤/≥, p≤0.1; ≈, p>0.1. Post-hoc statistics for main effects of condition and the interaction are presented above the appropriate boxes.

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CHAPTER 5:

GENERAL DISCUSSION

5.1. Synopsis

Ample evidence has linked adverse early-life experiences with an increased risk for affective disorders in later life (Carr et al., 2013, Heim and Binder, 2012, Mandelli et al., 2015, Maniam et al., 2014, Mazure, 1998, Sadowski et al., 1999, Seckl, 2004), but why some individuals are more susceptible to such environmental pathogens than other remains poorly understood. In many cases, individuals who were exposed to ELS manifest endophenotypes associated with affective disorders, even when no full disorder can be diagnosed (Aisa et al., 2007, Brunson et al., 2005, Chen and Baram, 2015, Gould et al., 2012, Maniam et al., 2014, Pesonen et al., 2013). Examples for such endophenotypes of affective disorders are changes in stress-coping behaviour, reduced cognitive function, and neuroendocrine dysregulation (Hasler et al., 2004, Hasler and Northoff, 2011, Heinzmann et al., 2014, Radley et al., 2011). While it was long believed that mental disorders are strongly dependent on genetic influences (Gershon et al., 1971), the contribution of environmental risk factor and pathogens, i.e. of proven environmental causes (Caspi and Moffitt, 2006), has become more and more recognized (Burmeister et al., 2008, Flint and Kendler, 2014, Klengel and Binder, 2015). Recent studies in genetic epidemiology have demonstrated that, in the vast majority of cases, psychiatric disorders are brought about by an interaction of genetic vulnerability and environmental hazards (Flint and Kendler, 2014, Heim and Binder, 2012, Klengel and Binder, 2015). In the context of G × E interactions, epigenetic modifications are a powerful process which can explain how the environment "gets under ones skin" and why some people are more sensitive to certain adversities than others (Hollins and Cairns, 2016, Karsten and Baram, 2013, Mifsud et al., 2011, Murgatroyd, 2014, Radley et al., 2011, Radtke et al., 2015). The study of G × E interactions is now a thriving field of research that has the potential to considerably advance our knowledge and understanding of the etiology, pathophysiology, and underlying mechanisms of affective disorders, and to guide the development of targeted and personalized treatment options and early interventions. In the presented work, we used a genetic animal model of affective disorders in combination with an environmental ELS manipulation, mimicking a common clinical situation, to investigate the consequences of this G × E interaction with the aim of shedding light onto the molecular and neuroendocrine underpinnings of endophenotypes of affective disorders.

We began by extending the established validity of the SR mouse model by experimentally testing its predictive validity in the response to antidepressant SSRI treatment (Chapter 2). We

were able to show that HR animals respond to chronic fluoxetine treatment with an amelioration of depression-associated endophenotypes at the level of behaviour, cognition, and HPA axis feedback regulation, paralleled by changes in hippocampal neurogenesis. In LR animals, on the other hand, chronic fluoxetine treatment worsened the atypical depression-like endophenotypes. Many clinical studies have described a similar divergence in the response to SSRI treatment between depression patients with melancholic/psychotic and atypical features (Duman, 2004, Korte et al., 2015, McIntyre, 2016). The presented data thus confers a high degree of predictive validity to the SR mouse model as an animal model for these opposing subtypes of MDD.

In a next step, we employed the three SR mouse lines to model the clinical situation of genetic differences in HPA axis sensitivity interacting with ELS (Chapter 3). We were able to show that, in the short-term, ELS affected the offspring of all three SR mouse lines in terms of physiological development and behaviour. However, only HR pups revealed additional alterations in HPA axis regulation and gene expression at this point in time, providing evidence for early programming effects of neuroendocrine systems by ELS. Our results suggest that the genetic predisposition for high stress reactivity rendered HR animals more vulnerable to ELS than animals of the other two mouse lines. Indeed, ELS-exposed HR pups had elevated basal plasma CORT levels on P9 compared to STD-housed HR mice, and this effect was absent in IR and LR animals, thus demonstrating that ELS elicited an HPA axis activation only in genetically susceptible animals. Interestingly, we were able to show that HR mice already displayed an increased stress reactivity at this very young age, even without ELS exposure, although overall the animals' GC levels in response to a stressor remained relatively low due to the SHRP. Until now it was uncertain whether the genetic predisposition would measurably increase the stress reactivity of HR offspring already during the first days of life, or whether the effect would emerged more gradually, increasing with repeated stress experience over the course of development. Our new findings confirm the innate and immediate nature of the HPA axis endophenotype in the SR mouse model and demonstrate that difference in stress hormone regulation between the three mouse lines are already present during early postnatal development.

In early adulthood, ELS-exposed HR mice showed an even further increase in stress reactivity compared to STD-raised HR mice, as well as hyperactive stress-coping behavior, and changes in the expression of stress-related genes in the PVN and the hippocampus, while IR and LR animals seemed largely protect from these long-term consequences of ELS. These results

impart that many late-onset consequences of ELS have their roots in an early neuroendocrine dysregulations and suggests a potential target for early intervention.

Finally, we examined how the observed long-term effects of ELS developed over the adult lifespan, and to what extent cognitive function was impaired by the adverse early-life conditions (Chapter 4). Our results revealed that the endophenotypic changes observed in HR mice during early adulthood at the level of stress-coping behaviour and neuroendocrine regulation did not diminish in late adulthood, but stayed stable over time. In addition, ELS led to impaired cognitive function in HR mice already during early adulthood. In LR mice, we observed no significant enduring consequences of ELS in early adulthood. However, in late adulthood, these animals seemed to improve in their cognitive abilities and revealed some late-onset changes in the regulation of stress-related genes in the hippocampus, as well as a small, but significant, increase in baseline GC levels.

In summary, in the presented research we further validated the SR mouse model as an animal model of affective disorders. In addition, we present data on a clinically relevant G × E interaction using the SR mouse model and a recently established paradigm of ELS, showing that endophenotypes related to MDD emerge preferentially in animals that are genetically predisposed for increased stress reactivity.

5.2. Predictive validity of the SR mouse model

When investigating the predictive validity of the SR mouse model, we detected line specific differences in the effects of chronic SSRI treatment. Interestingly, these findings in our animal model reflect what is seen in human patients, particularly in studies that stratified patients by depression subtype when assessing the treatment benefits of SSRIs. Namely, the clinical data suggests that the efficacy of SSRIs differs between melancholic/psychotic depression and atypical depression patients (Baghai et al., 2008, Duman, 2004, Korte et al., 2015, Thase, 2007). This makes intuitive sense, since these two subtypes of depression are near mirror images of each other (displaying either psychomotor agitation or retardation, insomnia or hypersomnia, depressed mood or reactive mood, weight gain or loss) and are likely to have different neurobiological underpinnings, which could be best targeted through different treatments (Antonijevic, 2006, Baghai et al., 2008, Gold and Chrousos, 1999, Gold and Chrousos, 2002). Evidence from human studies shows that patients with melancholic/psychotic depression often respond well to chronic SSRI treatment (Hirschfeld, 1999, Korte et al., 2015). SSRIs exert their

function by inhibiting the uptake of serotonin from the synaptic cleft and increasing the postsynaptic serotonin signal. Increased serotonin has a reinstating effect on neurotropic signaling, thereby contributing to increased hippocampal neurogenesis and synaptic plasticity (Surget et al., 2011), and to increased negative feedback from forebrain regions by promoting the insertion of GRs (Heydendael and Jacobson, 2008, Seckl and Fink, 1992). Both increased neurogenesis and negative feedback contribute to counteract the HPA axis hyper-responsiveness seen in many MDD patients (Rothschild et al., 1993, Surget et al., 2011, Wolfersdorf et al., 1995, Zanardi et al., 2000). The fact that HR animals responded to chronic SSRI treatment with reduced hyperactive stress-coping, improved negative GC feedback, and increased neurogenesis strongly suggests that similar neurobiological pathways underlie the endophenotypes observed in HR animals and melancholic/psychotic depression patients.

Atypical depression patients often do not benefit from SSRI treatment and appear to be more responsive to MAOA inhibitors (Korte et al., 2015, McIntyre, 2016, Thase, 2007). MAOA inhibitors function by inhibiting the monoamine oxidizing enzyme, which digests three different classes of monoamines (dopamine, norepinephrine and serotonin), thereby increasing the monoaminergic signal in the brain (Heydendael and Jacobson, 2009, Thase et al., 1995). It has been suggested that a reduced monoaminergic response, which is brought about by increased inflammatory processes (van Heesch et al., 2013), is responsible for the anhedonic symptoms in atypical depression (Korte et al., 2015). MAOA inhibitors are thought to improve depressive symptoms in atypical depression patients by normalizing the monoaminergic signal in the *Locus coeruleus* (Heydendael and Jacobson, 2009). Furthermore, an elevated monoaminergic signal can reduce GR expression in forebrain regions and dampen the negative feedback suppression of the HPA axis, thereby increasing the stress reactivity (Heydendael and Jacobson, 2008, Heydendael and Jacobson, 2009). In LR mice, several studies have demonstrated increased bodyweight and passive behaviour (Heinzmann et al., 2014, Knapman et al., 2010a, Touma et al., 2008, Touma et al., 2009), which could be indicative of inflammation and impaired monoamine reward signaling (Pan et al., 2013, Stice et al., 2010). In future studies, it may be beneficial to assess if LR animals show an amelioration of symptoms after treatment with MAOA inhibitors, as this would further validate the model and constitute a valuable tool to understand the mechanism underlying the treatment response in atypical depression.

Today, when treating MDD patients, it is still not standard to include information regarding individual specifiers of the depression subtype into the treatment decision. Rather, most MDD patients are prescribed an SSRI as a first line treatment, with additional drugs added or replacing SSRIs in second or third line. Our results in the SR mouse model strengthen the proposition that better treatment outcomes could be attained for MDD patients if more extensive phenotyping and subtype characterization were used a standard tool to inform the choice of pharmacological treatment. Furthermore, in clinical studies, stratification of MDD patients according to their respective subtype could greatly advance our knowledge regarding the efficacy of a range of pharmacological treatment options. Currently, many treatment effects are diluted or hidden in the data, due to the large heterogeneity of the study population (see also Heinzmann et al., 2014, Gold and Chrousos, 2013). Studies using stringent criteria to stratify their patient sample could thus provide further insights into the endophenotypes associated with the different subtypes of MDD and allow a better understanding of the underlying neurobiological mechanisms.

5.3. G × E interaction: Effects of ELS in the SR mouse model

Early-life adversity is a powerful negative stimulus, which can give rise to many long-lasting consequences in a developing individual (Carr et al., 2013, Chartier et al., 2010, Heim et al., 2002, Nishi et al., 2014, Sanchez et al., 2001). In our study, we found that as little as seven days of ELS experience, induced my erratic maternal behavior in the limited nesting and bedding material paradigm, was sufficient to elicited acute physiological and behavioral effects in the offspring of all three SR mouse lines. Specifically, at the end of the ELS period, all ELS-raised pups showed a delay in bodyweight gain relative to their respective STD-housed control groups, and displayed ELS-induced changes in emotional and stress-coping behavior. These short-term changes appeared to be independent of the animals' genetic predisposition. However, alterations at the level of neuroendocrine read-outs, including adrenal weight and plasma CORT levels, as well as changes in gene expression, were detected only in HR offspring, suggesting that the animals' genetic predisposition played a critical role in determining the extent of their vulnerability to ELS. Many rodent studies in the field of stress research rely on the inbred C57BL/6 mouse strain as model system (van Bogaert et al., 2006), and reliable main effects of ELS have been reported in these animals (Kohl et al., 2015, Naninck et al., 2015, Rice et al., 2008, Wang et al., 2012, Wang et al., 2013). Our data, however, illustrates that ELS affects genetically different individuals in different ways, and highlights that carefully considering

genetic variation is critical for understanding the etiology of affective disorders. There have been some previous studies demonstrating strain-dependent differences in the effects of ELS (Mehta and Schmauss, 2011, Rana et al., 2015, Stohr et al., 1998). The key advantage of the SR mouse model is that we can directly relate the observed differences in vulnerability to a specific neuroendocrine trait that differs between the lines, namely HPA axis reactivity. This is possible because all three breeding lines of the SR mouse model were derived from the same founding strain (CD1 mice), and were selected for breeding based on a single criterion, while between-strain analyses are comparing individuals that strongly differ in their genetic background, so that the origin of an observed difference cannot be pin-pointed.

In the presented studies, the short-term alterations in stress hormone regulation and gene expression measured in juvenile HR ELS mice were most likely of critical importance in paving the way for the lasting changes in stress-coping behavior, cognitive function, stress reactivity, and gene regulation that were observed in early and late adulthood. Indeed, LR mice, which showed no short-term effects of ELS on neuroendocrine function and gene expression, were largely protected from the adverse long-term effects of ELS.

5.4. Neuroendocrine programming through ELS

5.4.1. How can ELS produce enduring neuroendocrine programming effects in HR mice?

Integrating our findings with the existing literature, we propose the following mechanism for the ELS-induced programming effects in HR offspring. During the exposure to the 7-day ELS paradigm, it is likely that pups of all three breeding lines initiated a small neuroendocrine response to the environmental stressor (Dent et al., 2000). However, as the ELS exposure occurred during the SHRP, the pups' HPA axis reactivity was subdued, allowing only a minor increase in CRH release, which could not stimulate a measurable release of ACTH or a rise in plasma CORT levels due to the insensitive state of the pituitary and the adrenal glands (Levine, 2002, Schmidt et al., 2002). Importantly, in offspring of the HR mouse line, the overall sensitivity of the HPA axis is genetically increased (Heinzmann et al., 2014, Touma et al., 2008), causing HR pups to be hypersensitive to molecular stress signals even during the SHRP. Thus, a slight ELS-induced increase in the CRH signal could be sufficient to stimulate a release of ACTH, and a subsequent adrenal response, leading to elevated plasma CORT levels in HR pups. It should be noted that on P9, the adrenals of HR ELS pups were significantly larger than those of

STD-raised HR pups, reflecting their increased CORT synthesis and release. Critically, during the early postnatal phase of neonatal mice, GRs are not yet functionally integrated into the hippocampus (Goldman et al., 1973, Levine, 2002, Schmidt et al., 2003), so that no negative feedback signal can be produced to control the stress response and the return of CORT levels to baseline. This lack of negative feedback regulation explains the sustained elevation of basal CORT levels in HR pups, measured on P9. As soon as GRs became functionally integrated in the HPA axis (starting ~P12 (Meaney et al., 1985, Schmidt et al., 2003)), the high basal plasma CORT levels most likely provoked a substantial inhibitory feedback signal onto the CRH-releasing neurons in the PVN, so that the tonic HPA axis activation was gradually suppressed and the baseline CORT levels returned to normal, low levels. Yet, a sustained neuroendocrine activation, occurring during a sensitive window of brain development when CORT levels should have been low, can give rise to a lasting hyper-sensitivity of the HPA axis (Bale and Epperson, 2015, Korosi and Baram, 2010). This was manifested by a marked increase in stress reactivity in adult HR mice that experienced ELS.

Epigenetic processes are prominently involved in shaping the changes in neuroendocrine function and gene regulation (Heim and Binder, 2012, Meaney et al., 2007, Murgatroyd, 2014, Weaver et al., 2004). For instance, ELS-induced demethylation of the Fkbp5 GRE promotor has been shown to produce long-lasting changes in stress-dependent gene expression, leading to a dysregulation of the stress hormone system (Klengel et al., 2013). The functional role of microRNAs is also being investigated in this context (Hollins and Cairns, 2016). In particular, candidate microRNAs that are known to target the expression of HPA axis-related genes, such as the miR-34 family, which is involved in regulating the sensitivity of CRHR1 and its expression (Andolina et al., 2016, Haramati et al., 2011), miR-18 and miR-124, which regulate the expression of GR protein (Vreugdenhil et al., 2009), or miR-132, which is dependent on GR activation and decreases BDNF expression (Bredy et al., 2011), may be relevant regulators involved in ELS-induced neuroendocrine programming.

5.4.2. What is the role of the PVN in HPA axis programming?

At the end of the ELS period, we found a reduction in the levels of hypothalamic CRH mRNA in HR ELS pups, resembling similar findings of previous studies (Levine, 2002, Rice et al., 2008, Schmidt et al., 2006). Subsequent measurements revealed that this effect was maintained into early and late adulthood. Strikingly, studies exploring neuroendocrine programming effects of early-life handling of rodent pups have reported that changes at the level of hypothalamic CRH

expression appeared as the first in a cascades of gene regulation changes, leading to reduced ACTH expression, reduced CORT secretion, and increased hippocampal GR expression (Korosi and Baram, 2010, Levine et al., 1967, Plotsky and Meaney, 1993). It was demonstrated that this cascade could also be triggered by the administration of a selective CRH-R1 receptor antagonist in the early postnatal period, showing that the hypothalamic CRH signal during early-life is causally involved in down-stream programming of the HPA axis (Fenoglio et al., 2006). In the case of early-life handling, evidence suggests that the signal leading to reduced hypothalamic CRH expression comes, indirectly, from increased activation of the paraventricular nucleus of the thalamus (PVT)(Fenoglio et al., 2006). The PVT inhibits CRH-stimulating activity of the central amygdala (ACe) and the bed nucleus of the stria terminalis (BNST) (Hsu and Price, 2009) and leads to alterations in the activation of transcription factors regulating CRH expression in PVN neurons (Korosi and Baram, 2010, Korosi et al., 2010). It remains to be tested whether a similar signaling pathway is involved in the lasting downregulation of hypothalamic CRH after ELS in HR mice. Studies of immediate early gene expression in the implicated brain regions may provide useful insights.

An alternative pathway, which may be involved in the acute changes in hypothalamic CRH levels, is GC-induced GABA-ergic negative feedback from the anterior pituitary, where GRs become functionally active early in the postnatal development (Schmidt et al., 2005, Walker et al., 1986). Also, feedback suppressing the expression of CRH directly at the level of CRH-positive neurons in the PVN may play a role, as studies have reported abundant expression of GRs in the parvocellular neurons of PVN during the early postnatal period (van Eekelen et al., 1991, Yi et al., 1994), though these receptor appear to become functionally active only after ~P8 (Yi et al., 1993).

Taken together, research suggests that early-life changes in hypothalamic CRH regulation are centrally involved in the subsequent programming of the HPA axis and that inhibitory signaling from the PVT, the pituitary, and the PVN itself may contribute to the acute changes in hypothalamic gene expression.

5.5. Future outlook

The research presented in this thesis has generated many interesting and thought-provoking findings regarding the interaction of genetic differences in stress reactivity with ELS, and it also raises many questions for future studies. For instance, we have shown here that a genetic predisposition for high or low stress reactivity interacts with adverse early-life experiences, shaping the short- and long-term outcomes. In future studies, it would be of interest to explore how exactly innate differences in stress reactivity are involved in shaping the response to adulthood stressors, using, for example, a chronic mild stress paradigm or exposure to addictive substances in adulthood. As the HPA axis develops during the early postnatal life (Levine, 2002, Meaney et al., 1985, Schmidt et al., 2003), it seems unlikely that adulthood stressors would be able to reprogram the stress response in an equally powerful way as ELS, which occurs during a critical period of brain and neuroendocrine development. However, genetic differences in stress reactivity may nonetheless contribute to profound differences in the vulnerability to adult stress exposure. Previous studies have reported a G × E interaction at the level of hedonic and anxiety-related behavior, associated with differences in emotional reactivity (Stedenfeld et al., 2011), but the underlying mechanism remain unclear. In the field of addiction research, a dysregulated stress reactivity has been proposed as a risk factor for substance addiction and relapse (Koob and Kreek, 2007, Lovallo, 2006). Thus, the SR mouse model could be employed as a promising tool to shed light onto the role of genetic differences in stress reactivity in the consequences of adulthood stress exposure. Readouts of such a G × E interaction study could include behavioral, neuroendocrine, and molecular assays, as well as an in-depth analysis of potential epigenetic processes, such as methylation changes on key regulatory genes, or changes in mircoRNA levels, which can confer even wide-spread alterations in gene regulation (Hollins and Cairns, 2016).

A further open question is whether it would be possible to rescue, or even to prevent, the emergence of adverse long-term consequences induced by ELS in HR mice by early or late intervention. As discussed previously, a potential window for early intervention could be timed directly after or during the ELS exposure in the postnatal period. The primary aim of such an early intervention would be to prevent changes in HPA axis programming and gene expression by normalizing basal CORT and hypothalamic CRH levels during important periods of postnatal brain development. This may realign the future development and forestall any downstream effects at the level of stress-coping behavior, cognitive function, and stress-reactivity. As chronic

antidepressant treatment with SSRIs has demonstrated good efficacy in adult HR animals, SSRIs may be a promising tool to intervene again lasting neuroendocrine changes during early-life.

However, the administration of a psychoactive substance to developing mouse pups necessitates a careful consideration of the dosage and of potential side-effects of the drug, which may be different from the side-effects in adult animals. During brain development serotonin serves important functions as a trophic factor involved in cell migration, axonal growth, and differentiation of neurons (Lauder, 1990, Whitaker-Azmitia, 2001), and even small changes in serotonergic signaling can lead to abnormalities in brain development (Lauder et al., 2000). Furthermore, studies concerning human affective disorders, such as MDD, are essentially seeking solutions for human subjects. Thus, when investigating the effects of perinatal SSRI exposure, it is essential to consider differences in the developmental timing of the serotonergic system in humans and in rodents. It is estimated that the third trimester of human gestation corresponds to the early postnatal period (~P2-7) in rodents (Ansorge et al., 2004, Vitalis et al., 2013), complicating the translation of findings from mouse pups to human infants.

Nevertheless, several studies have investigated the effects of perinatal exposure to SSRIs, as this is the most commonly prescribed treatment for pre-and postpartum depression in women (Bennett et al., 2004). The results show that *in-utero* exposure to SSRIs can cause withdrawal symptoms and psychomotor deficits in human infants and neonate mice (Gentile and Galbally, 2011, Hayes et al., 2012, Simpson et al., 2011, Zeskind and Stephens, 2004). Furthermore, increased HPA axis reactivity, as well as increased anxiety and social avoidance, have been described in young children and juvenile animals (Casper et al., 2003, Klinger et al., 2011, Oberlander et al., 2008, Zimmerberg and Germeyan, 2015). The long-term consequences of perinatal SSRI exposure in humans remain largely unknown (Glover et al., 2015), but animal models have revealed long-lasting effects on depression-like and anxiety-related behavior of pre- and postnatal exposure to SSRIs (Ansorge et al., 2008, Hansen et al., 1997). In addition, animal studies have shown that perinatal SSRI exposure can adversely impact on hippocampal neuroplasticity by reducing the expression of neurotrophic factors, such as BDNF (Karpova et al., 2009). Interestingly, when adverse early-life conditions are included in the study design, the benefits of the antidepressant treatment seem to outweigh the drawbacks. For instance, a recent animal study described that the adverse effects of prenatal stress exposure on HPA axis regulation and depression-like behavior could be reversed by chronic low-dose SSRI treatment during the pre- and postnatal period (Salari et al., 2016). In line with this, different studies

showed that prenatal stress-induced impairments in hippocampal neurogenesis and structural plasticity could be rescued by postnatal administration of SSRIs (Ishiwata et al., 2005, Rayen et al., 2011). These studies reported only minor or no negative effects of SSRI treatment alone, suggesting that timing, dosage, and route of administration are critical in this respect. Based on these promising findings, it may be possible to rescue the ELS-induced endophenotype of HR mice through postnatal treatment with low doses of SSRIs. To test this in an experiment, fluoxetine could be administered to the dam starting on P1, via the drinking water or using osmotic mini-pumps (previous studies employed low concentrations ranging from 5-8 mg/kg/day (Rayen et al., 2011, Salari et al., 2016)), allowing the fluoxetine and its active metabolite to pass to the offspring while nursing (Gentile et al., 2007). The short-term effects of such antidepressant treatment in ELS- and STD-housed pups could then be determined on P9, at the level of gene expression, neuroendocrine regulation, and behavior. Lasting changes and a potential rescue effect of fluoxetine on ELS-induced endophenotypes of affective disorders should be assessed in adult animals.

A different line of approach could be to attempt to counteract the lasting consequences of ELS in HR mice by SSRI treatment during adulthood. This has the advantage that considerations of interfering with early developmental processes of the serotonergic system can be neglected. However, treating mature animals has the disadvantage that any dysregulated neuroendocrine and molecular processes are already deeply entrenched, and have profoundly shaped downstream behavior over time (Lewis et al., 2014). Further, plasticity, required for change, may be harder to achieve in adults than in developing organisms (Kolb and Whishaw, 1998). Nevertheless, it has been demonstrated that SSRI treatment can reduce HPA axis hyper-responsiveness in human adults, who experienced childhood trauma (Rinne et al., 2003). Thus, effective antidepressant treatment in adults can normalized HPA axis dysregulation (Holsboer and Barden, 1996, Schule et al., 2003), probably by increasing GR mRNA and protein levels in the hippocampus (Pariante and Miller, 2001, Seckl and Fink, 1992). In addition, SSRIs can contribute to enhanced plasticity by increasing serotonin levels in the brain, which is involved in the expression and secretion of BDNF (Martinowich and Lu, 2008, Surget et al., 2011). Whether a neuroendocrine normalization can be obtained by SSRI treatment in adult HR animals after ELS, and whether their hyperactive stress-coping behavior and cognitive deficits can also be ameliorated in this way, remains to be tested.

Lastly, one major drawback of the presented research is that it included only male test subjects. Unfortunately, this gender bias is still found in the vast majority of preclinical and clinical studies (Hayden, 2010, Holdcroft, 2007, Wald and Wu, 2010). There are, of course, several good reasons for why many researchers preferentially opt for male subjects. For instance, possibly the main reason is that males do not go through complex hormonal cycles, which would otherwise need to be taken into account in the design and analysis of the study (Becker et al., 2005, Wizemann, 2001). For many research questions, using males therefore dramatically reduces the number of experimental subjects required, by increasing the homogeneity of the study population. Furthermore, the influence of gonadal hormones and sex chromosomes on behavior, neuroendocrine function, and gene expression can be very broad and many aspects remain poorly understood (Bale and Epperson, 2015), making it difficult to adequately control for potentially confounding effects. In addition, every new finding contributing to the body of existing research is built on previous findings and needs to be integrated into the current knowledge in order to move the field forward to new discoveries. Keeping the gender of experimental subjects constant increases the comparability of the results and often enables scientists to extrapolate from pervious findings (Eliot, 2011). In spite of these apparent advantages of male experimental subjects, more and more scientists and grant organizations are recognizing the importance of including females into their studies (Cahill, 2006, Clayton and Collins, 2014). This is especially critical for the study of affective disorders, as these afflict women twice as often as men (Altemus et al., 2014, Nestler et al., 2002). Therefore, in future research, the interaction of genetic predisposition in the SR mouse model and ELS should also be investigated in female animals. As sex-dependent difference in the neuroendocrine effects of perinatal stress have repeatedly been described (Bale and Epperson, 2015, Garcia-Caceres et al., 2010, Glover and Hill, 2012, Weinstock, 2007, Mueller and Bale, 2008), it is likely that females will show a different response pattern to postnatal ELS exposure than their male siblings. Evidence from prenatal depression studies indicates a sexually dimorphic effect of prenatal stress, as it is generally proposed to increase the risk for neuroendocrine dysregulation and affective disorders in females, while males appear to be more likely to develop learning and memory difficulties and autism-spectrum disorders (Bale, 2011, Weinstock, 2007, Davis and Pfaff, 2014), but not all findings are consistent (Entringer et al., 2009, Glover and Hill, 2012). Other studies have investigated gender differences in susceptibility to postnatal stress (Arp et al., 2016, Elton et al., 2014, Horovitz et al., 2012, Lajud and Torner, 2015, Naninck et al., 2015, Oomen et al., 2011), but also these results are not yet fully conclusive and highlight the

importance of several influencing factors, including age, type of stressor, duration of stress exposure, and level of analysis or readout (Loi et al., 2014). To increase our understanding of the complex three-way interaction of genes, gender, and environment, a study exploring the consequences of ELS in female mice in the SR mouse model would be a promising future research project.

5.6. Conclusion

In conclusion, the results presented in this thesis demonstrate a high degree of construct and predictive validity in the SR mouse model, further establishing its value as a research tool to explore the molecular processes underlying the pathophysiology of MDD, as well as the neurobiological underpinnings of the antidepressant treatment response in melancholic/psychotic and atypical depression. In addition, using the SR model, we were able to show that mice with a genetic predisposition for high stress reactivity display a heightened vulnerability for lasting adverse consequences after ELS, affecting the neuroendocrine system and gene expression, as well as stress-coping behavior and cognitive function in early and late adulthood. These long-term consequences were preceded by acute changes in neuroendocrine readouts and gene expression in the PVN in vulnerable animals only, suggesting that a short-term neuroendocrine dysregulation during the SHRP may be critically involved in programming the lasting endophenotypes. To further elucidate the processes underlying the developmental programming in this G × E interaction and to test their reversibility, we suggest studying the effects of an early or late intervention with pharmacological treatment. Moreover, valuable insights may be gained by changing the timing (e.g. postnatal or adulthood) and type (e.g. chronic mild stress, social defeat, or substance addiction) of the stress manipulation, as well as by repeating the experiments using female animals.

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7. APPENDIX

7.1. Abbreviations

ACe Central Amygdala

ACTH Adreno-Corticotropic Hormone

ANOVA Analysis of Variance

AUC Area under the Curve

AVP Arginine vasopressin

BDNF Brain-derived Neurotrophic Factor

BLA Basolateral Amygdala

BnST Bed Nucleus of the Stria Terminalis

CAR Cortisol Awakening Response

CNS Central Nervous System

CORT Corticosterone

CRH Corticotrophin-Releasing Hormone

dDG Dorsal Dentate Gyrus

DEX Dexamethasone

DG Dentate Gyrus

dHip Dorsal Hippocampus

DSM Diagnostic and Statistical Manual of Mental Disorders

ELS Early-life Stress

FKBP5 FK506 binding protein 5

FST Forced Swim Test

GABA *Gamma*-Aminobutyric Acid

GC Glucocorticoid

G × E Gene × Environment

GR Glucocorticoid Receptor

GRE Glucocorticoid Response Element

GWAS Genome-wide Association Studies

HPA Hypothalamic-Pituitary-Adrenal

HPRT Hypoxanthine-Guanine Phosphoribosyltransferase

HR High Reactivity

ITI Inter-trial Interval

IR Intermediate Reactivity

LR Low Reactivity

LTP Long-Term Potentiation

MAOA Monoamine Oxidase A

MAOI Monoamine Oxidase Inhibitor

MDD Major Depressive Disorder

MR Mineralocorticoid Receptor

MWM Morris Water-Maze

OFT Open Field Test

ORT Object Recognition Test

P Postnatal Day

PFA Paraformaldehyde

PFC Prefrontal Cortex

PTSD Posttraumatic Stress Disorder

PVN	Paraventricular Nucleus of the Hypothalamus
PVT	Paraventricular Nucleus of the Thalamus
qPCR	Quantitative Polymerase Chain Reaction
REM	Rapid Eye Movement
RIA	Radioimmunoassay
SD	Standard Deviation
SHRP	Stress Hyporesponsive Period
SNP	Single Nucleotide Polymorphism
SNS	Sympathetic Nervous System
SR	Stress Reactivity
SRT	Stress Reactivity Test
SSRI	Selective Serotonin Reuptake Inhibitor
STD	Standard
TBP	TATA-Binding Protein
TCA	Tricyclic Antidepressants
TST	Tail Suspension Test
USV	Ultrasonic Vocalization
vDG	Ventral Dentate Gyrus
vHip	Ventral Hippocampus
WCM	Water Cross-Maze
WHO	World Health Organization

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7.3. Affidavit

I hereby confirm that the dissertation “Genetic risk factors and early-life stress interact to shape endophenotypes of affective disorders: Behavioral, neuroendocrine, and molecular consequences of a gene × environment interaction” is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

Munich, date _____

Signature _____

7.4. Declaration of author contributions

Chapter 2

Antidepressant treatment differentially affects the phenotype of high and low stress reactivity mice

All listed authors contributed to this manuscript: Alexandre Surget, Petra Van Nieuwenhuijzen, Jan Michael Heinzmann, Alana Knapman, Silja McIlwrick, Willy Paul Westphal, Chadi Touma, and Catherine Belzung. The study was designed by Alexandre Surget, Chadi Touma and, Catherine Belzung. The acquisition of the data was accomplished by Petra Van Nieuwenhuijzen, Jan Michael Heinzmann, Alana Knapmann, and Willy Paul Westphal. Data analysis and statistics were done by Alexandre Surget and Silja McIlwrick. Alexandre Surget drafted the manuscript, which was revised by Catherine Belzung, Chadi Touma and Silja McIlwrick. All authors approved to all modifications and approval of the final version of the manuscript.

Chapter 3

Genetic predisposition for high stress reactivity amplifies effects of early-life adversity

All listed authors contributed to this manuscript: Silja McIlwrick, Alexandra Rechenberg, Mariana Matthes, Jessica Burgstaller, Thomas Schwarzbauer, Alon Chen, and Chadi Touma. The study was designed by Silja McIlwrick and Chadi Touma. Silja McIlwrick performed the experiments and prepared the manuscript. Alexandra Rechenberg assisted with the sample preparation for qPCR. Mariana Matthes assisted with the USV experiment. Jessica Burgstaller assisted with the analysis of the maternal behaviour and USV recordings. Thomas Schwarzbauer assisted with the recording of maternal behaviour. Chadi Touma edited the manuscript and supervised the study. Alon Chen gave advice regarding the format of the manuscript and data presentation. All authors approved to all modifications and approval of the final version of the manuscript.

Chapter 4

Late-onset consequences of early-life stress are moderated by differences in genetic stress reactivity

All listed authors contributed to this manuscript: Silja McIlwrick, Tobias Pohl, Alon Chen, and Chadi Touma. The study was designed by Silja McIlwrick and Chadi Touma. Silja McIlwrick performed the experiments and prepared the manuscript. Tobias Pohl assisted with behavioral testing in the late adulthood animal cohort. Chadi Touma edited the manuscript and supervised the study. Alon Chen gave advice regarding the format of the manuscript and data presentation. All authors approved to all modifications and approval of the final version of the manuscript.

Date, Signature of Lab Head and Thesis Candidate

7.5. List of all publications

van de Giessen E, **McIlwrick S**, Veltman D, van de Nbrink W, Booij J. Obesity an addiction? Imaging of neurotransmitter systems in obesity *Pet and Spect in Psychiatry*. 641-656. DOI: 10.1007/978-3-642-40384-2_27.

McIlwrick S, Rechenberg A, Matthes M, Burgstaller J, Schwarzbauer T, Chen A, Touma C. (2016). Genetic predisposition for high stress reactivity amplifies effects of early-life adversity. Psychoneuroendocrinology. 2016 Aug; 70:85-97. DOI: 10.1016/j.psyneuen.2016.04.023.

Surget A, Van Nieuwenhuijzen PS, Heinzmann JM, Knapman A, **McIlwrick S**, Westphal WP, Touma C, Belzung C. (2016). Antidepressant treatment differentially affects the phenotype of high and low stress reactive mice. Neuropharmacology. 110. 37-47. DOI: 10.1016/j.neuropharm.2016.07.007.

McIlwrick, S., Pohl, T., Chen, A., Touma, C. (2016). Late-onset consequences of early-life stress are moderated by differences in genetic stress reactivity. Frontiers in Cellular Neuroscience. Submitted September 2016