Implication of individual differences in glucocorticoid responsiveness to stress in the development of psychopathology-like behaviour and underlying neurobiology

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Preface

In the following pages I present a selection of the work conducted in the development of my doctoral thesis. Following a brief general introduction there is a review chapter, serving as an addition to the general introduction. It provides commentary around the complexity inherent in considering the interaction between stress physiology, developmental timepoints and the developmental programming of psychopathology-like behavior, in that case with specific reference to pathological aggression. Thereafter follow three research chapters focused on addressing how individual and constitutive differences in physiological reactions to adversity influence the neurobehavioral outcome of that adversity.

Summary

The period comprising late childhood and adolescence is a critical window in brain development. It is a time of both neuroendocrine and neuroanatomical lability, a circumstance which renders individuals highly sensitive to stress. Indeed, experience of adversity early in life increases vulnerability to psychopathology, though not all individuals exposed to such stress go on to develop psychopathological alterations. The mechanisms via which vulnerability to psychopathology is differentially translated between individuals are not yet well understood.

Previous work from our laboratory has shown that exposing Wistar rats to an intermittent, unpredictable schedule of psychogenic stressors during the period equivalent to late childhood and puberty increases the expression of several psychopathology-like behaviors, when assessed at adulthood. In common with findings in humans, results have hinted that not all individuals exposed to this stress protocol develop along the same trajectory, particularly with regard to aggression. Additional findings have implicated the release and actions of glucocorticoids, the end product of hypothalamic-pituitary-adrenal (HPA) axis activation, as a factor mediating some of the neurobehavioral alterations induced by peripuberty stress. This thesis has therefore focused on assessing whether individual differences in glucocorticoid responsiveness to stress may influence individual differences in neurobehavioral outcome following stress.

In a first study, we exposed outbred, male Wistar rats to peripuberty stress and measured several socio-affective behaviors at adulthood, as well as examining brain structure by means of *ex vivo* magnetic resonance and diffusion tensor imaging. By applying a profiling approach we were able to discern two distinct neurodevelopmental trajectories arising from peripubertal stress. One trajectory lead to the development of pathological aggression and reductions in mean diffusivity in infralimbic cortex, amygdala, hippocampus and subiculum. The other trajectory was associated with increased anxiety-like behavior and reduced social motivation but no evidence of altered brain structure was observed in the regions examined. In addition, we assessed glucocorticoid responsiveness to peripuberty stress at various timepoints across the protocol and found the propensity to show impaired habituation to be associated with development of a more aggressive profile later in life.

Human studies have found impairment in habituation of glucocorticoid responses to the same stressor, shown by approximately 35% of individuals, to be both heritable and associated with increased self-report of psychopathology-related indices. Taking this and the findings outlined above into consideration, we next asked whether constitutive differences in the ability to habituate to repeated stress might be implicated in vulnerability to develop psychopathology. To answer this question, we developed a new animal model. Using a selective breeding approach in outbred Wistar rats, we generated lines enriched for stress habituation and lack of stress habituation, as indexed by glucocorticoid responsiveness on the last day of a three day stress protocol. Once the lines were successfully established, we assessed the socio

-affective behavior and neuroendocrine phenotype of rats drawn from the lines, but not exposed to stress. We found that rats with constitutive impairment in stress habituation displayed enhanced aggression, anxiety-like, and depression-like behaviors, a pattern that was stable across generations. We additionally observed differential expression of genes implicated in HPA axis activation within both central and peripheral nodes of the HPA axis. This was found in the context of a distinct neuroendocrine phenotype in which rats with constitutively impaired HPA axis habituation showed enhanced corticosterone reactivity to acute stress relative to the other line, yet no evidence for general HPA axis hyperactivity.

In a final experiment, we studied the interaction of two risk factors, early life stress and HPA axis dysregulation, in the subsequent development of psychopathology-like behavioral alterations. The rat lines, selected either for low or high glucocorticoid responsiveness to repeated stress (i.e. strong or impaired corticosterone habituation, respectively) were exposed to peripuberty stress. Socio-affective behaviors and basal activation of several stress-sensitive brain regions were assessed at adulthood. Results indicated that both factors enhanced levels of anxiety-like and aggressive behavior, as well as increasing basal activity in several subregions of the prefrontal cortex in a manner that was associated with increased behavioral inhibition. Peripuberty stress had a differential impact on aggression in the two rat lines, enhancing aggression in the stress-habituating low-line rats but not in the already high-aggressive, high-line rats.

In summary, we have established the incidence of individual differences in neurobehavioral trajectory following peripuberty stress, and found these differences to be associated with differential patterns of glucocorticoid responsiveness to the stress. Constitutively impaired stress habituation increased psychopathology-like behavior in its own right, a trajectory which did not become more pronounced with exposure to peripuberty stress. In contrast, constitutively strong habituation enhanced sensitivity to the programming effects of peripuberty stress.

Keywords: Stress, early life stress, peripuberty, adolescence, HPA axis, corticosterone, stress habituation, adaptation, allostatic load, individual differences, aggression, anxiety, prefrontal cortex, hippocampus, amygdala, MRI, DTI

Résumé

La fin de l'enfance et l'adolescence sont des périodes critiques dans le développement du cerveau. C'est une période de labilité à la fois neuroendocrine et neuroanatomique, une situation qui rend chaque individu hautement sensible au stress. En effet, il a été montré que de vivre des adversités tôt dans la vie pouvait être un risque important de développer des maladies psychologiques, bien que certains individus exposés à de tels stress ne développeraient pas de troubles mentaux. Les mécanismes de cette vulnérabilité à l'adversité pouvant différer entre chaque individu ne sont pas encore bien compris.

D'anciens travaux de notre laboratoire ont montré qu'exposer des rats Wistar à des épisodes de stress sporadiques et imprédictibles, pendant la période équivalent à la fin de l'enfance et la puberté (appelé par la suite peripuberté), mènent à l'état adulte à des altérations du comportement lié à l'humeur. Comme chez l'homme, les résultats montrent que tous les individus ne réagissent pas de la même façon, surtout pour les comportements agressifs. Des résultats complémentaires ont impliqué la libération et l'action des glucocorticoïdes, le produit final de l'activation de l'axe hypothalamo-hypophyso-surrénalien (HHS), comme facteurs clés médiant ces altérations du comportement induites par le stress peripuberté. Cette thèse se concentre donc sur l'évaluation des différences individuelles dans la réactivité des glucocorticoïdes au stress, pouvant expliquer les différences de comportement suivant ce stress.

Dans une première étude, nous avons exposé des rats mâles Wistar au stress peripuberté et mesuré plusieurs comportements sociaux affectifs à l'âge adulte. Nous avons aussi évalué la structure du cerveau par des techniques *ex vivo* de résonance magnétique et d'imagerie de tenseurs de diffusion. En appliquant une approche de profilage, nous avons été capable de discerner deux trajectoires neurodéveloppementales distinctes suite à ce stress. L'une mène au développement de l'agressivité pathologique et à des réductions de la diffusivité moyenne dans le cortex infralimbique, l'amygdale, l'hippocampe et le subiculum. L'autre est associé à une augmentation des comportements anxieux et à une réduction de la motivation sociale, mais aucune évidence d'altérations du cerveau n'a été trouvé dans les régions étudiées. De plus, nous avons évalué la réactivité des glucocorticoïdes au stress peripuberté à différents points pendant le protocole et avons montré une propension à une habituation altérée associée au développement du profil plus agressif plus tard dans la vie.

Des études chez l'homme ont montré que ces troubles de l'habituation de la réponse aux glucocorticoïdes lié au stress, qui touchent environ 35 % de la population, sont héréditaires et associés à une augmentation du risque de développer des troubles psychologiques. Prenant en considération ceci et les découvertes présentées ci-dessus, nous nous sommes ensuite demandés si les différences constitutives dans la capacité à s'habituer au stress répété pouvaient être impliquées causalement dans la vulnérabilité au développement des troubles psychologiques. Pour répondre à cette question, nous avons développé un nouveau modèle animal. En utilisant un élevage sélectif de rats Wistar, nous avons généré des lignées avec une plus grande ou une absence d'habituation au stress, comme indexé par la

sécrétion des glucocorticoïdes dans le dernier jour d'un protocole de 3 jours de stress. Une fois le bon établissement de ces lignées, nous avons examiné les comportements sociaux-affectifs et le phénotype neuroendocrinien des rats issus de ces lignées, mais non exposés au stress. Nous avons trouvé que les rats présentant un défaut constitutif dans l'habituation au stress ont une augmentation du comportement agressif, ainsi que des comportements de type anxieux et dépressifs, et ces traits se stabilisent le long des générations. Nous avons aussi observé une expression différentielle des gènes impliqués dans l'activation de l'axe HHS, aussi bien centraux que périphériques. Ces différences de phénotype neuroendocrinien ont montré que les rats avec des différences d'habituation de l'axe HHS ont aussi une plus grande réactivité de la corticostérone suite à un stress aigu comparé aux autre lignées, mais sans une hyperactivité générale de l'axe HHS.

Dans une dernière expérience, nous avons étudié l'interaction de deux facteurs de risque : le stress pendant l'enfance et la dérégulation de l'axe HHS, et leur implication dans le développement de troubles mentaux. Des lignées de rats, présentant une réactivité basse ou haute aux stress répétés (i.e. forte ou absence d'habituation de la corticostérone, respectivement), ont été exposées au stress peripuberté. Les comportements sociaux-affectifs et l'activation basale de plusieurs régions du cerveau sensibles au stress ont été évalués à l'état adulte. Les résultats ont montré que ces deux facteurs augmentent les comportements agressifs et anxieux, ainsi que l'activation basale de plusieurs sous-régions du cortex préfrontal, dans le sens d'une augmentation de l'inhibition comportemental. Le stress peripuberté a un effet différent sur l'agressivité chez les deux lignées, en augmentant l'agressivité chez les rats avec une faible habituation (« low line ») mais sans modifié l'agressivité déjà forte des rats avec une haute habituation (« high line »).

En résumé, nous avons établi l'effet des différences individuelles dans les réponses neurocomportementales suite au stress pendant la puberté, et avons trouvé que ces différences sont associées à différents motifs de réactivité des glucocorticoïdes au stress. Le trouble constitutif de l'habituation à la réactivité des glucocorticoïdes suite aux stress répétés augmente les comportements psychopathologiques.

Mots clés : stress, stress pendant l'enfance, peripuberté, adolescence, axe HHS, corticostérone, habituation au stress, adaptation, charge allostatique, différences individuelles, agression, anxiété, cortex préfrontal, hippocampe, amygdale, IRM, ITD.

General Introduction

Introduction

The concept of stress

Selye (1936) first described a non-specific, three-stage, organism wide response observed following the exposure of rats to diverse noxious stimuli. He referred to the first stage, occurring within 48 hours of any such stimulus, as the "general alarm reaction of the organism". The reaction in its entirety, from alarm, to resistance, to exhaustion following longer-term exposures, was termed "general adaptation syndrome". Later he used the term "stress response" to describe the alarm reaction, referring to the "non-specific neuroendocrine response of the body" (Selye, 1950).

In recent years, definitions of what may constitute a stressor (i.e. a stimulus causing a stress response) have been attempted by many, but, owing to the breadth of both the syndrome itself and of the disciplines of those studying it, no single, unifying definition exists. However, a broad and generally accepted characterization within the field of neuroscience is that stress equals "an actual or anticipated disruption of homeostasis or an anticipated threat to well-being" (Ulrich-Lai & Herman, 2009).

Allostasis and mechanisms of allostatic load

In addition to outlining 'alarm' and 'resistance' phases of the stress response, Selye also described, in conditions of unrelenting exposure to a stressor, a state he called 'exhaustion' whereby many of the negative effects of the initial alarm phase reasserted themselves. The appearance of this exhaustion-like state was suggested to show that the adaptability of organisms to changes in their surroundings was finite. Moreover, it appeared that the magnitude of individual adaptability appeared to depend largely upon genetic factors (Selye, 1950). More recently, this has been conceptualized in different terms (McEwen & Stellar, 1993). The response to stress is thought to help restore homeostasis and mediate adaptation following cessation of a stressor, a process that has been termed allostasis. Such responses, however, have a physiological cost. Repeated, prolonged or inadequate stress responses may lead to damage, termed allostatic load. Much like Selye's 'exhaustion', over time allostatic load may come to have irrevocable effects, being beyond the capacity of the individual to cope, a state called allostatic overload. Accumulation of allostatic load is thought to form the pathological basis of many stress-related disorders (McEwen, 2007). Figure 1 illustrates ways in which allostatic load may be accumulated via dysregulation of 'normal' stress responses. It is unlikely that these mechanisms act within isolation within the lifetime of an individual. Conceivably, a period of stress may program the responsiveness of the individual to other stressors.

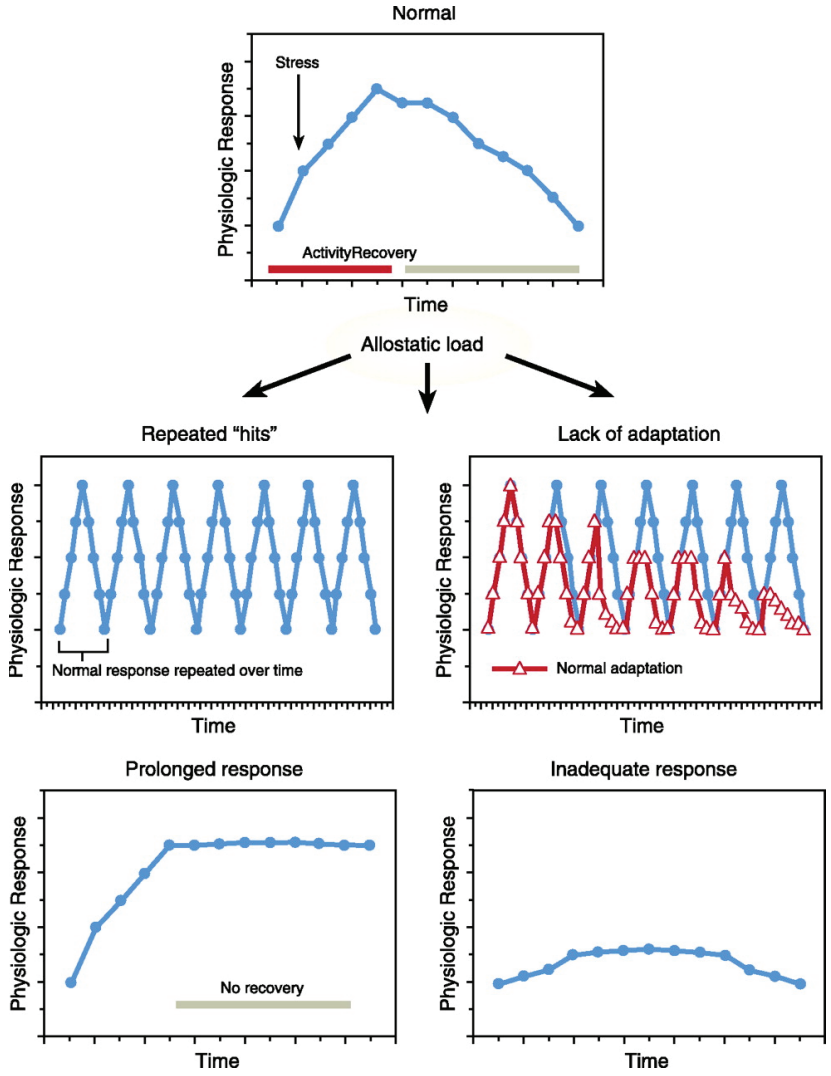

Figure 1 The top panel illustrates a 'normal' stress response. The panels below represent the four types of allostatic load - middle *left*: repeated "hits" from multiple stressors; *middle right: impaired* adaptation; *bottom left*: prolonged response which leads to enhanced exposure to stress mediators; *bottom right:* insufficient cortisol response which fails to buffer the activity of other mediators (i.e. increased levels of cytokines). The first three types of allostatic load may all give rise to excessive exposure to the catabolic effects of glucocorticoids. From McEwen (1998)

The neurobiology of stress

Upon perception of a stressor, physiological systems are activated which allow an organism to react and adapt to the presence of the stressor. This change is orchestrated via complex interaction across several parts of the central nervous system (Joëls & Baram, 2009). Many factors may influence the nature of the stress response; including the type of stress and its duration, prior experience, as well as the individual's genetic background (Fig. 2A). The effects of stress on the central nervous system are mediated by a range of substances, including neuromodulators, steroid hormones and peptides. The diversity in these mediators allows for similar, necessary, diversity in responses (Joëls & Baram, 2009).

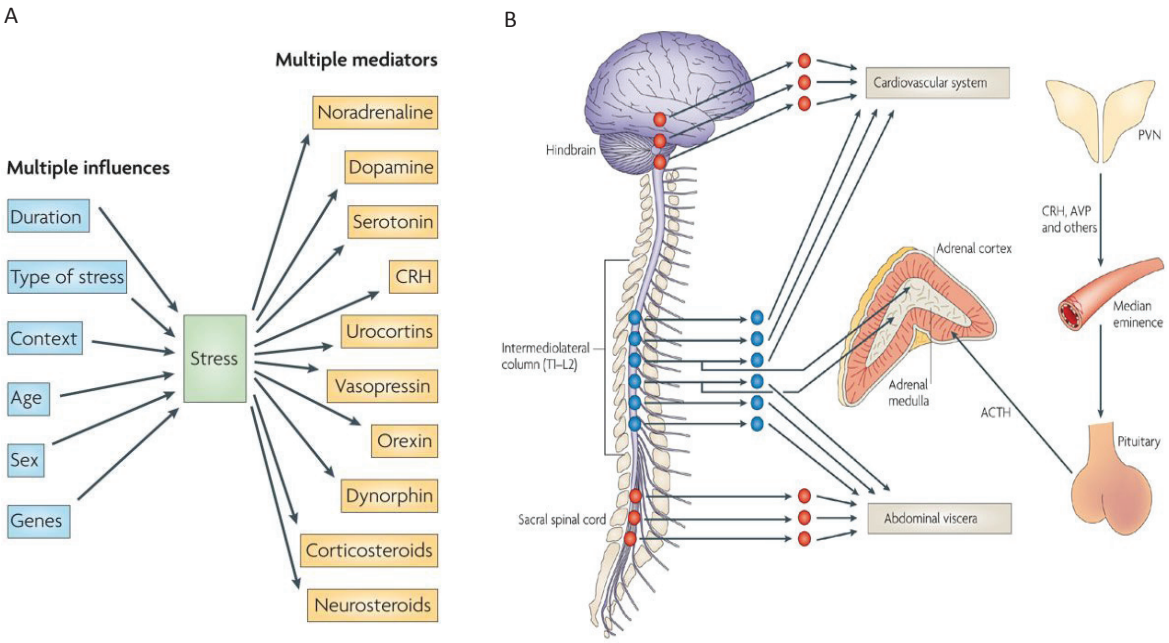

Figure 2 Many factors may influence the stress response, including the duration and type of stress, as well as the individual's genetic background (A: from Joëls & Baram, 2009). The effects of stress on the central nervous system are mediated by a range of substances, including neuromodulators, steroid hormones and peptides. The diversity in these mediators in part allows for the diversity in responses to stress. The sympathetic branch of the autonomic nervous system (ANS; B: left-side) and hypothalamic-pituitary-adrenal (HPA; B: right-side) axes are the principle systems involved in reacting to stress, as well as maintaining or reinstating homeostasis during and after stress. (B: from Ulrich-Lai & Herman, 2009).

The autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axes are the principal systems whose activation allows for maintenance and reinstatement of homeostasis during, and after, exposure to a stressor (Fig. 2B). Perception of a stressor results in activation of sympathetic neurons of the ANS, which project to the internal organs and the adrenal glands. This activation rapidly increases circulating levels of adrenaline and noradrenaline, leading to increased heart rate and contraction force, vasoconstriction, and energy mobilization, together enabling the classic 'fight or flight' type of stress response. These effects are buffered in turn by activation of the parasympathetic branch of the ANS, whose effects are, in general, opposite to sympathetic activation. Exposure to a stressor additionally activates the HPA axis, though its mediators mobilize within a somewhat less rapid timeframe. This response is initiated by the release of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus of the hypothalamus (PVN). These peptides synergize to stimulate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary which, in turn, triggers the production and release of glucocorticoids (primarily cortisol in humans; corticosterone in most rodent species) from the adrenal glands into the circulation (Ulrich-Lai & Herman, 2009). Glucocorticoids exert many effects, including promoting the mobilization of stored energy, suppression of the immune system

and potentiation of sympathetically mediated effects via their actions at mineralocorticoid (MR) and glucocorticoid (GR) receptors (de Kloet, 2014; de Kloet et al., 2008; Joëls et al., 2013). Activation of the HPA axis and ANS thus have largely complementary actions throughout the body.

In the brain, glucocorticoids have many additional effects, acting as modulators of brain structure and function via genomic and non-genomic actions at both MRs and GRs (de Kloet et al., 2008; Groeneweg et al., 2011; Sandi, 2004). Activation of MR and GR in diverse brain regions, including the prefrontal cortex, hippocampus, and amygdala, influences activity within those regions, thereby influencing the continued activity of the HPA axis (see Fig. 3 for indication of receptor distribution; Shirazi et al., 2015). The hippocampus and the prelimbic part of the prefrontal cortex have been implicated in negative feedback regulation of the HPA axis, whereas the amygdala and the infralimbic part of the prefrontal cortex are thought to have a stimulatory role on the HPA axis (Ulrich-Lai & Herman, 2009). In the PVN, as well as in the pituitary gland, GR activation acts to inhibit continuation of the stress response by inhibiting expression of CRH and ACTH, respectively. GR activation thus regulates activity of the HPA axis, and its readiness to respond to new challenges. This negative feedback has been found to act via both fast and slow mechanisms (Tasker & Herman, 2011).

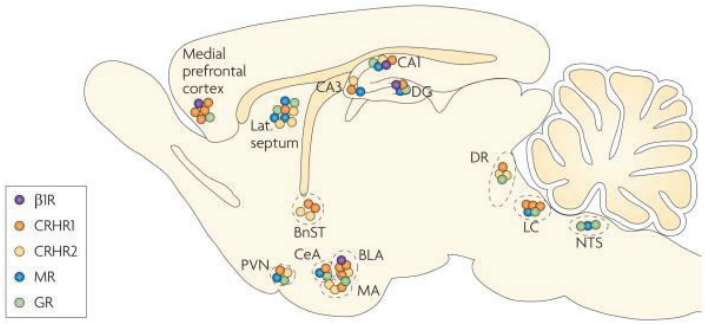

Figure 3 β1-adrenoceptors for noradrenaline (b1Rs), CRH receptor 1 (CRHR1), CRHR2 and the mineralocorticoid and glucocorticoid receptors (MRs and GRs, respectively) cluster in the brain. These clusters can be found in diverse regions including: the prefrontal cortex, the amygdala, the hippocampus, and the paraventricular nucleus of the hypothalamus (PVN), all regions that have been found to be highly sensitive to various effects of stress. Abbreviations: BLA, basolateral amygdala; BnST, bed nucleus of the stria terminalis; CeA, central amygdala; MA, medial amygdala; NTS, nucleus tractus solitarii (from Joëls & Baram, 2009)

In accordance with the role of GR activation as a primary mediator of negative feedback inhibition of the HPA axis, GR expression appears to be programmed according to circumstances (de Kloet, et al., 2005). Additional factors may act to modulate the sensitivity of GR including: posttranslational modification (Nicolaides et al., 2010); interactions with various co-chaperones in the cytoplasm (Hartmann et al., 2012; Touma et al., 2011); and interactions with co-regulator proteins once bound to DNA (de Kloet et al., 2009; Zalachoras et al., 2016). Factors impacting GR sensitivity may have a significant impact on regulation of HPA axis activity. One such example can be found in GR co-chaperone, FK506 binding protein 5 (FKBP5; *Fkbp5*) which, when associated with GR, reduces the affinity of the receptor for its ligand (Wochnik et al.,

2005). This has the effect of decreasing nuclear translocation of GR, thereby impacting the ability of glucocorticoid signal to induce transcriptional regulation (Binder, 2009). When GR does translocate, it leads to an upregulation of FKBP5, thereby leading to further enhancement of GR resistance (Vermeer et al., 2003). In humans, polymorphisms in the gene encoding FKBP5 have been associated with greater induction of FKBP5 mRNA following GR activation (Klengel et al., 2013), having the functional effect of prolonging stress responses (Ising et al., 2008; Touma et al., 2011). Crucially, polymorphisms within FKBP5 have been associated with increased risk for stress-related psychiatric disorders, such as depression, PTSD, and pathological aggression, when occurring in interaction with early life adversity (Appel et al., 2011; Bevilacqua et al., 2012; Binder et al., 2008; Bryushkova et al., 2016; Xie et al., 2010).

Early life stress and programming of the HPA axis

The responsivity of the HPA axis changes throughout the life span, according to the developmental stage of the individual (Romeo, 2016). Importantly, evidence indicates that HPA axis responsiveness later in life can be programmed by early life experiences (Matthews, 2002; Tarullo & Gunnar, 2006). The experience of many types of adversity early in life can alter the development of the HPA axis, and appears to predispose individuals toward the development of psychopathology (Gunnar, 2015; Trickett et al., 2010). Adults maltreated as children exhibit alterations both in circadian HPA axis rhythmicity, as well as stressor -induced responses, which can vary depending on several factors (Heim & Nemeroff, 2001: Tarullo & Gunnar, 2006). Early life maltreatment can influence adult HPA axis function in both directions, such that response to a stressor may be hypoactive in one individual and hyperactive in another. This variability has been associated with the type of maltreatment experienced, the timing of the maltreatment, as well as by current psychiatric diagnosis (Tarullo & Gunnar, 2006; Gunnar, 2015). A similar role for early life experience in HPA axis programming has been reported across animal models (Aisa et al., 2008; Brunton & Russell, 2010; Sanchez, 2006; Weaver et al., 2004), thereby indicating the usefulness of such models in dissection of the interaction.

Early life stress and development of psychopathology

A large body of literature implicates experience of early adversity as representing a vulnerability toward the development of psychiatric disorders later in life, including: mood disorders, anxiety disorders, and (anti)social disorders (Agid et al., 1999; De Bellis & Thomas, 2003; Essex et al., 2011; Famularo et al., 1992; Green et al., 2010; Heim & Nemeroff, 2001; Pechtel & Pizzagalli, 2011; Viding & McCrory, 2012; Weder et al., 2009). Childhood adversity is estimated to account for at least 30% of psychiatric disorders, which rises to 45% if disorders with childhood onset are also taken into consideration (Green et al., 2010). Adversity can take many forms, including: poverty, a lack of stability in parenting (e.g. neglect, parental substance abuse or mental illness), life-changing events (e.g. a serious accident/illness, family breakdown, death of a parent), as well as incidences of explicit abuse, which in itself can take several forms. Adversities may be inter-related, and the likelihood of one or more forms of adversity occurring in

conjunction is high. Importantly, early adversity does not only impact mental wellbeing but also the physical health, with individuals exposed to multiple adversities having considerably lower life expectancy than unexposed individuals (Brown et al., 2009).

Childhood and adolescence as a critical period in brain development

Throughout development there are windows of plasticity in the brain, during which brain structure and function are sensitive to the programming effects of the environment (Andersen & Teicher, 2008; Spear, 2000). Late childhood and adolescence represents such a window. It is a period characterized by maturational changes in brain regions, such as, prefrontal cortex, hippocampus, and amygdala, that are jointly implicated in the modulation of HPA axis function, and in socio-affective behaviors (Andersen & Teicher, 2008; Casey et al., 2008; Giedd, 2004; Gogtay et al., 2004; Paus et al., 2008; Spear, 2000). The plastic changes ongoing during this window include increased myelination, synaptic overproduction, and synaptic pruning (Liston & Gan, 2011; Paus et al., 2008; Spear, 2000), all of which are sensitive to disruption by stress hormones (Chetty et al., 2014; Liston & Gan, 2011; Pattwell et al., 2016). Moreover, the period around puberty is marked by a profound upward shift in the responsiveness of the HPA axis (Romeo, 2003). Taken together, this combination of factors makes peripuberty a time particularly sensitive to the programming effects of stress on brain structure and function, and therefore on behavior.

Effects of child and adolescent stress on brain structure

Studies specifically measuring the impact of adversity in childhood and adolescence on brain structure are relatively few but support the view that the course of brain development can be altered by stress during this time. Magnetic resonance imaging studies have reported reduced regional volumes of orbitofrontal and medial prefrontal cortical regions following early adversity (Baker et al., 2013; Cohen et al., 2006; Holz et al., 2015). In contrast, enlargement of amygdala has been found following stress late in childhood (Pechtel et al., 2014). Interestingly, the relative magnitude of alterations was found to be associated with the reported severity of childhood stress (Baker et al., 2013; Pechtel et al., 2014).

Regional differences in brain volume found in magnetic resonance studies are thought to reflect hypo- and hypertrophy of neuronal processes in the affected region. Examination of neuronal morphology in rats after exposure to chronic stress around puberty reported findings that complement findings in humans. Relative to a non-stressed control group, hypotrophy of neuronal processes in hippocampus and prefrontal cortex (Eiland et al., 2012; Henckens et al., 2015; Isgor et al., 2004), and hypertrophy of the amygdala have been reported following stress (Eiland et al., 2012; Henckens et al., 2015).

An animal model of early life stress induced psychopathology: Overview of findings observed using the peripuberty stress model

As outlined above, in humans the time around puberty has been recognized as a period where individuals

may be particularly vulnerable to the effects of stress. Despite this, relative to other critical windows in development, basic research into the effects of stress during puberty and adolescence is limited, and animal models few. This has meant that understanding of the mechanisms underlying different developmental trajectories following stress during this period has remained relatively poor. Seeking to expand the variety and validity of animal models available to address this key area of research, this laboratory developed a peripuberty stress model (Toledo-Rodriguez & Sandi, 2011). The stress protocol involves exposing rats to unpredictable, psychogenic stress on seven intermittent days between postnatal (p) day 28 to p42. In rats this two-week period comprises late childhood and early adolescence, and culminates with puberty. The stressors used in this protocol are fear-inducing, and include exposure to an elevated platform and, separately, exposure to predator odor, both of which are delivered under bright lighting conditions. For the specific details of the protocol please refer to chapter 2. In the following paragraphs we outline the principal behavioral and neurobiological findings obtained from adult male rats exposed to peripuberty stress earlier in life.

That the protocol is an effective stressor has been indicated by three important findings. Firstly, assessment of the magnitude of corticosterone released on the first and final days of the protocol revealed unequivocal increases in plasma corticosterone concentration in rats exposed to the stress (Marquez et al., 2013). Moreover, peripuberty stress was found to result in reduced weight gain in exposed rats (Tzanoulinou et al., 2014a), as well as in a delay of puberty onset (Marquez et al., 2013). At the behavioral level, peripuberty stress has been found to result in long-term alterations in social, cognitive, and affective behaviors when measured in adult male rats, and these findings are outlined in Table 1.

Behavioral test	Observation	Interpretation	References
Elevated Plus Maze	↓ time in open arms	↑ anxiety-like behavior	Cordero et al., 2016 ; Marquez et al., 2013; Tzanoulinou et al., 2014a
Novelty exposure	↑ time exploring a novel object	↑ novelty-seeking	Tzanoulinou et al., 2014b
Sucrose preference	↓ sucrose consumption	↑ depression-like behavior	Marquez et al., 2013
Forced swimming	↑ time floating	↑ depression-like behavior	Marquez et al., 2013; Veenit et al., 2014
Social preference	↓ time exploring a juvenile rat	↓ social motivation	Marquez et al., 2013 ; Poirier et al., 2014 ; Tzanoulinou et al., 2014a ; Tzanoulinou et al., 2014b ; Veenit et al., 2014
Resident-intruder	↑ time of offensive behavior	↑ inter-male aggression	Cordero et al., 2016 ; Marquez et al., 2013; Tzanoulinou et al., 2014b
Male-female cohabitation	↑ time of offensive behavior to female	↑ aggression to females	Cordero et al., 2012; Cordero et al., 2016
Five-choice serial reaction time task	Slowed task acquisition and ↑ errors of omission once well trained	↑ deficit in attention	Tzanoulinou et al., 2016

Table 1 Overview of the main behavioral alterations observed following exposure to peripuberty stress in male rats.

As well as giving rise to decreased social motivation (Márquez et al., 2013; Poirier et al., 2014; Tzanoulinou et al., 2014b; Tzanoulinou et al., 2014a; Veenit et al., 2014), peripuberty stress has been found to increase the aggressive behavior of rats toward adult conspecifics (Cordero et al., 2012; Cordero et al., 2016; Marquez et al., 2013; Tzanoulinou et al., 2014b). Peripubertally stressed rats not only exhibit increased levels of aggressive behavior towards opponents of a similar size but have also been found to show atypically high levels of aggression toward smaller, larger, or even anaesthetized intruders, as well as toward females (Cordero et al., 2012; Marquez et al., 2013). The pattern of indiscriminate highly-aggressive responding, whereby the rat appears to disregard the level of threat posed by each opponent, has led to the characterization of peripubertally stressed rats as abnormally aggressive. This characterization has been supported by the finding that peripuberty stress exposed rats more frequently targeted the vulnerable body parts of the intruder when biting, and continued to attack even when the opponent showed signs of submission (Marquez et al., 2013). Exposure of peripubertal rats to the whole protocol was necessary to observe behavioral alterations (Tzanoulinou et al., 2014b).

In the brain, exposure to peripuberty stress has been found to result in elevated amygdala activation under basal conditions, as indexed by uptake of radio-labelled 2-deoxyglucose, a modified form of glucose that is readily taken up by cells but cannot be metabolized (Marquez et al., 2013). Hints toward increased activation of prefrontal cortex under basal conditions were also found. Further examination of the basis of changes in activity suggested that alterations potentially resulted from a shift in the balance between excitation and inhibition in these regions following peripuberty stress, such that excitation comes to predominate. Reductions in the expression of inhibition-related genes and proteins were found in both amygdala (Tzanoulinou et al., 2014a; Tzanoulinou et al., 2014b) and in several subregions of the prefrontal cortex (Tzanoulinou et al., 2016) following peripuberty stress. Moreover, behavioral deficits observed following peripuberty stress, and found in association with reduced inhibition in prefrontal cortex, were ameliorated by a treatment that enhanced the expression of inhibition-related gene, *Nlg2* (Tzanoulinou et al., 2016), in prefrontal cortex thereby implying a causal link between altered inhibition and behavioral dysfunction following stress.

In an effort to understand the role of HPA axis activation induced by peripuberty stress in subsequent behavioral alterations a number of experiments have been carried out. Mimicking peripuberty stress induced HPA axis activations via the administration of corticosterone according to the same schedule revealed that peripuberty stress effects on (anti)social behaviors could be recapitulated by corticosterone alone (Veenit et al., 2013). Interestingly, anxiety-like and depression-like behaviors were unaltered by this treatment. Experiments which attempted to block the different components of HPA axis following stress were able to shed some light on the dissociation. When glucocorticoid receptors were blocked with an antagonist prior to each exposure to peripuberty stress, then the effects of the stress on adult social, but not affective, behaviors could be blocked (Veenit, unpublished observations). If type 1 CRH receptors were blocked with an antagonist in the week following the completion of the peripuberty stress protocol, then the effects of the stress on both social and affective behaviors could be blocked (Veenit et al., 2014).

Taken together these three studies strongly implicate HPA axis activation induced by stress as playing a key role in the development of psychopathology-like behaviors later in life, with corticosterone seeming to play a particularly important role in the programming of aggressive behavior.

This thesis continues in chapter 1 to discuss in greater depth the role of developmental aberrations in HPA axis activity in the development of psychopathology, taking pathological aggression as a case in point.

Chapter 1

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

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Abstract

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

Introduction

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

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

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

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

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

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

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

When translating developmental research between studies in humans and preclinical rodent models, it is important to note that there are important differences in the timing of the HPA axis development

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

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

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

2.1 Rodents selectively bred for extremes in HPA axis activity

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

Mouse lines selected for extremes in HPA axis responsiveness to stress have been generated recently (Touma et al., 2008). Specifically, C57Bl/6 mice were selected and bred according to their plasma corticosterone response to 15 minutes of restraint stress, producing high-reactive (HR), low-reactive (LR) and intermediate-reactive (IR) lines (Touma et al., 2008). Once the lines were established, although they did not show differences in corticosterone levels at circadian nadir, HR mice had significantly higher diurnal corticosterone than the IR and LR lines (Touma et al., 2008). Following exposure to a stressor, and as compared to LR mice, HR animals were more reactive and showed higher activation of the paraventricular hypothalamic nucleus. Moreover, HR mice exhibited higher corticosterone responses to an ACTH injection and impaired negative feedback inhibition following a combined dexamethasone/CRH test (Heinzmann et al., 2014; Touma et al., 2008). The IR line displayed intermediate responses in these measurements. In one study, mice from these lines were tested for their aggressive behavior in the resident-intruder test. In this test, an unfamiliar mouse ('intruder') is introduced into the homecage of the experimental animal ('resident'). In this study, analyses were focused on the time the resident mouse

took to attack the intruder – i.e., latency to attack, used as a proxy of aggressiveness – following the placement of the latter in the resident's cage. LR mice were the fastest to attack and 92% of them performed an attack within 300s vs only 42% of HR mice. The IR line behaved at an intermediate level, with 70% performing an attack within 300s (Touma et al., 2008). Therefore, low HPA axis responsiveness was linked to enhanced reactivity to attack an intruder conspecific and, hence, aggressiveness, in this study, while high HPA axis responsiveness had a negative link with aggression.

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

2.2 Inbred rat strains

The second approach that we have chosen to discuss in this section is the comparison of phenotypes presented by inbred rat strains, which are generated by mating siblings across many consecutive generations. This process results in a strain in which only one version of each gene is present, and all animals are therefore genetically identical, somewhat akin to twins. Specifically, we discuss here strains of rats that present differences in the functioning of their HPA axis and that have been tested for their aggressive responses.

Such a comparison can be established, for example, between Fischer 344 (F344) and Lewis inbred rat strains, which were both derived from the Sprague Dawley strain. As noted by several studies, although

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

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

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

be displayed by the Lewis and SHR strains showing, respectively, the lowest and highest HPA axis reactivity, while low aggression levels correspond to the strains (i.e., F344 and WKY) showing intermediate HPA axis responses.

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

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

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

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

One of the best studied mouse lines in this context are the ones originally selected from wild house mice according to their short (SAL) or long (LAL) latency to attack a conspecific mouse (van Oortmerssen & Bakker, 1981). SAL mice displayed higher number of attacks and higher duration of aggressive behavior than LAL mice (Caramaschi et al., 2008a). Importantly, SAL mice have been described as abnormally

aggressive as they attack females and anesthetized intruders, and ignore submissive postures of their opponents (Caramaschi et al., 2008a). Analysis of their HPA axis function indicates abnormal reactivity in SAL mice. Thus, although no differences between SAL and LAL mice were described under basal conditions (Veenema et al., 2003), SAL mice showed a flatter circadian corticosterone rhythmicity; the typical upshift of corticosterone during the dark phase being blunted in comparison to LAL mice (Korte et al., 1996). Furthermore, following exposure to novelty, administration of ACTH or forced swim stress, SAL mice displayed blunted corticosterone response relative to LAL mice (van Riel et al., 2002; Veenema et al., 2003), and mild psychosocial stress-induced corticosterone increases were short, as opposed to longer-lasting responses observed in LAL mice (Veenema et al., 2003).

Lines of rats derived from wild-caught Norway rats were selected according to their low ('domesticating') or high (maintenance of 'wild') aggressiveness toward a glove (Naumenko et al., 1989). Domesticated rats showed no aggressiveness toward humans by the 10th generation of selection (Plyusnina & Oskina, 1997). In terms of social behavior, wild rats emitted considerably more fighting bouts in shock-induced fighting tests than tame rats, but, at the 19th generation of selection did not display more inter-male aggression when not provoked by shock, nor were they more frequently muricidal (Naumenko et al., 1989). Later generations of the lines showed relatively higher inter-male aggressive behavior and lower social interaction in wild rats relative to domesticated rats (Gulevich et al., 2015). Regarding their HPA axis, wild line rats display higher basal corticosterone levels than tame rats (Gulevich et al., 2015; Naumenko et al., 1989). This finding was sustained when studying fecal matter obtained in the absence of any human interaction, which would presumably constitute a stressor, particularly to the wild line (Albert et al., 2008). Additionally, wild line rats showed higher corticosterone responses to novelty than domesticated rats, and had higher adrenal weight, indicative of both situational and general hyperactivity of the HPA axis (Naumenko et al., 1989; Plyusnina & Oskina, 1997).

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

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

In addition to genetic selection, early life experiences can also have profound consequences on the development of the HPA axis. In particular, exposure to stressful experiences during different stages of development are known to have long-term consequences on HPA axis function and behavior. Early life

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

4.1 Prenatal stress

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

4.2 Early postnatal stress

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

In Wistar rats, maternal separation during the first two weeks of life led to a pattern of changes in endocrine and behavioral responses differential according to developmental stage (Veenema et al., 2006).

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

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

In monkeys, juveniles reared in isolation were found to display elevated baseline cortisol levels, though acute stress-induced cortisol levels was not different to controls at adulthood (Meyer & Bowman, 1972; Sackett et al., 1973). Young monkeys, that were maternally-separated at birth, hand-reared for the first month and subsequently raised with same-age peers for the next 5 months, displayed higher levels of impulsive aggressive behaviors during play-fighting (Higley et al., 1996). Monkeys with this early life history were toward the bottom of the social hierarchy when housed with mother-reared peers (Suomi, 1997) and when challenged by a period of social separation, peer-reared monkeys exhibited extreme behaviors and higher HPA axis responses (Higley et al., 1991; Higley & Suomi, 1989). Furthermore, studies on monkeys maltreated by the mother during infancy have reported increased plasma cortisol levels in infant monkeys and exaggerated aggressive behaviors during adolescence (Howell et al., 2013). Conversely, other studies of peer-reared monkeys found low basal cortisol and low HPA axis response to stress as well as no differences in basal and stress-induced levels of cortisol (Clarke, 1993; Winslow et al., 2003; Champoux et al., 1989; Feng et al., 2011). Thus, no clear picture of the effects of peer-rearing stress on the HPA axis is evident. Recent studies have focused on explaining some of this variability, determining genetic factors and emphasizing the importance of gene-environment interactions linking stress, HPA axis and aggression (Novak et al., 2013). (Novak & Suomi, 2008) applied a rearing model in which monkeys were raised with an inanimate surrogate mother and provided daily exposure to playmates. Surrogate/ peer-reared monkeys were more aggressive and displayed abnormal aggressive behaviors, as they did not respond to submissive postures of their opponents (Novak & Suomi, 2008). Furthermore, monkeys exhibited lower levels of circulating cortisol and showed blunted HPA axis response to a period of social separation (Capitanio et al., 2005; Davenport et al., 2003; Shannon et al., 2005; Shannon et al., 1998).

Overall, the picture arising from early stress protocols in different species emphasizes, once more, a

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

4.3 Peripubertal and adolescent stress

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

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

Bullying, or social abuse, is modelled in rodents via means of repeated social subjugation. Social subjugation of juvenile rats, by daily exposure to an aggressive adult, was shown to lead to enhanced basal corticosterone levels as well as exaggerated aggressive behaviors after both physical and social provocation, including towards larger opponents (Cunningham & McGinnis, 2008). In hamsters, juveniles

(P26-38) exposed for 20 minutes daily in the homecage to an aggressive adult male (Delville et al., 1998), while not showing alterations in basal corticosterone levels, had increased stress-induced corticosterone responses (Wommack & Delville, 2003). Subjugated hamsters attacked less intruders of similar size, but exhibited increased aggressive behavior (specifically, more biting) towards smaller opponents (Delville et al., 1998; Wommack & Delville, 2003; Wommack et al., 2003). Subjugated animals also showed premature transition from play-fighting behavior to adult-like patterns of attack, and displayed high levels of aggression at adulthood (Wommack et al., 2003). Other studies reported that hamsters subjugated during puberty (P26-38) showed high levels of aggression toward intruders and blunted release of cortisol (Ferris et al., 2005).

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

Given all the findings reported above, we can argue that the relationship between stress and the development of alterations in HPA axis functions and aggressive behaviors that emerges from this data is complex. Again, rats stressed at peripuberty and/or adolescence tend to develop higher HPA axis reactivity and increased aggression. An exception seems to be for the peripubertal stress model in which lower HPA axis reactivity was linked to increased aggression. Critically, the effects seem to be highly dependent on the developmental period when stress is given, but also depend on the protocol and species used. Given the limited number of studies, further research is needed to disentangle the impact of different types of stress over time and at varying intervals of brain development in relation to aggressive behavior.

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

In addition to genetic factors and early life stress, the HPA axis can be affected during developmental periods by exposure to a range of substances. We have a special focus here on drugs of abuse and antidepressants. The rationale to review the literature on drugs of abuse rests on the well-known, close

and bidirectional interaction of the HPA axis and the mesolimbic dopamine system, the latter being a major site of action for these drugs (Koob & Kreek, 2007; Ungless et al., 2010). Moreover, mesolimbic dopamine plays a critical role in motivation towards both social and non-social stimuli (Salamone & Correa, 2012). Antidepressants are included in this section as there is documented evidence that they can affect neurodevelopmental trajectories of individuals.

5.1 Cocaine

Evidence indicates that prenatal cocaine exposure blunts HPA axis reactivity to novel and stress inducing stimuli in rats (Johns & Noonan, 1995; Johns et al., 1994), whilst also leading to enhanced aggressiveness (Johns & Noonan, 1995; Johns et al., 1994; Wood & Spear, 1998). Conversely, chronic cocaine exposure during adolescence appeared to give rise to a hyperactivity of the HPA axis in response to stress exposure (Alves et al., 2014) as well as leading to enhanced aggressiveness in both rats (Alves et al., 2014) and hamsters (Harrison et al., 2000; Jackson et al., 2005; Knyshevski et al., 2005).

5.2 Alcohol

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

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

5.3 Cannabinoids

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

2006).

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

5.4 Antidepressants

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

Exposure to addictive substances and medicines *in utero* can lead to both hypo and hyperactivity of the HPA axis later in life. Whether alterations in HPA axis activity in response to challenge represent general hypo- or hyper-function of the axis remains unknown. Drug-induced alteration of HPA axis function is associated with both increase and decreases in aggressive behavior depending on the drug in question. Effects of drug exposure during adolescence, on the other hand, render a more coherent picture. Across drug classes, evidence, though scant, indicates that adolescent exposure leads to enhanced HPA axis response to stressors, as well as enhanced aggression.

6. Conclusions

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

Thus, although a general unifying picture cannot be extracted from the reviewed data, there are certain commonalities that ought to be emphasized. We found several examples suggesting that aberrations towards abnormally low or abnormally high HPA axis functionality taking place during development are associated to increased aggression, frequently characterized by pathological features. Thus, the reviewed literature suggests the existence of a U-shape function between developmental HPA axis reactivity and the emergence of aggressive phenotypes (Figure 1). Other data from the animal literature, not reviewed here, show that the direct manipulation of glucocorticoids at adulthood, leading to both abnormally low (Haller et al., 2004; Haller et al., 2001) or high (Haller et al., 1997; Kruk et al., 2004) glucocorticoid levels can lead to pathological aggression. This is found alongside alterations in the activity of brain regions and circuits implicated in the control of aggression (Haller, 2014a, 2014b). However, a critical issue is whether aberrant HPA axis has a causal implication in the development of aggressive phenotypes.

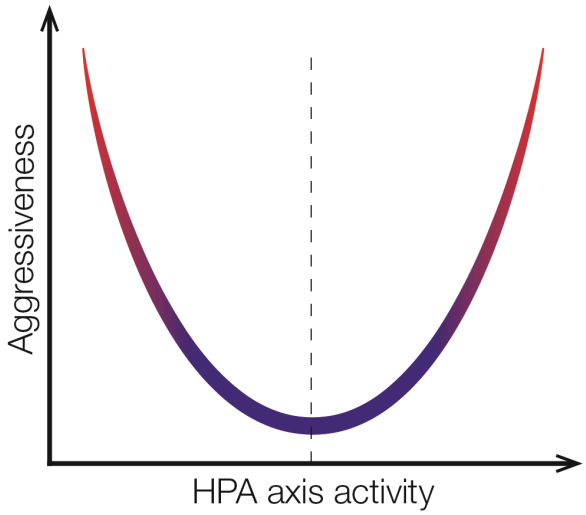

Figure 1. U-shaped relationship between HPA axis functioning and aggressive behavior.

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

Research linking both aspects from a developmental perspective is scarce and it is thus difficult to outline a comprehensive view that implies any particular link between extremes in HPA axis variation and features of pathological aggression. Indeed, the HPA axis is not a single unit and various outcomes may arise from a unique modification in the system. For example, a decrease in corticosterone production may lead to differential behavioral outcomes whether it is associated with a hypersensitivity to a regulator of aggression or in a negative feedback of the systems. Discrepancies between studies and outcomes, in addition to the already mentioned differences in protocols and species, may arise from the inappropriately focal picture observable using single-point analyses of HPA axis function employed by many studies in the field. Traditionally, in line with the 'hypoarousal theory' of violence (Raine, 1996), blunted activation of the stress systems has been proposed to be particularly associated with symptoms of psychopathy. However, enhanced HPA axis reactivity was also related to pathological aggression in several rodent models (e.g., post-weaning social isolation; lines bred for high anxiety, and lines bred for maintenance of wildness), potentially mimicking emotional-impulsive types of aggression. Importantly,

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

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

recent evidence in humans suggests that, even within individuals high in psychopathic traits, there might be subtypes presenting not only blunted, but also high HPA responses to stress (Johnson et al., 2015).

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

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Author contributions

S.W, A.P & D.H made equal contributions to the writing of the manuscript

C.S. wrote and edited the manuscript

Aims & objectives

Aims

As outlined in both the general introduction, and in Chapter 1, in common with the perinatal period, the period comprising late childhood and adolescence represents an additional developmental window during which individuals are highly sensitive to the effects of stress. However, not all individuals exposed to stress during this period go on to develop psychopathological problems, suggesting that additional qualities held by the individual may determine the outcome of exposure to stress. The factors that determine a path leading from early life stress to psychopathology are not well understood. This is doubly true for our understanding of factors determining individual differences in response to stress exposure. The sensitivity and responsiveness of the HPA axis has been posited as one factor, differing between individuals, which may mediate the outcome of stress exposure. Achieving better understanding of the role of the HPA axis in contributing to differential developmental trajectories in response to the same early life experience may allow insights leading to improved development, and targeting, of intervention strategies.

The study of gene x environment interactions in the development of psychopathology in humans is often complicated, not only by the inherent complexity of mechanisms translating environmental inputs into behavioral and neurobiological alterations but also by uncontrollable, extraneous factors. The increased controllability afforded by the use of animal models may therefore be employed in the dissection of such interactions. The research performed in the context of this thesis aimed to further investigate the long-term behavioral and neurobiological effects of peripubertal stress. However, rather than delving deeper into mechanistic processes, the investigations presented here focused on the determination of the involvement of differential HPA axis responsiveness in the development of differential neurobehavioral outcomes following the same early life experience. While the investigations presented here were interested broadly in socio-affective behaviors, our particular interest focused on aggression. We additionally sought to assess neurobiological alterations potentially underlying behavioral changes.

Objectives

Objective 1

Early life stress has been associated with the development of pathological aggression in some, but not all, individuals exposed to it. The determinants and correlates of this individual difference are not well defined. Studies of the brain structure of individuals diagnosed with aggression-related psychopathologies have shown pathological aggression to be associated with variation in brain volume in regions important in socio-affective function. It has been demonstrated that exposure to peripubertal stress in rats leads to alterations in aggressive behavior such that it becomes pathological in nature. This enhancement in aggression has been found alongside behaviorally-consequent shifts in activation in the same brain regions. The impact of peripuberty stress upon brain structure has not yet been studied, however. Though peripuberty stress has been found to impact later aggressiveness in many studies, some have indicated

that, in accordance with findings in humans, there were individual differences in the development of this response. Our first objective was therefore to determine whether the appearance of individual differences in behavioral outcome following peripuberty stress could be substantiated. To determine aggressive rats, considering both quantitative and qualitative measures of aggression, we applied a profiling approach. We additionally studied the behavioral phenotype of stress-exposed rats in other, non-aggression related behavioral tests. In addition, we used magnetic resonance imaging, in combination with diffusion tensor imaging, to survey neuroanatomical alterations potentially associated with altered neurobehavioral outcomes. The findings of this study are presented in chapter 2.

Objective 2

The HPA axis is a key mediator of adaptive physiological responses that enable individuals to cope with challenging situations. Dysregulation of its activity has been widely implicated in the development of stress-related psychopathologies, though whether a dysregulated HPA axis can lead to psychopathology in the absence of stress is unclear. Habituation of HPA axis responses across repeated exposure to the same stressor is a common adaptation. Impairment in this process, expressed only in subset of people, has been suggested to represent a risk factor for development of psychopathology. Given the ethical and practical difficulties of exposing individuals to repeated stress, study of the mechanisms via which impaired stress habituation might lead to psychopathology has been stunted. The additional lack of an appropriate animal model has exacerbated this problem. Our second objective was therefore to develop such a model in rats, thus allowing for the controlled assessment of whether this factor truly engenders risk for psychopathology. We applied a selective breeding strategy, in order to generate lines enriched for stress habituation and lack of stress habituation, as indexed by glucocorticoid responsiveness to repeated stressors, as well as a control line intermediate for the trait. Once lines were developed we assessed the socio-affective behavioral profile, as well as the neuroendocrine profile, of rats drawn from each of the lines but not exposed to early life stress. Details of the development of the lines, as well as findings regarding their behavioral and neuroendocrine profile are reported in chapter 3.

Objective 3

As previously discussed, experience of adversity early in life and dysregulation of hypothalamus-pituitary-adrenocortical (HPA) axis activity are risk factors often independently associated with the development of psychopathological disorders. Having addressed these risk factors in isolation in the previous objectives, here we asked whether in combination these factors would interact to shape the development and expression of psychopathology differentially. To answer this question we applied peripuberty stress, or control conditions, to the newly-developed stress-habituating and stress-non-habituating rat lines. We assessed the long-term effects of the different early life conditions upon

socio-affective behavior and brain activity in regions frequently found to be sensitive to pathological alterations following stress. The findings of this study are presented in chapter 4.

Chapter 2

Not all rats have an equal response to peripubertal stress: implication of individual differences in glucocorticoid responsiveness to repeated stress in the development of an aggressive phenotype and associated neuroanatomical alterations

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Abstract

Experience of early life adversity is implicated in the development of aggressive and anti-social behavior later in life but how negative early life experiences are translated to produce an aggressive phenotype in only a subset of individuals is not well understood. Magnetic resonance imaging (MRI) studies of individuals diagnosed with aggression-related psychopathologies show that pathological aggression is associated with variation in brain structure in areas important in socio-emotional function, and that this variation may potentially be exacerbated by experience of early life stress. However, studies such as these cannot easily parse the relationship between early life stress, brain structure and the development of an aggressive phenotype. Applying a combination of MRI and diffusion tensor imaging (DTI) to an animal model of pathological aggression induced by early-life stress, the peripuberty stress model, we asked whether individual differences in the development of an aggressive phenotype following stress exposure were associated with individual differences in brain structure in rats. We additionally sought to examine whether differences in the magnitude and/or adaptation of the stress-responsive hypothalamic-pituitary-adrenal axis during peripuberty stress exposure might be associated with the development of aggression later in life. We show here that exposure to peripubertal stress in rats leads to changes in tissue microstructure, but not macrostructure, within several aggression-related brain regions *only* in those individuals displaying an aggressive phenotype. Peripubertally stressed rats not displaying aggressive behavior were affected in terms of other non-aggression related behaviors but this phenotype was not associated with any observable neuroanatomical alterations in the brain regions examined. Moreover, attenuation of adaptation of the glucocorticoid response to stress across stress exposure was found to be associated with higher levels of aggression and reduced volume of infralimbic cortex in stress-exposed rats. This study thus establishes a strong link between peripubertal stress exposure and structural deviations in brain regions in association with pathological aggression, and points toward differential glucocorticoid adaptation to repeated stress as a potential underlying mechanism. It additionally highlights the importance of considering individual differences in behavioral response to stress when determining neurobiological correlates.

Introduction

The influence of early life experiences on the development of psychopathology has been the subject of intense research in recent years. Aggression, a core symptom of many psychiatric disorders, has become the focus of growing attention, and a burgeoning body of literature implicates experience of early life adversity with the development of aggressive and anti-social behavior later in life (Beach et al., 2011; Caspi et al., 2002; Fanning et al., 2014; Lee et al., 2014; Viding & McCrory, 2012; Weder et al., 2009; Widom & Maxfield, 1996). There are clear individual differences in vulnerability to early life stress exposure, with only a proportion of individuals experiencing adversity going on to develop pathological aggression (Caspi et al., 2002; Green et al., 2010; Odgers et al., 2008). How negative early life experiences are translated in some individuals to produce an aggressive phenotype is not well understood, and achieving better understanding would represent a clear benefit to advancement in the prevention and treatment of pathological aggression.

Magnetic resonance imaging (MRI) studies of individuals diagnosed with aggression-related psychopathologies show that, relative to control groups, pathological aggression is associated with variation in regional volume in areas important in socio-emotional function, including prefrontal cortex, hippocampus, and amygdala (Barkataki et al., 2006; Coccaro et al., 2016; Coccaro et al., 2015; Dolan et al., 2002; Raine et al., 2000; Zetzsche et al., 2007). Whether differences in volume are associated with alterations in tissue microstructure has not yet been reported. Interestingly, volumetric data obtained from non-clinical populations suggest that relative severity of exposure to early life adversity is correlated with degree of volume differences in the very same regions (Cohen et al., 2006; Hanson et al., 2015; Lupien et al., 2011). Recently, some additional findings have indicated that differences in volume in prefrontal cortex and hippocampus may be greater in aggressive individuals with experience of early life stress versus those without, as illustrated for example in patients with borderline personality disorder (Morandotti et al., 2013; Sala et al., 2011). However, studies such as these cannot exclude the contribution of additional factors (e.g., socioeconomic, cultural, nutritional, or other differences) to the relationship between early life stress, brain structure and the development of an aggressive phenotype, and addressing this question would largely benefit from the use of controlled animal studies.

Alterations in regional brain structure are thought to be associated with concomitant alterations in regional functionality (Draganski et al., 2004; Maguire et al., 2000). Prefrontal cortex, hippocampus and amygdala are part of a cortico-limbic circuitry not only structurally but functionally implicated in aggression, both in humans and in animals (Haller, 2014; Kohl et al., 2015; van der Kooij et al., 2014; White et al., 2016). All three regions undergo continuous development early in life, rendering them susceptible to the impact of stress (Casey et al., 2008; Spear, 2000). Though the consequence of early stress on brain and behavior has been well studied using animal models, research has tended to focus on stress applied during the prenatal and early-postnatal periods. Increasing appreciation of peri- adolescence as an additional

window during which environmental factors may influence developmental outcome has led to surge in experiments studying the effects of stress applied during this time. Our laboratory has developed a model that involves exposing rats to a sub-chronic, variable regimen of fear-inducing stressors across peripuberty, encompassing pre-puberty and puberty periods (Marquez et al., 2013). Exposure to peripuberty stress gives rise to changes in behavior in multiple domains but most notably leads to an increase in aggression such that it becomes pathological in nature (Cordero et al., 2013; Marquez et al., 2013; Tzanoulinou et al., 2014). Alongside differences in aggressive behavior, behaviorally-consequent shifts in activation have been demonstrated in several brain regions, including prefrontal cortex and amygdala (Marquez et al., 2013). The influence of peripubertal stress exposure on brain structure has not yet been studied, though application of alternative variable stress regimens during adolescence suggest that stress may impact neuronal morphology within similar regions (Eiland et al., 2012; Isgor et al., 2004).

It is noteworthy that although the finding that aggressive behavior is enhanced in rats following exposure to peripuberty stress has been replicated a number of times (Cordero et al., 2012; Cordero et al., 2016; Cordero et al., 2013; Marquez et al., 2013; Tzanoulinou et al., 2014), in accordance with findings in humans, there is substantial variability in later aggressiveness between individuals exposed to the stress (Tzanoulinou et al., 2014; Cordero et al., 2016). This implicates the peripubertal stress model as a potentially useful one with which to investigate the basis of individual differences in stress-induced aggression more generally. The correlates of variability in aggression remain to be determined but the literature suggests that individual differences in hypothalamic-pituitary-adrenal (HPA) axis activity in response to stress exposure represent a plausible candidate (Veenit et al., 2013).

Here, using a combination of MRI and diffusion tensor imaging (DTI), we asked whether individual differences in the development of an aggressive phenotype following peripuberty stress exposure were associated with individual differences in brain structure. Rather than rely on a single measure of aggression, we adopted a profiling approach to allow a more holistic assessment of the aggressiveness of individual rats, taking into consideration both quantitative and qualitative indices. Previous application of this approach has enabled the determination of neurobiologically meaningful subtypes of response to trauma (Cohen et al., 2004; Ritov et al., 2016). We focused our investigation on medial prefrontal cortex, amygdala, and hippocampal formation, brain regions that are: i) subject to ongoing development during adolescence (Spear, 2000; Casey et al., 2008); ii) demonstrated to be affected by peripubertal stress (Marquez et al., 2013), as well as other models of unpredictable stress applied during adolescence (Isgor et al., 2004; Eiland et al., 2012); iii) of importance in the performance of aggression (Haller, 2014; van der Kooij et al., 2014; Köhl et al., 2015). Furthermore, in order to enquire whether differences in the magnitude and/or adaptation of the stress-responsive HPA axis during peripuberty stress exposure might be associated with the development of aggression later in life, we analyzed the link between the emerging phenotype and glucocorticoid responsiveness to the stress. This is the first study to apply such an approach to the study neuroanatomical correlates of individual differences in the long-term aggressogenic response to early life stress.

Materials & Methods

Subjects

Experimental subjects were male offspring of Wistar Han rats, bought from a commercial supplier (Charles River, France) and bred in our animal facility. At weaning on p21, pairs of rats from different litters were matched according to weight and mixed among home cages. Animals in the same home-cage were always assigned to the same experimental group. Juveniles used in the social preference test and females used as cohabitants were of the same strain, purchased from the same supplier. Rats were maintained on a 12-h light–dark cycle (lights on at 0700h), in a temperature- and humidity-controlled environment (21 ± 1 °C; 55% humidity ± 5%), with *ad libitum* access to laboratory chow and water. Experimental subjects remained undisturbed, except for weekly cage changes, until experimental procedures began at adulthood (designated as p90). Prior to the first experiment, all rats were handled on three occasions, for two minutes per occasion. Experiments were performed between 0800 and 1200h, except where otherwise stated. All procedures were conducted in accordance with the Swiss National Institutional Guidelines on Animal Experimentation and approved by a license from the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

Peripuberty Stress protocol

The stress protocol was performed as previously described (Marquez et al., 2013). Following exposure to an open field (50 x 50 x 30cm) for five minutes on p28, the stress protocol consisted of the presentation of two different stressors, each one lasting 25 minutes (see Fig. 1 for exact schedule). These were either; exposure to the synthetic fox odor trimethylthiazoline (TMT; Phero Tech Inc., Canada) or to an elevated platform (EP). TMT exposure was administered in a plastic box (38 x 27.5 x 31 cm) via a scent-charged cloth. The box was placed under bright light (210–250 lx). The elevated platform (12 x 12cm, elevated 95cm from the ground) was also under direct bright light (470–500 lx). Following each stress session, animals were returned to neutral cages for 15 minutes. A transparent Plexiglas wall perforated with holes separated pairs of cagemates during this time. Following the holding period, animals were returned to their home cage. The stressors were applied during peripuberty, on seven intermittent days between p28 –p42, following a variable schedule. To assess the effect of exposure to peripuberty stress on HPA axis activity we took several blood samples from stressed rats across the course of the protocol. Specifically, we obtained tail blood at the offset of stress on the first day (p28), the third day (p30) and the final day (p42) of the protocol.

Behavioral procedures

As indicated above, this study focuses on the identification of neurodevelopmental trajectories that lead to differential aggression following exposure to peripubertal stress. However, in order to gain a better understanding of the behavioral phenotype associated with differences in aggression, animals were

additionally characterized in a broader battery of behavioral tests. The sequence of behavioral tests was chosen with the aim of submitting animals to tests inducing low to increasing stress levels. The full battery included exposure to a novel environment, the elevated plus maze, a test of social preference, the resident-intruder test, and forced-swimming (see Fig. 1 for details).

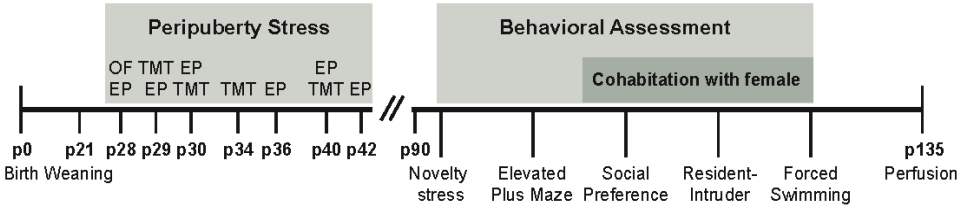

Figure 1 Experimental design. Animals were weaned at p21 and assigned to Control or Peripuberty Stress (PPS) groups. The stress protocol consisted of exposure to an open field (OF) on p28, followed by an elevated platform (EP), with predator odor (trimethylthiazoline; TMT) also used as a stressor. Stressors were presented in an intermittent and variable fashion, as depicted in the schema. Control animals were handled briefly on the days on which their experimental counterparts were exposed to stress. Behavioral testing started at p90, with a delay of one week imposed between each test in the series of tests.

Novelty stress

Following 20 minutes of exposure to a dimly-lit (30 lx) novel environment (circular plastic container; 35cm high, 25cm diameter), blood samples were obtained by tail-nick. A second tail-blood sample was obtained from the same tail-nick following 30 minutes in a neutral holding cage. Rats were then returned to their homecage. The containers were cleaned with 5% ethanol solution and dried between animals.

Elevated Plus Maze

Anxiety-like behavior was evaluated using the EPM test (Pellow & File, 1986). The apparatus consists of two opposing open arms (50 x 10cm) perpendicular to two enclosed arms (50 x 10 x 50cm) that extend from a central platform (10 x 10cm), elevated 65 cm above the floor. Light levels were maintained at 14-16 lx on the open arms and 5-7 lx on the closed arms. At the start of the test, the rat was placed on the central platform facing a closed arm and allowed to explore the maze for five minutes. In between animals, the apparatus was cleaned with 5% ethanol solution and dried. Behavior was monitored using a ceiling-mounted video camera and analyzed with a computerized tracking system (Ethovision 9; Noldus IT, Netherlands). The time spent and entries in the open and closed arms, and distance moved, were automatically recorded.

Social Preference test

The social preference test was performed as described by Tzanoulinou and colleagues (2014). Briefly, the test was performed in a rectangular, three-chambered box that included a central compartment and two side compartments. After five minutes of habituation to the central chamber, retractable doors were removed and the rat was allowed to explore the whole apparatus for 10 minutes. Side compartments were each equipped with a central, floor-fixed, transparent, perforated cylinder that contained either an unfamiliar male juvenile rat or an unfamiliar object. The apparatus was cleaned with 5% ethanol solution and dried between each trial. Each trial was video-recorded (MediaCruise, Canopus Co. Ltd, Japan) and manually scored offline by an experimenter blind to experimental group. The percentage of time spent exploring (snout <2cm from the cylinder) either the juvenile or the novel object was recorded, and a social preference ratio calculated according to the formula: time spent exploring the juvenile/time spent exploring the juvenile + object.

Resident-intruder test

Prior to the night of the resident-intruder (RI) test, experimental rats cohabited with a female partner for 10 days in order to encourage territoriality. The female was removed 30 minutes before the onset of the test, and replaced afterwards. The test was performed during the beginning of the dark cycle (between 1900 and 2200h). The resident was exposed in its home cage to a smaller (5-10% lighter), unfamiliar male intruder of the same strain for 30 minutes. Each intruder was used only once. Encounters were video-recorded and scored offline by an experimenter blind to the experimental group, assisted by Observer software (Noldus IT, Netherlands). The following parameters were quantified in terms of frequency and duration: attack, offensive upright, lateral threat, keeping down, biting, social investigation, non-social investigation and auto-grooming. The cumulative duration of the first four behaviors were summed to provide a measure of total offensive behavior. Latency to the first offensive event initiated by the resident was also recorded.

Further detailed video analysis was performed to identify the signaling, targeting, and intensity of biting attacks, as described by Toth and colleagues (2012). Specifically, a bite was considered to be signaled when it occurred in the context of an ongoing bout of offensive behavior. Bites were scored as targeted toward vulnerable (head, throat and belly) or non-vulnerable (back or flanks) parts of the opponent. Bites were also scored as hard or soft, depending on the response elicited by the bite. A hard bite was scored when the bite evoked a strong startle response from the opponent. Soft bites elicited little or no response from the opponent. The proportion of each of the following was calculated for all bites performed by one rat: i) unsignalled versus signaled bites; ii) bites targeted to vulnerable versus non-vulnerable areas; iii) hard versus soft bites. For bite-related measurements the number of rats in the control group reduced to eight, since three control rats did not perform any bites and to include them in the analysis with scores of zero would have biased results.

Forced swimming test

Whilst still cohabitating with females, rats were submitted to a forced-swimming test (FST) to evaluate depression-like behavior (Porsolt et al., 1978). Animals were placed in a plastic beaker (25 cm diameter x 46 cm) containing 30 cm of water (25°C) for 15 minutes. A second session was performed 24h later for 5 minutes. Both sessions were recorded using a ceiling mounted video camera, and the time spent immobile (making only those movements necessary to keep the snout above the water), swimming or climbing was quantified manually with the aid of in-house software (Clicker; EPFL, Switzerland) by an experimenter who was blind to the experimental condition.

Profiling for aggression

There are many behaviors exhibited during a social encounter which are considered to engender aggression. These can be both 'normal' (i.e. within species-typical norms) and 'abnormal' in nature (Haller, 2014). In order to measure holistically the development of an aggressive phenotype following stress exposure, an individual profiling approach was applied (Cohen et al., 2004; Ritov et al., 2015). Here, several indices of behavior observed during the resident-intruder encounter, including those considered to reflect both normal and abnormal aggression, were profiled. Classification criteria were defined according to the extremes (20^{th} or 80^{th} percentile, depending on index) of the control group's distribution for each measure, including: total duration of offensive behavior, frequency of offensive behaviors, latency to first offence, frequency of abnormal bites (either unsignalled or to a vulnerable body part), proportion of all biting attacks that were unsignalled, proportion of all biting attacks targeted toward vulnerable parts, and proportion of all biting attacks that elicited a strong response. Every rat that scored above (or below in the case of the sole 20^{th} percentile measure; latency to first offence) the cutoff for a particular measure was scored as being 'aggressive' in that measure. When a rat accrued five such scores, out of a possible seven, it was considered as an 'aggressive' rat overall.

Aggression *z* scores were calculated from the seven variables described above. The *z* scores were integrated to derive a single aggression score, subsequently used as a continuous variable against which tissue volume and mean diffusivity (MD) values for each region of interest were correlated (see Supplementary information for further details).

Perfusion

Two weeks after the last behavioral test, rats were anesthetized with a lethal dose of pentobarbital (Esconarkon, Streuli Pharma, Switzerland, 150 mg/kg body weight) and transcardially perfused according to the optimal method described by Cahill and colleagues (2012). Heads were stored in 4% PFA overnight, after which they were rehydrated for a minimum of one week prior to scanning in phosphate-buffered saline (PBS) containing 0.05% sodium azide.

Ex vivo DTI-MRI

Prior to scanning, the jaw and skin of each head was removed to allow placement into a 39 mm diameter birdcage radiofrequency coil (Rapid GmbH). Samples were immersed in fluorinated liquid (Galden, Solvay) to reduce susceptibility artifacts. Magnetic resonance (MR) images were then acquired with a 7-Tesla pre-clinical scanner (Agilent Technologies) in a single overnight session. Two sets of MR images were acquired: a high-resolution T2-weighted 3D Fast Spin-Echo (3DFSE) image for structural analysis and a Diffusion Tensor Imaging (DTI) protocol. The MR parameters were as follows:

3DFSE: Effective TE 60ms, TR 2000ms, matrix 192x128x192, isotropic 0.15mm voxels, 1 average, acquisition time 1h 44 min.

DTI: R 5000ms, TE 35ms, 10 averages, matrix 128x96x96, voxel 0.2x0.2x0.5mm, 40 continuous slices, 30 diffusion directions with b=2000 s/mm2 and 4 b=0 images, acquisition time 3h 54 min.

Image processing and statistical analysis

Volumetric and relaxometry analyses within Regions of Interest (ROIs)

The MR images were first converted to NIFTI format from the manufacturer's proprietary format using in house software. Structural brain images were analyzed by a region of interest (ROI) method that allowed automated comparison of the grey and white matter changes between peripuberty stress subgroups and control rats in predefined areas. The selected ROIs fulfilled the following criteria: i) subject to ongoing development during adolescence (Spear, 2000; Casey et al., 2008); ii) previously demonstrated to be affected by peripubertal stress (Marquez et al., 2013), as well as other models of unpredictable stress applied during adolescence (Isgor et al., 2004; Eiland et al., 2012); iii) of demonstrable importance in the performance of aggression (Haller, 2014; van der Kooij et al., 2014; Köhl et al., 2015). Medial prefrontal cortex, hippocampal formation, and amygdala all met these criteria. We additionally selected a subcortical control region, still developing during adolescence but not implicated in aggressive behavior, globus pallidus. FSEMS anatomical images from each rat were initially registered to a structural template using a rigid body registration and then subjected to unified segmentation tool in SPM8 (Ashburner & Friston, 2005), which corrects the intensity uniformity and then performs image segmentation by classifying different tissues [i.e. grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF)] according to a set of tissue probability templates (Valdés-Hernández et al., 2011). Following segmentation, the tissue class images (GM, WM and CSF) were used to create a population-specific template (PST) by running the diffeomorphic anatomical registration using exponentiated lie algebra (DARTEL) template-creation tool in SPM8 (Ashburner, 2007). Next, only the segmented grey and white matter tissue class images were used and spatially and nonlinearly normalized (warped) using the outputs from the previous DARTEL step. The DTI parameter maps were calculated using FSL (dtifit) and consisted of Mean Diffusivity (MD) and Fractional Anisotropy (FA). Template rat brain was manually parcelated into

regions of interest (ROIs) using J-IM software (Xinapse Systems, UK). The ROI's were then converted into binary image masks and the quantitative data (FA and MD) extracted from each ROI from each (template-registered) image using MARSbar tool box in SPM8 (Brett et al., 2002).

Immunofluorescence

After scanning, brains were removed from the skull and re-fixed in 4% PFA overnight, before being cryoprotected in 30% sucrose solution and frozen at -80°C. Subseries of coronal sections (30 µm thick), including medial prefrontal cortex, were cut on a cryostat and subsequently processed for immunofluorescence. Free-floating sections were triple labeled for myelin basic protein (MBP), NeuN, and DAPI. The floating sections were rinsed briefly with PBS then blocked for 1h in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and 5% normal donkey serum (Jackson ImmunoResearch) and then incubated overnight at 4°C with rabbit anti-MBP (Abcam, AB40390, 1:200) and mouse anti-NeuN (Millipore, MAB377, 1:100). The sections were washed in PBS and incubated for 2h at room temperature with the secondary antibodies: donkey-anti-rabbit IgG Alexa 568 conjugate (Lifetechnologies, A10042, 1:1000) and goat-anti-mouse IgG Alexa 488 conjugate (Lifetechnologies, A11029, 1:800). After washing in PBS the sections were incubated 10 minutes in DAPI (Sigma, 1:10000), rinsed and mounted with Fluoromount-G (Southern Biotech).

Images were captured with a Zeiss LSM700 confocal microscope using a 20X/0.8 objective. Sample images were captured at the same coordinates for each animal on each coronal slice. A mosaic of 16 images was captured for one hemisphere per slice, covering 750µm of the infralimbic cortex, starting from the medial hemispheric boundary. LSM images were stitched together using the Grid-Collection Stitching plug-in for FIJI (Preibisch et al., 2009). MBP fibers were delineated using Moments thresholding (Tsai, 1985) followed by a median filter of 2px on the binary mask. Positive pixels of the fiber mask were summed vertically through the width of the image. This summed pixel value was normalized by the image height. The resulting counts were then binned into 6 equal bins (layers) representing absolute distance from the medial edge of the cortex. Neuronal cells were delineated by scaling the original images by 0.2, applying a grayscale closing operation followed by a Laplacian of Gaussian filter of sigma= 4. Local minima within the Laplacian image were detected with a tolerance of 0.05. These maxima were filtered in order to keep only the ones whose average intensity was 1.3 times higher than their local background on the original NeuN image. Similarly to MBP, the number of NeuN positive cells detected following filtering was counted according to layer. In both cases, values from all sections from one animal were averaged to provide a single value per animal.

Corticosterone measurement

Measurements of free corticosterone were obtained from all blood plasma samples, via use of an enzymatic immunoassay kit performed according to manufacturer's instructions (Enzo Life Sciences,

Switzerland). Levels were calculated using a standard curve method.

Statistics

Data were analyzed using SPSS 17.0 (Chicago, USA). One rat was excluded from the control group in all measures as it was a statistical outlier (defined as more than three standard deviations from the mean) in the key measure of several behavioral tests. Results are presented as the mean ± SEM. Variables derived from the resident-intruder test were analyzed using two-tailed Mann-Whitney tests. Statistical testing of other behavior tests, MRI-DTI data and immunofluorescence were performed using Kruskal-Wallis tests. Bonferroni-corrected, Mann-Whitney post-hoc tests were used to explore significant results, with comparisons performed between the control group and each of the two peripuberty stress groups. A 2-way repeated measures ANOVA was used to analyze measurements of corticosterone concentration obtained from plasma taken across the stress protocol, with peripuberty stress subgroup as the between-subjects factor and postnatal day as the within-subjects factor. Similarly, a 2-way repeated measures ANOVA was used to analyze measurements of MBP and NeuN immunostaining, with peripuberty stress subgroup as the between-subjects factor and layer of infralimbic cortex as the within-subjects factor. Correlations of corticosterone measures against bite frequency and regional volume, and aggression score against mean diffusivity, were performed using Spearman's method. Statistical significance level was set at $p<0.05$. A p-value was considered as tending toward significance when $0.05 \leq p \leq 0.1$.

Results

Exposure to peripuberty stress gave rise to an aggressive phenotype

We first evaluated whether rats exposed to peripuberty stress showed differences in aggression relative to the non-stressed control group, and independent of an individual differences approach. In accordance with previously published data from our lab (Cordero et al., 2013; Marquez et al., 2013; Tzanoulinou et al., 2014), peripubertally stressed rats displayed an aggressive phenotype (Aggression *z score*: U=13, $p<0.01$). Breaking down the cumulative *z* score into individual variables, group differences in total percentage of time engaged in offensive behavior (Fig. 2A: U=49, n.s.) and frequency of offensive behaviors (Fig. 2B: U=59.5, n.s.) were not evident. However, peripubertally stressed rats engaged in offensive behavior more readily (Fig 2C: U=32, $p<0.05$), and the biting attacks they performed were of proportionately greater intensity (Fig 2G: U=16, $p<0.05$). Peripubertally stressed rats also displayed non-significant trends toward higher proportion of biting attacks targeted to vulnerable body parts of the opponent (Fig 2F: U=24, $p<0.1$), as well as higher frequency of attacks characterized as abnormal in terms of targeting or signaling (Fig 2E: U=28, $p<0.1$). Biting attacks were equally well signaled to the opponent (Fig 2H: U=43, n.s.).

Individual differences in development of an aggressive phenotype suggest two subtypes of behavioral response to peripuberty stress

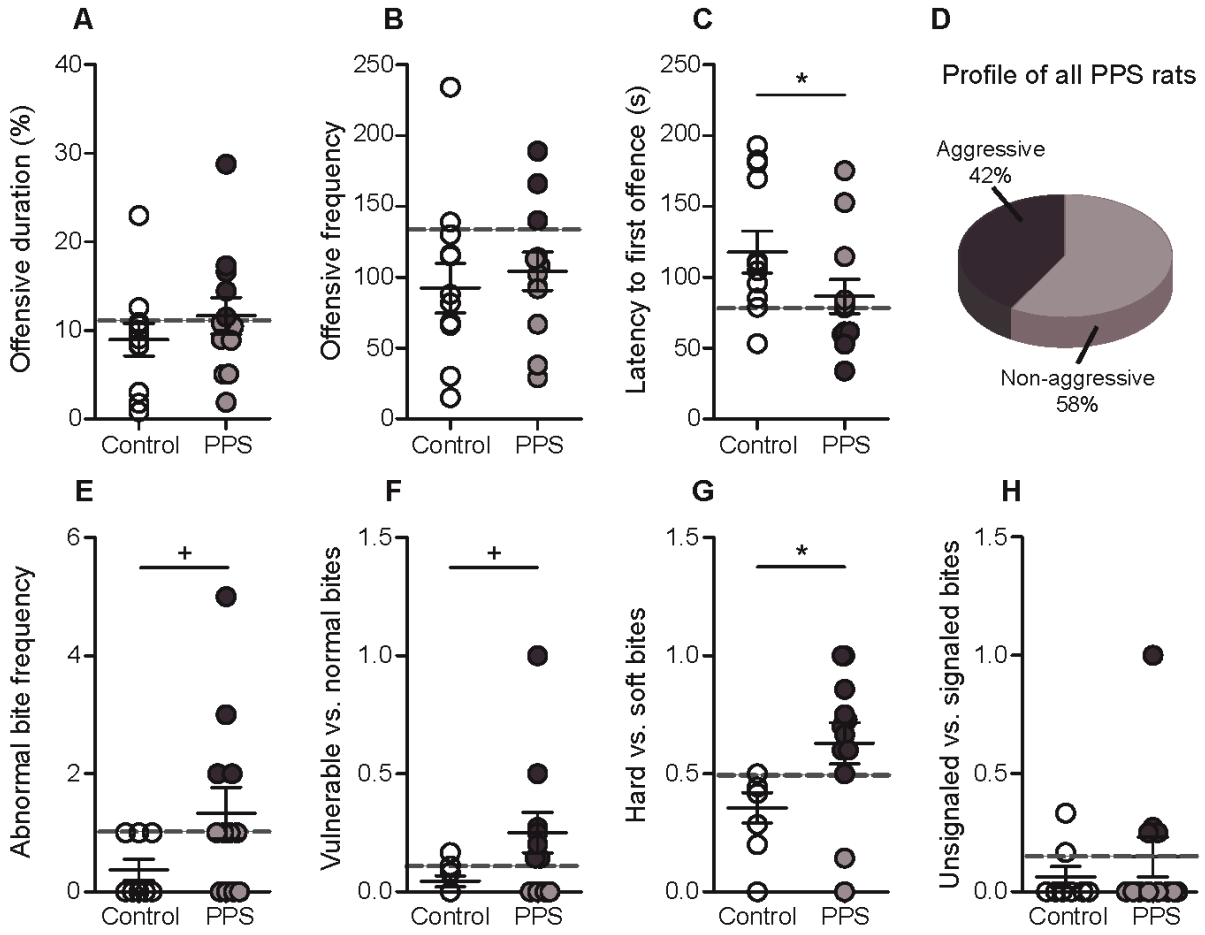

Figure 2 There were individual differences in the development of an aggressive phenotype following exposure to peripuberty stress (PPS). When exposed to an unfamiliar intruder, adult PPS rats did not differ at the group level from the control group in terms of the total amount of time spent engaged in offensive behavior (A), nor in the frequency of offensive behaviors (B). However, PPS rats did offend more readily (C). Compared to control rats, the attacks of PPS rats tended to be more frequently abnormal in nature (E), with a non-significant trend to target vulnerable body parts more readily (F). A higher proportion of biting attacks performed by PPS rats were 'hard', eliciting a strong startle response from the opponent (G). Control and PPS rats showed similar signaling of their intent to attack (H). Significant differences between groups are indicated by asterisks (Mann-Whitney tests; + = $p<0.1$; * = $p<0.05$; see text for further details). Large inter-individual variability was evident in all aspects of aggressive behavior. Profiling was conducted using the values of the control group as a reference. Dashed lines indicate the 80th (A, B, E, F, G, H) or 20th (C) percentile for each variable considered within the profile. A rat was considered to be aggressive overall when it exceeded the cutoff in a minimum of five of these indices. This yielded two subgroups amongst PPS-exposed rats, the non-aggressive (n=7) and aggressive (n=5) individuals (D).

As predicted and as previously observed (Tzanoulinou et al., 2014; Cordero et al., 2016), we observed variability in performance of aggression between individuals exposed to peripuberty stress. In order to discern individuals more affected in terms of aggression following stress, we applied a profiling approach according to the distribution of scores from the control group following previous contributions to the literature (Cohen et al., 2004; Ritov et al., 2015). Classification was made according to the upper 80th percentile of the control distribution for the variables; specifically: total duration of offensive behavior (>10.92%), frequency of offensive behaviors (>130), latency to first offence (<78.8s), frequency of abnormal bites (>1), proportion of all biting attacks that were unsignalled (>0.17), proportion of all biting attacks targeted toward vulnerable parts (>0.11), and proportion of all biting attacks that were 'hard' (>0.5). Rats were classified for each variable (Fig. 2A-C, 'normal' aggression; Fig. 2E-H, 'abnormal' aggression). Every rat achieving an aggressive score in five of the seven variables was classified as an aggressive rat overall. This delineated two subpopulations within the peripubertal stress group, depicted in Fig. 2D, one defined as 'aggressive' (n=5) and the other as 'non-aggressive' (n=7).

Non-aggressive peripubertally stressed rats were affected by stress in other non-aggression related behavioral domains

Behavioral responses of the two identified behavior profiles were compared to control, non-stressed animals in a variety of behavioral tests. When exposed to the elevated plus maze, a test of anxiety-like behavior, a difference between the three groups with regard to the percentage of time spent on the open, unprotected arms of the maze was found (Fig. 3B: $H(2)=6.447$, $p<0.05$). Post-hoc tests revealed that control and aggressive groups did not differ in this respect ($U=27$, n.s.), rather, the non-aggressive group showed a reduction in time spent on the open arm relative to control ($U=13$, $p<0.025$). No difference in general locomotion was found on the plus maze, with each group travelling a similar distance within five minutes ($H(2)=2.01$, n.s.). A similar difference between subgroups was observed in the social preference test (Fig 3C: $H(2)=8.658$, $p<0.05$). Specifically, non-aggressive rats showed a reduction in social preference compared to the control group ($U=5$, $p<0.005$), whereas the aggressive group did not ($U=17$, n.s.). The decrease in the preference ratio observed in non-aggressive rats was driven by increased exploration of the object ($U=8$, $p<0.025$), as well as a tendency towards differential exploration of the juvenile between groups ($H(2)=5.162$, $p<0.1$). In contrast, experience of peripuberty stress did not lead to any difference in corticosterone responsiveness to novel environment, a mild form of stressor (Fig. 3A: $H(2)=1.528$, n.s.). Additionally, we found no difference between groups in the time spent immobile during the first exposure to forced swimming ($H(2)=1.02$, n.s.), nor the second (Fig. 3D: $H(2)=0.287$, n.s.). See Table 1 for further details of parameters from each behavioral test.

Behavioral test	Parameter measured	Control		Non-aggressive		Aggressive		Group comparison [a]		
		mean	sd	mean	sd	mean	sd	H	p	
Elevated plus maze	Closed arm %	56.97	12.26	66.42	10.48	57.48	13.75	3.64	0.16	n.s.
	Centre %	25.22	5.97	26.22	8.15	26.50	7.51	0.64	0.72	n.s.
	Open arm %	17.82	11.23	7.35	4.28	16.02	9.64	6.45	0.04	*
	Closed visits #	14.09	5.63	14.71	3.73	12.40	2.51	0.77	0.68	n.s.
	Centre visits #	21.18	4.56	20.71	6.37	18.00	1.22	2.00	0.37	n.s.
	Open visits #	7.55	2.02	6.29	3.99	6.00	2.24	2.70	0.26	n.s.
	Open latency (s)	17.33	51.75	29.91	40.49	10.44	20.62	1.91	0.39	n.s.
	Distance (cm)	1631.61	183.15	1752.80	201.99	1648.10	107.47	2.01	0.37	n.s.
Sociability test	Preference ratio %	82.49	5.11	70.40	6.52	75.66	10.75	8.66	0.01	**
	Juvenile %	25.64	6.15	20.21	5.51	25.31	4.21	5.16	0.08	T
	Juvenile visits #	59.64	9.20	55.14	10.90	63.00	18.12	0.83	0.66	n.s.
	Juvenile latency (s)	11.54	8.71	11.87	9.78	30.42	38.99	0.20	0.90	n.s.
	Object %	5.35	1.58	8.28	1.81	8.09	3.59	7.42	0.03	*
	Object visits #	26.45	7.98	29.43	8.48	26.80	10.71	0.65	0.72	n.s.
	Object latency (s)	28.25	33.01	23.67	35.83	28.06	34.47	0.10	0.95	n.s.
Forced-swimming test	Day 1 float %	59.00	15.06	56.66	4.90	53.66	10.53	1.02	0.60	n.s.
	Day 1 float latency (s)	109.15	33.99	96.22	35.88	97.76	33.32	1.24	0.54	n.s.
	Day 2 float %	42.81	14.81	42.32	9.28	38.73	13.52	0.29	0.87	n.s.
	Day 2 float latency (s)	66.31	36.82	60.41	35.47	85.66	34.38	1.46	0.48	n.s.

[a] Group comparisons between the three experimental groups were calculated using the Kruskall-Wallis test, df=2

Table 1 Results obtained for Control, PPS non-aggressive, PPS aggressive rats in behavioral tests assessing anxiety-related behavior, social preference, and depression-like behavior. (Kruskal-Wallis tests: T = p<0.1; * = p<0.05; ** = p<0.01, n.s. = not significant)

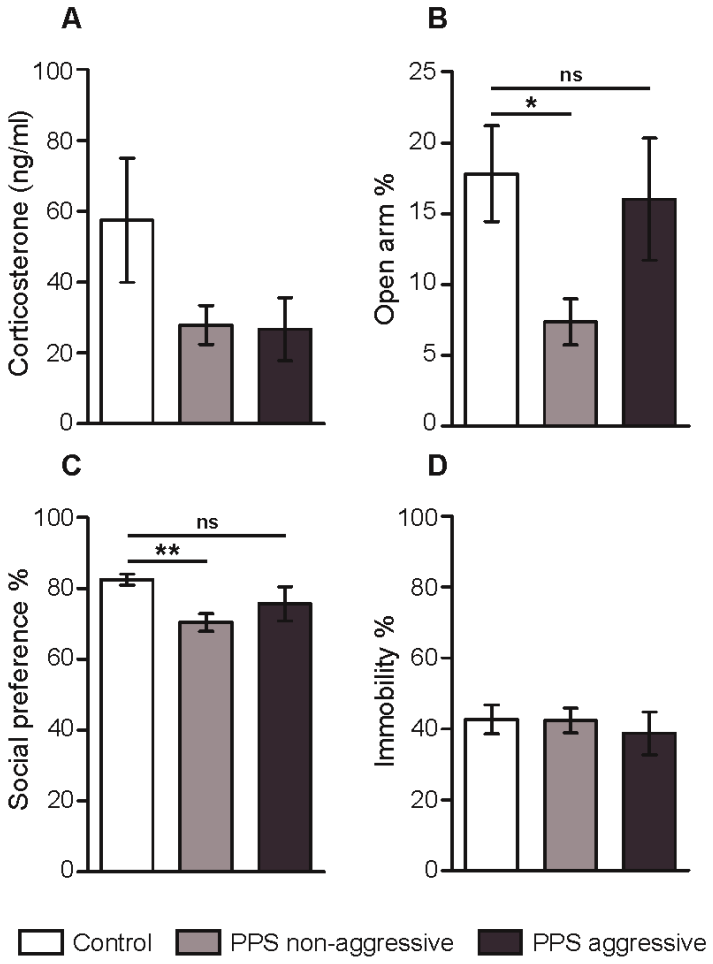

Figure 3 Individual differences in the behavior of following exposure to peripuberty stress (PPS) were found in other measures of emotionality. Non-aggressive PPS rats spent less time on the open arm of an elevated plus maze (B) and showed reduced preference for a social target in a test of sociability (C) relative to the control group. These differences were not evident in PPS rats classified as aggressive. No differences were found between either of the peripubertal stress groups and the control rats in corticosterone response to novelty stress (A), or in immobility during the second exposure to forced swimming (D). Significant differences between groups are indicated by asterisks (Mann-Whitney post-hoc tests: ns = non-significant; * = $p<0.025$; ** = $p<0.005$; see text for further details).

Individual differences in aggression following peripuberty stress were not associated with differences in brain macrostructure in stress-sensitive brain regions

Ex vivo structural MRI revealed no difference between the groups in total brain volume ($H(2)=1.71$, n.s.), nor in cortical thickness ($H(2)=1.615$, n.s.). Analyses indicated that volumes did not differ between subgroups within any of the ROIs measured (prelimbic cortex: $H(2)=0.875$, n.s.; infralimbic cortex: $H(2)=1.068$, n.s.; amygdala: $H(2)=0.16$, n.s.; hippocampus: $H(2)=1.168$, n.s.; subiculum: $H(2)=0.454$, n.s. & GP: $H(2)=0.125$, n.s.).

Individual differences in aggression following peripuberty stress were associated with differences in brain microstructure in stress-sensitive brain regions

In addition to assessing volumes with structural MRI, we obtained measures of tissue microstructure using diffusion tensor imaging in the same ROIs. We found a, largely, bilateral reduction of mean diffusivity in medial prefrontal cortex, hippocampal formation and amygdala, but not in the globus pallidus, of aggressive rats (Fig. 4A, B). In medial prefrontal cortex, group differences were found in infralimbic cortex (Left: $H(2)=7.963$, $p<0.05$; Right: $H(2)=7.963$, $p<0.05$), but only tended to differ in prelimbic cortex (Left: $H(2)=4.673$, $p<0.1$; Right: $H(2)=5.98$, $p<0.1$). Post-hoc comparisons revealed that the differences in infralimbic cortex were related to a reduction of mean diffusivity in the aggressive group relative to the control group (Left: $U=6$, $p<0.025$; Right: $U=7$, $p<0.025$). The non-aggressive group did not differ from the control group (Left: $U=29$, n.s.; Right: $U=35$, n.s). The same pattern of differences was evident in hippocampus (Left: $H(2)=9.248$, $p<0.01$; ctrl/aggr: $U=3.5$, $p<0.005$; ctrl/non-aggr $U=28$, n.s.; Right: $H(2)=6.243$, $p<0.05$; ctrl/aggr: $U=5$, $p<0.025$; ctrl/non-aggr: $U=31$, n.s) and subiculum (Left: $H(2)=9.801$, $p<0.01$; ctrl/aggr: $U=0$, $p<0.0005$; ctrl/non-aggr $U=37$, n.s.; Right: $H(2)=7.89$, $p<0.05$; ctrl/aggr: $U=3$, $p<0.025$; ctrl/non-aggr: $U=27$, n.s), but only unilaterally in amygdala (Left: $H(2)=7.815$, $p<0.05$; ctrl/aggr: $U=8$, $p<0.025$; ctrl/non-aggr $U=30$, n.s.). No significant group differences were observed in globus pallidus (Left: $H(2)=0.765$, n.s.; Right: $H(2)=0.153$, n.s.).

As indicated above, we also derived an integrated aggression *z* score for each rat from measures obtained during the resident-intruder test. These *z* scores were found to correlate with mean diffusivity (MD) values obtained for each region. See supplementary figures 1 & 2 for further details.

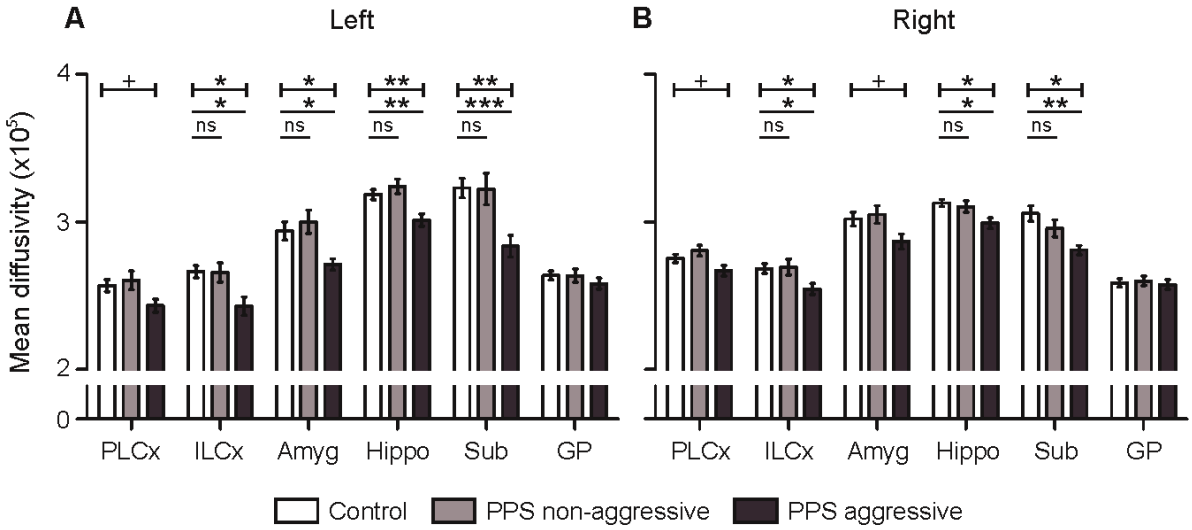

Figure 4 Development of an aggressive phenotype following peripuberty stress exposure was associated with reductions in mean diffusivity in subcortical brain regions often associated with aggression but not in a control region not associated with aggression. Several of these differences were common between the left (A) and right (B) hemispheres of the brain. Significant differences between groups are indicated by asterisks (Kruskal -Wallis tests (uppermost line): + = p<0.1; * = p<0.05; ** = p<0.01; Mann-Whitney post-hoc tests: ns = non-significant; + = p<0.05; * = p<0.025; ** = p<0.005; *** = p<0.0005; see text for further details). Abbreviations: PLCx = prelimbic cortex; ILCx = infralimbic cortex; Amyg = amygdala; Hippo = hippocampus; Sub = subiculum; GP = globus pallidus.

The reduction in the mean diffusivity in infralimbic cortex of the aggressive subgroup was not related to abundance of myelin, nor to the number of neurons, within the region.

Differences in mean diffusivity detected during diffusion-tensor imaging may derive from a number of alterations in tissue microstructure. In order to start addressing whether there was any distinct histological basis for the differences we had observed, we applied immunostaining for myelin basic protein, a major component of mature oligodendrocytes, and for NeuN, a neuronal nuclear antigen, to several sections of infralimbic cortex taken from each rat (Fig. 5). In analyzing the immunofluorescent images, we further split sections into equally sized zones, starting from the medial edge of the hemisphere and working toward the corpus callosum. Analysis revealed that though abundance of each protein varied across layers of cortex in an expected fashion (m.e. of layer: MBP: $F_{(5, 95)}$=116.9, p<0.0001; NeuN: $F_{(2,19)}$=285.32, p<0.0001), this did not differ as a function of experimental group (layer*group: MBP: $F_{(10,95)}$=1.30, n.s.; NeuN: $F_{(10,95)}$=0.911, n.s.). No effect of experimental group was found in the case of either protein when considering the region as a whole (m.e. group: MBP: $F_{(2,19)}$=2.004, n.s.; NeuN: $F_{(2,19)}$=0.547, n.s.).

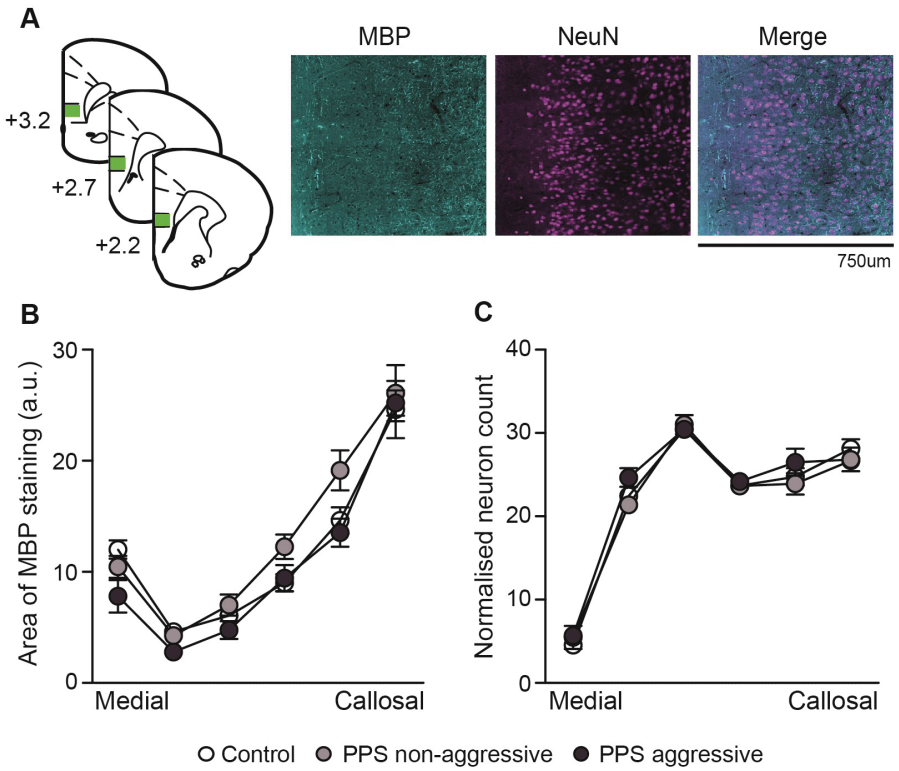

Figure 5 Differences in mean diffusivity found in infralimbic cortex are not related to changes in the abundance of myelin basic protein (B), nor in number of cells positive for NeuN, a neuron-specific nuclear marker (C). Position relative to bregma at which infralimbic cortex was sampled from each rat, alongside a representative staining (A).

Differences in the pattern of corticosterone responsiveness to peripuberty stress are associated with aggression phenotype, and also with volume of infralimbic cortex

Analysis of the corticosterone concentration within the blood plasma obtained at several timepoints during the peripuberty stress exposure revealed a difference in the pattern of glucocorticoid responsiveness to stress across the different days (Fig 6A: day*subgroup interaction: $F_{(2,20)}=6.378$, $p<0.01$). The non-aggressive group had higher corticosterone levels than the aggressive on both p28 and p42 (p28: $t_{(10)}=3.834$, $p<0.01$; p42: $t_{(10)}=3.108$, $p<0.05$), though when days were collapsed, the groups did not differ in levels of corticosterone ($F_{(1,10)}=3.193$, n.s.). Irrespective of subgroup, corticosterone levels declined across exposure to stress ($F_{(2,20)}=13.946$, $p<0.001$).

Though the corticosterone response to stress of the aggressive rats was lower on first exposure to stress, levels in this parameter did not show significant correlation with bite frequency during the resident-intruder test (Fig 6B: $\rho=-0.49$, n.s.). We found that when the corticosterone response to the third stress (p30) was considered in relation to the first response (p28, i.e. a measure of response adaptation over repeated stress exposures), there was significant correlation with bite frequency in the resident-intruder test (Fig 6C: $\rho=0.7$, $p<0.05$). This indicated that rats whose HPA axis response to repeated stress habituated in a more dramatic fashion were less aggressive at adulthood. Corticosterone adaptation values were also found to correlate with volume of infralimbic cortex (Fig 6D: $\rho=-0.58$, $p<0.05$), such that

greater adaptation was associated with larger infralimbic cortex volume. Adaptation of HPA axis response to stress did not correlate with any further measures relating to brain structure (data not shown).

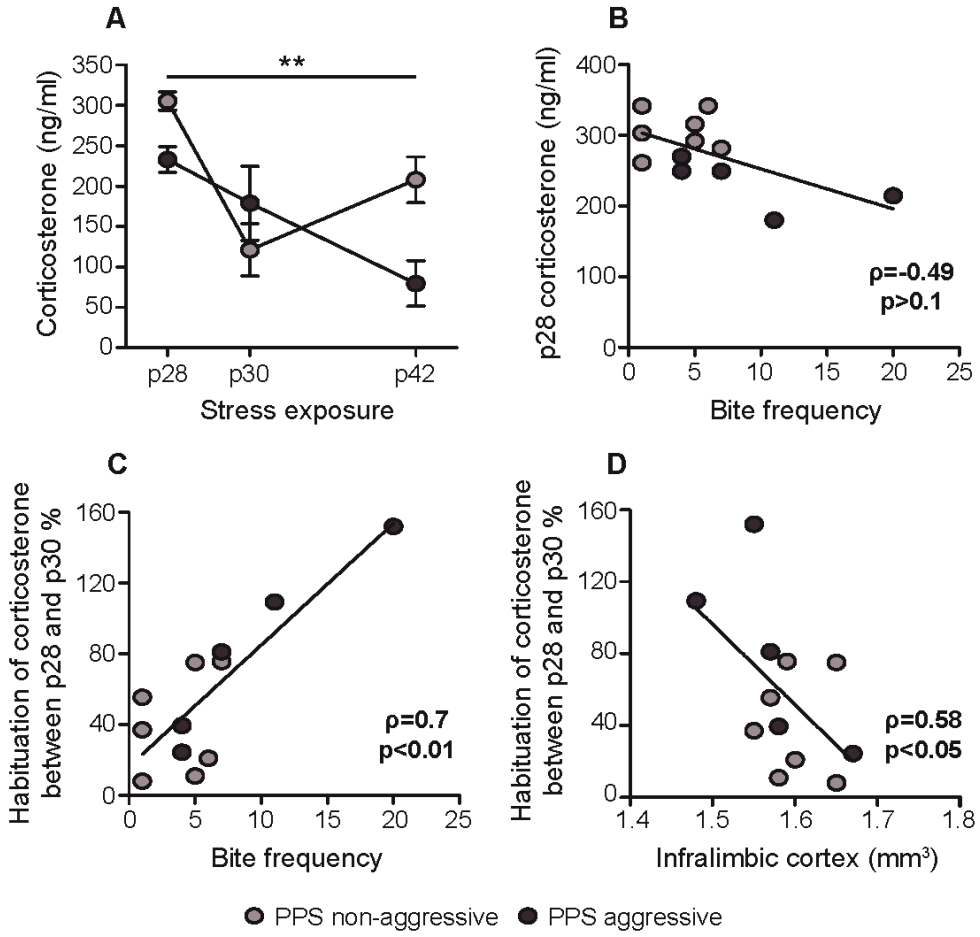

Figure 6 Development of an aggressive phenotype following peripuberty stress (PPS) exposure is associated with differential corticosterone responsiveness to repeated stress exposure. Rats from the PPS aggressive subgroup had lower corticosterone at the offset of stressors on p28 and p42 than those from the PPS non-aggressive subgroup, and this pattern was reversed at the p30 timepoint. Significant group by postnatal day interaction is indicated by asterisks (Repeated measures ANOVA: ** = $p<0.01$; see text for further details). Corticosterone response at the offset of stressors on p28 was not significantly correlated with bite frequency in the resident-intruder test, an index of aggression (B). However, habituation of corticosterone response to stress between p28 and p30 was associated with aggression (C). It was also associated with volume of infralimbic cortex (D). The habituation percentage was calculated with the formula (p30 CORT/p28 CORT)*100 and a score of 100 therefore represents a total lack of habituation in the corticosterone response to stress on p30. Spearman's correlations and associated p-values are shown on graphs (ρ = rho).

Discussion

We show here that exposure to peripubertal stress in rats leads to reductions in mean diffusivity within several aggression-related brain regions *only* in those individuals displaying an aggressive phenotype. We also show that peripubertally stressed rats not displaying aggressive behavior were affected in terms of other non-aggression related behaviors, specifically showing increased anxiety-like behavior and reduced sociability. However, this phenotype was not associated with any observable neuroanatomical alterations in the brain regions examined.

It is important to note that, when considered at the group level, the behavioral findings resulting from peripubertal stress, in terms of increased aggression and anxiety, and reduced sociability, are in line with previous findings from our laboratory (Cordero et al., 2012; Marquez et al., 2013; Tzanoulinou et al., 2014). Importantly, by applying an aggression profiling approach, we show critical individual differences in the long-term response to peripubertal stress. This approach supports earlier contributions to the literature that have emphasized the importance of examining individual differences when asking questions regarding neurobiology associated to a behavioral outcome (Cohen et al., 2004; Anacker et al., 2016; Ritov et al., 2016). In this instance, we shy away from use of the terms 'resilience' and 'susceptibility', given that both groups show increases in psychopathology-like behavior. Instead, we suggest that stress-exposed individuals may develop along different neuroanatomical trajectories, trajectories potentially determined by pre-existing individual factors.

We focused our MRI and DTI analyses on a number of candidate brain regions, including different subdivisions from the medial prefrontal cortex (mPFC), amygdala, and hippocampus; all of them brain regions known to be subject to ongoing development during adolescence, functionally affected by peripubertal stress, and of demonstrable importance in the performance of aggression in both humans and animals (Spear, 2000; Gregg & Siegel, 2001; Casey et al., 2008; Andersen & Teicher, 2008; Marquez et al., 2013; Haller, 2014; van der Kooij et al., 2014; Köhl et al., 2015; White et al., 2016). Interestingly, we did not find differences in the volume of any of these regions relative to the control group in either subgroup of peripuberty stress exposed rats. This is in contrast to volumetric studies of humans dealing with aggression-related psychopathologies, as several studies have indicated reductions in the volume of prefrontal cortex (Raine et al., 2000; Sala et al., 2011), hippocampus (Dolan et al., 2002; Barkataki et al., 2006; Zetzsche et al., 2007; Sala et al., 2011; Morandotti et al., 2013; Coccaro et al., 2015) and amygdala (Coccaro et al., 2015). It could be argued that the origins of stress-related changes in brain structure are unlikely to be the same as those in individuals with aggression-related psychopathologies. However, at least in the case of prefrontal cortex, volume decrements in aggressive, borderline personality disordered individuals appeared to be exacerbated by a history of early adversity (Sala et al., 2011; Morandotti et al., 2013), suggestive of a synergistic effect.

Volume differences in human MRI studies are thought to reflect hypo- and hypertrophy of neuronal processes in the affected region. Studies of neuronal morphology changes following chronic stress in

animal models support this concept (Cook & Wellman, 2004; McEwen & Magarinos, 1997; Vyas et al., 2002; Isgor et al., 2004; Eiland et al., 2012). However, in animal studies where both morphology and volume were measured in the same individual, changes in morphology were not reflected by concomitant changes in regional volume, suggesting that these parameters are not simply interchangeable (Henckens et al., 2015).

Importantly, we found reductions of mean diffusivity in infralimbic cortex, amygdala, hippocampus and subiculum, but not in globus pallidus, of aggressive rats. In investigating the histological basis of diffusivity reductions observed, we performed immunofluorescence in one of the affected brain regions, infralimbic cortex. We assessed the number of neuronal cells, as well as myelination using MBP, the major constituent of mature oligodendrocytes. Though the immunostaining was successful in both cases, and we detected expected variation across depths of the infralimbic cortex, no differences were found between experimental groups. Though this would seem to indicate a lack of relationship between diffusivity and these measures, we should note that measuring myelination as we have here does not allow for the determination of thickness of myelin sheaths. Since this property can affect diffusion, we cannot rule out myelination-related diffusivity changes entirely. Reductions in mean diffusivity are therefore likely to be derived from other changes to tissue microstructure. Studies in which tissue properties were assessed jointly with DTI and with histology indicated that mean diffusivity measures, as well as deriving from myelination and neuronal density, may also derive from general cellularity, as well as density of neurites (Khan et al., 2016; Tu et al., 2016). Our reductions in mean diffusivity might therefore reflect decreased alignment of neurites, increased complexity of neuronal processes or an increase in the number of glial cells (Beaulieu, 2002; Delgado y Palacios et al., 2011; Evans, 2013; Hemanth Kumar et al., 2014; Khan et al., 2016). Moreover, more than one alteration may occur in conjunction, complicating determination of the source of fluctuation in diffusivity (Tu et al., 2016).

We additionally asked whether individual differences in glucocorticoid responsivity to stress during peripuberty might be associated with the development of an aggressive phenotype. We found that the pattern of corticosterone released across repeated episodes of peripuberty stress differed between the aggressive and non-aggressive stress-exposed rats, and that this pattern was associated with their later aggressiveness. Specifically, rats showing greater initial adaptation of corticosterone response across stressors were less aggressive at adulthood. Association of HPA axis adaptation to stress with structural measures additionally revealed that individuals with stronger initial corticosterone adaptation to stress had larger volumes of infralimbic cortex. This is broadly in accordance with other studies, which have found that repeated stress exposure had lesser impact on brain structure in a stress-habituating versus a non-stress-habituating rat strain (Bourgin et al., 2015), amygdala in that case. This suggests that the degree of changes to brain structure induced by stress may depend on absolute exposure to glucocorticoids.

The brain regions we studied here are particularly responsive to the programming effects of stress and are

still in the process of maturation during the peripubertal period (Spear, 2000; Andersen & Teicher, 2008; Romeo et al., 2013). Glucocorticoids are potent modulators of many biological processes, including neuroanatomical plasticity (de Kloet et al., 2005; Eiland & Romeo, 2013; McEwen, 2016), and could conceivably play a role in inducing changes in brain structure that are associated with an aggressive phenotype. Interestingly, experiments using DTI to determine the impact of the timing of stress exposure on brain microstructure have implicated the pre-puberty window as being a time of heightened vulnerability to alterations relative to later timepoints (Zalsman et al., 2015). Many developments are ongoing during this narrow window. Synaptic overproduction, synaptic pruning, and myelination are particularly prominent (Andersen & Teicher, 2008; Liston & Gan, 2011) and are all sensitive to disruption by stress exposure (Liston & Gan, 2011; Pattwell et al., 2016).

A limitation of the study at hand is that we cannot determine the causal relationships between aggressive behavioral phenotype, responsiveness to stress, and brain structure. Indeed, a longitudinal study of mice, in which MRI scans were performed before and after exposure to chronic social defeat stress, indicated that pre-existing differences in hippocampal structure, as well as magnitude of stress-induced volume change, predicted susceptibility to the behavioral effects of stress (Tse et al., 2014). However, the results of this study and their similarity to patterns observed in humans with pathological aggression (Sala et al., 2011; Morandotti et al., 2013) strongly support the usefulness of this approach to model these interactions.

In summary, we present evidence here of two distinct neurodevelopmental trajectories arising from peripubertal stress in rats, one of them leading to the development of pathological aggression and reductions in mean diffusivity in infralimbic cortex, amygdala, hippocampus and subiculum. Interestingly, these brain regions have been highlighted in structural and functional human studies as altered in individuals showing abnormal levels of aggression. Our study establishes a causal link between peripubertal stress exposure and structural deviations in these brain regions in association with pathological aggression, and points toward differential glucocorticoid adaptation to repeated stress as a potential underlying mechanism. Future studies addressing the reversibility of the structural and behavioral phenotypes following manipulation of stress adaptation during early life are warranted to investigate causality between the different parameters.

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Author contributions

S.W.: performed and organised behavioral and immunofluorescence experiments and the stress protocol, scored videos, analysed data, wrote the initial version of the manuscript

T.C.W., M.B., R.W.: performed and analysed MRI experiments

S.C.R.W. & D.C: provided the concept and infrastructure for MRI experiments

C.S.: provided the concept and feedback for the experiments, and corrected the manuscript

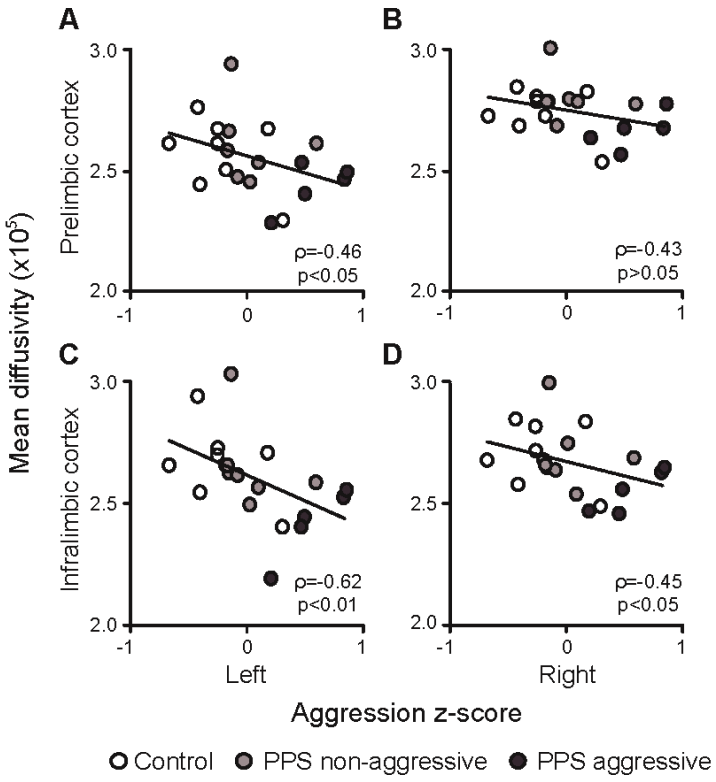
Supplementary information

Methods

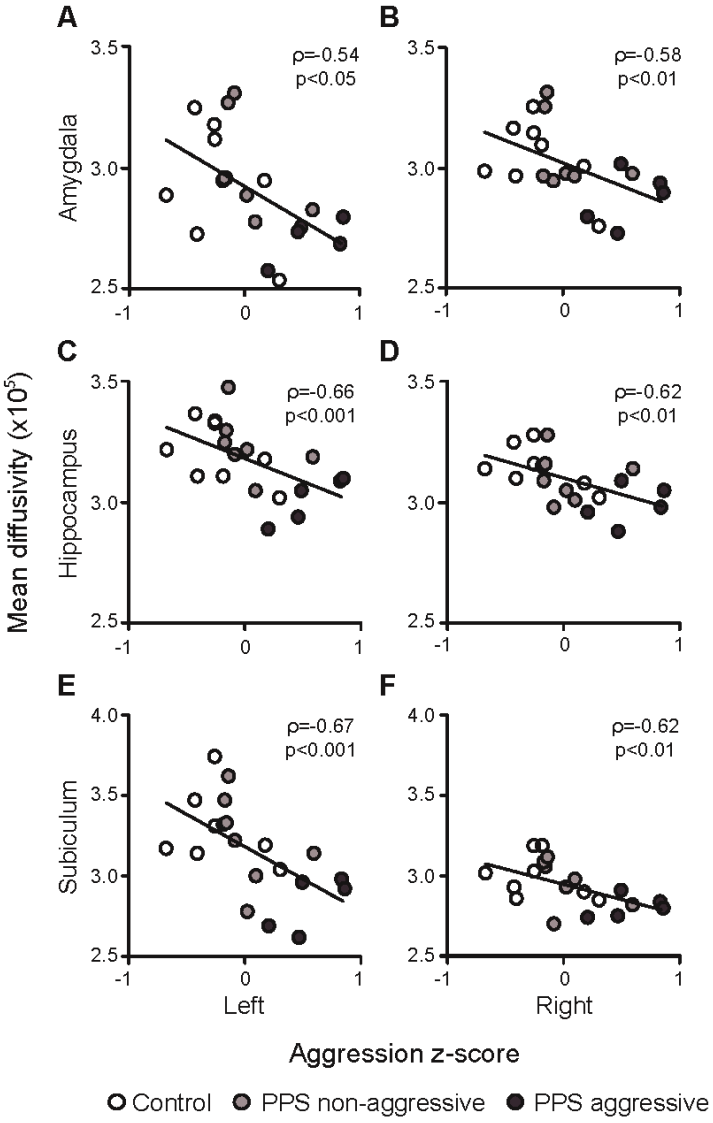
Integrated aggression *z* score

To assess aggressive behavior, *z* scores were calculated from the seven variables by which rats were initially profiled. These variables included: total duration of offensive behavior, frequency of offensive behaviors, latency to first offence, frequency of abnormal bites, proportion of all biting attacks that were unsignalled, proportion of all biting attacks targeted toward vulnerable parts, and proportion of all biting attacks that elicited a strong response. The values for each individual variable were converted to *z* scores using the formula: ((score – mean of all scores) / standard deviation of all scores). These *z* scores were integrated to derive a single aggression score, subsequently used as a continuous variable against which mean diffusivity values for each region of interest were correlated.

Results



Supplmentary Figure 1 Individual level of aggression in the resident-intruder test is associated with mean diffusivity in subregions of the medial prefrontal cortex. Positive correlations between aggression *z* score and mean diffusivity were found in left (Fig. S1A) but not right (Fig. S1B) prelimbic cortex, and bilaterally in infralimbic cortex (Figs. S1 C & D). Spearman's correlations and associated p-values are shown on graphs (ρ = rho).



Supplementary Figure 2 Individual level of aggression in the resident-intruder test is associated with mean diffusivity in subcortical regions of the limbic forebrain. Positive correlations between aggression *z* score and mean diffusivity were found bilaterally in amygdala (Figs. S2 A & B), hippocampus (Figs. S2 C & D), and in subiculum (Figs. S2 E & F). Spearman's correlations and associated p-values are shown on graphs (ρ = rho).

Chapter 3

Constitutional differences in glucocorticoid responsiveness to repeated stress are associated with differences in psychopathology-like behavior in rats

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Abstract

The hypothalamus-pituitary-adrenal (HPA) axis coordinates responses that enable an individual to cope with stressful challenges. Its end products, glucocorticoids, additionally mediate adaptation following cessation of a stressor. Dysregulation of this process is thought to play a key role in stress-related psychopathology, such as depression and post-traumatic stress disorder. Reduction of glucocorticoid responses across repeated exposure to the same stressor is a common, though not universally expressed, adaptation. Human studies have found impairment in adaptation of this kind to be both heritable and associated with increased self-report of several depression-related indices, suggesting that it may represent a risk factor for development of psychopathology. However, whether individual differences in stress habituation are causally implicated in vulnerability to psychopathology is not easily studied, owing to the lack of appropriate animal models. In light of this, using a selective breeding program in rats, we generated lines enriched for stress habituation and lack of stress habituation as indexed by glucocorticoid responsiveness, as well as a control line intermediate for the trait. Here we present findings indicating the high level of variation in glucocorticoid responsiveness to repeated stress in an outbred Wistar rat population, as well as the response to selection for extremes in this trait. Under stress free conditions, rats with constitutive impairment in stress habituation displayed enhanced aggression, anxiety-like, and depression-like behaviors, as well as alterations in the expression of genes within both central and peripheral nodes of the HPA axis and enhanced reactivity to acute stress exposure. Together, these findings strongly link constitutive differences in stress adaptability with vulnerability to develop psychopathology-like alterations. The developed rat lines therefore represent a promising model with which to further examine the relationship between stress adaptability and stress-related pathophysiology more generally, especially with respect to underlying mechanisms. Finally, this model could be used to assess the therapeutic potential of treatments enhancing stress habituation in psychopathology.

Introduction

The hypothalamus-pituitary-adrenal (HPA) axis coordinates metabolic, behavioral and physiological responses that enable an individual to cope with stressful challenges (Shirazi et al., 2015). Activation of the HPA axis involves a cascade of responses that starts with the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) by the paraventricular nucleus of the hypothalamus (PVN). The release of CRH and AVP activates adrenocorticotrophic hormone (ACTH) secretion from the pituitary which, in turn, triggers the production and release of glucocorticoids (primarily cortisol in humans; corticosterone in most rodents) from the adrenal glands into the circulation (Ulrich-Lai & Herman, 2009). Circulating glucocorticoids exert a plethora of effects, both genomic and non-genomic, via their actions through mineralocorticoid (MR) and glucocorticoid (GR) receptors (de Kloet, 2014; de Kloet et al., 2008; Joëls et al., 2013). Activation of GR in different brain areas, notably including the PVN, additionally acts to inhibit continuation of the stress response, with this negative feedback acting via both fast and slow mechanisms (Tasker & Herman, 2011).

Glucocorticoid actions are thought to help restore homeostasis and mediate adaptation following cessation of a stressor, a process that has been termed allostasis. Such responses, however, come at a cost, and repeated, prolonged or inadequate stress responses may lead to physiological damage, termed allostatic load, thought to be the pathological basis of many stress-related disorders (McEwen, 2007). In addition to its implication in numerous physiological disorders, dysregulation of HPA axis activity is associated with several psychopathologies (Ehlert et al., 2001; McEwen, 1998; Tarullo & Gunnar, 2006; Walker et al., 2008; Yehuda et al., 2009). Indeed, impaired ability to shut off HPA axis responses to stress has been causally implicated in depression (see de Kloet et al., 2005 for review), whereas over-efficacious negative feedback inhibition, leading to insufficient HPA axis stress responses, has been implicated as a risk factor in development of post-traumatic stress disorder (Yehuda & LeDoux, 2007).

There is important individual variability in both sensitivity to stress and in vulnerability to develop stress-related psychopathologies which may relate to disruption of allostatic processes. In healthy adult humans, a common adaptation to repeated exposure to the same stressor (homotypic stress) is reduction of the HPA axis response across stress exposures (Deinzer et al., 1997; Federenko et al., 2004; Gerra et al., 2001; Pruessner et al., 1997). Adaptation of this kind minimizes the impact of frequently experienced stressors, whilst leaving the HPA axis able to respond to novel, heterotypic stressors, and impairment in this process could leave an individual open to the accumulation of allostatic load (McEwen, 1998). It is noteworthy that habituation to homotypic stress is not uniformly expressed, with approximately 35% of individuals showing no decrement in cortisol response following repeated psychosocial stressors (Gerra et al., 2001; Kirschbaum et al., 1995; Wüst et al., 2005), a propensity that is highly heritable (Federenko et al., 2004). Interestingly, correlation of physical and psychological indices with cortisol response to repeated stressors indicated that those individuals who habituated to a lesser extent (i.e. those who failed to adapt) reported increased values in indices which, when taken together, could be considered to

engender a depression-like phenotype (Kirschbaum et al., 1995; Preussner et al., 1997; Kudielka et al., 2006). These findings support a potential role for impaired stress habituation in the accumulation of allostatic load, and suggest that it may represent one mechanism via which differential vulnerability to develop stress-induced psychopathology is translated. Critically, causality cannot be inferred from such studies, nor can the contribution of additional factors (e.g., prior stress history, socioeconomic status, or other variables) be excluded. The use of controlled animal studies could therefore be of benefit in addressing whether reduced adaptability to stress is indeed causally related to psychopathology-like phenotypes, as well as in the investigation of underlying neurobiological mechanisms.

Rats represent a useful model system for this purpose since evidence suggests that they habituate to repeated stress in a similar fashion to humans (for review see Grissom & Bhatnagar, 2009, though see also Rabasa et al., 2015). Moreover, in a parallel to human findings, rats exposed to stress regimens giving rise to HPA axis habituation appear to suffer less extreme physiological and behavioral consequences when compared to those exposed to stress regimens not leading to habituation (Flak et al., 2012; Jankord et al., 2011). At present, however, there is a lack of a specific and reliable animal model of individual variation in HPA axis adaptation with which to investigate the influence of stress adaptability in vulnerability to psychopathology. Indeed, comparisons of behavior between congenic stress-habituating (Lewis) and stress -non-habituating (Fischer 344) inbred rat strains have demonstrated equivocal results, with an equal number of studies finding differences in psychopathology-like behavior as not (Berton et al., 1997; Cadoni et al., 2015; Chaouloff et al., 1995; Cohen et al., 2006; Dhabhar et al., 1997; Ramos et al., 1997; Rex et al., 1996; Wu & Wang, 2010). The lack of stability in differences between these strains renders molecular-genetic comparisons potentially unreliable.

A more suitable approach may be the use of selective breeding, to produce lines that differ for the specific trait of interest, in this case stress habituation, followed by examination of phenotypes pertaining to risk for psychopathology. This approach has been successfully established in rats with regard to several psychopathology-like behavioral traits (anxiety-like: Liebsch et al., 1998; depression-like: Bignami, 1965; aggression: Naumenko et al., 1989). Moreover, since human studies show that HPA axis adaptability is highly heritable (Federenko et al., 2004) and selection for aspects of HPA axis activity has already been successful in several species (Edens & Siegel, 1975; Pottinger & Carrick, 1999; Satterlee & Marin, 2006; Touma et al., 2008), it suggests that a similar approach could prove fruitful if applied to stress habituation.

Taking these factors into consideration, we embarked on a selective breeding program, with the aim to generate lines enriched for stress-habituating and stress-non-habituating individuals. Our goal in the development of this resource was to allow investigation of the relationship between stress adaptability and psychopathology-like behavioral phenotypes, as well as underlying neurobiological mechanisms. Here we present findings indicating, first, a high level of variation in corticosterone response to repeated stress encountered in the outbred Wistar rat strain, as well as the response to selection for extremes in this trait.

We additionally present results from a characterization of the behavioral and endocrine phenotype of rats drawn from these selection lines.

Materials & Methods

Animals

Selective breeding procedure

Wistar Han rats were obtained from a commercial breeder (Charles River, France: 30 male & 30 female; parental generation; PG) and bred in our animal facility. The entire offspring of these pairings (F0) was subject to a 'stress adaptation test' (SAT). The SAT is a truncated version of the peripubertal stress protocol developed in our laboratory (Toledo-Rodriguez & Sandi, 2011) which, though clearly stressful, has been shown to be insufficient in begetting behavioral alterations associated with the longer protocol (Toledo-Rodriguez & Sandi, 2007; Tzanoulinou et al., 2014). Tail blood samples were taken at two timepoints on two separate days of the protocol; immediately after, and 30 minutes after, cessation of exposure to the stressors. Three breeding lines were established according to the outcome of the SAT. Rats with extremely low (<100ng/ml) or extremely high (>200ng/ml) secretion of corticosterone on the final day of the SAT, i.e. animals expressing habituation or non-habituation of the HPA axis response to repeated stress, were selected for the 'low' and the 'high' breeding line, respectively. A third breeding line, 'inter', was established consisting of animals with intermediate corticosterone values in the SAT.

Ten males and ten females from F0 were selected as founder pairs for each breeding line. Their offspring (F1) and the majority of animals from each subsequent generation were also tested in the SAT and selected for breeding based on their corticosterone response on post-natal day (p) 30. Selection was strictly within line, i.e. an animal from the low-line could only ever be selected to be a breeder within the low-line. To minimize effects of genetic drift, animals were mated within a system that strictly excluded sibling matings. Moreover, in order to balance the potential contribution of each litter to the next generation, litter size was reduced to a maximum of 12 pups at p2. Care was taken to ensure as much variability in pairings as possible; for example, if two animals from the same litter went forward to breed the next generation then they were not paired with animals coming from a single, alternate litter.

Stress Adaptation Test (SAT)

The protocol was based on multiple exposures to fear-induction procedures. Measures of acute stress reactivity, stress recovery (within session), and stress adaptation (across sessions) could be obtained whilst minimizing the stress exposure required to do so. Following exposure to an open field (50 x 50 x 30cm) for five minutes on p28, the stress protocol consisted of the presentation of two different stressors, each one lasting 25 minutes. These were either; exposure to the synthetic fox odor trimethylthiazoline (TMT) or to an elevated platform (EP). TMT exposure was administered in a plastic box

(38 x 27.5 x 31 cm) via a scent-charged cloth. The box was placed under a bright light (210–250 lx). The elevated platform (12 x 12cm, elevated 95cm from the ground) was also under direct bright light (470–500 lx). Following each stress session, animals were returned to neutral cages for 15 minutes. A transparent Plexiglas wall perforated with holes separated pairs of cagemates during this time. Following the holding period, animals were returned to their home cage. The stressors were applied during juvenility, on three consecutive days across p28–p30, during the light phase and following an unpredictable schedule. Tail blood samples were taken on p28 and p30, once at the offset of stress and again 30 minutes later.

Subjects

Experimental subjects were male offspring taken from the first breeding of pairs from the lines described above, not exposed to any stressors. Female Wistar Han rats (used as cohabiting partners) and male Wistar Han rats (used as intruders) were purchased from a commercial breeder (Charles River, France). Experimental cohorts were obtained from F4 for initial behavioral experiments (n=12/line), from F6 for replication of behavior (low- and high-line, n=12/line; intermediate-line, n=8), and from F8 for examination of endocrine factors (n=12/line). Between 10 and 12 breeding pairs were established per line to produce each generation of animals. In each generation, animals from 8–10 litters were used as, typically, 1–2 females per cohort did not become pregnant during cohabitation with the male. At weaning on p21, pairs of male rats from different litters were matched according to weight and mixed among home cages. Rats were maintained on a 12-h light–dark cycle (lights on at 0700h), in a temperature- and humidity-controlled environment (21±1 °C; 55% humidity ±5%), with *ad libitum* access to laboratory chow and water. They remained undisturbed, except for weekly cage changes, until experimental procedures began at adulthood (designated as p90). Experiments were performed between 0800 and 1200h, the circadian trough in corticosterone production, except where otherwise stated. All procedures were conducted in accordance with the Swiss National Institutional Guidelines on Animal Experimentation and approved by a license from the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

Assessment of behavioral consequences of line selection

Elevated Plus Maze

Anxiety-like behavior was evaluated using the EPM test (Pellow & File, 1986). The apparatus consists of two opposing open arms (50 x 10cm) perpendicular to two enclosed arms (50 x 10 x 50cm) that extend from a central platform (10 x 10cm), elevated 65 cm above the floor. Light levels were maintained at 14-16 lx on the open arms and 5-7 lx on the closed arms. At the start of the test, the rat was placed on the central platform facing a closed arm and allowed to explore the maze for five minutes. In between animals, the apparatus was cleaned with 5% ethanol solution. Behavior was monitored using a ceiling-mounted video camera and analyzed with a computerized tracking system

(Ethovision 9; Noldus IT, Netherlands). The time spent in the open and closed arms, and distance moved, were recorded. Two animals (one intermediate & one high line rat) were removed from the analysis of the F6 experiment because they fell from the maze before five minutes had elapsed.

Resident-intruder test

Prior to the night of the resident-intruder (RI) test, experimental rats cohabited with a female partner for 10 days in order to encourage territoriality. The female was removed 30 minutes before the onset of the test, and replaced afterwards. The test was performed during the beginning of the dark cycle (between 1900 and 2200h). The resident was exposed in its home cage to a smaller (5-10% lighter), unfamiliar male intruder of the same strain for 30 minutes. Each intruder was used only once. Encounters were video-recorded and scored offline by an experimenter blind to the experimental group, assisted by Observer software (Noldus IT, Netherlands). The following parameters were quantified in terms of frequency and duration: attack, offensive upright, lateral threat, keeping down, biting, social investigation, non-social investigation and auto-grooming. The cumulative duration of the first four behaviors were summed to provide a measure of total offensive behavior. Latency to first offensive act initiated by the resident was also recorded.

Forced-swimming test

Whilst still cohabitating with females, rats were submitted to a forced-swimming test (FST) to evaluate depression-like behavior (Porsolt et al., 1978). Animals were placed in a plastic beaker (25 cm diameter x 46 cm) containing 30 cm of water (25°C) for 15 minutes. A second session was performed 24h later for 5 minutes. Both sessions were recorded using a ceiling mounted video camera, and the time spent immobile (making only those movements necessary to keep the snout above the water), swimming or climbing was quantified manually with the aid of in-house software (Clicker; EPFL, Switzerland) by an experimenter who was blind to the experimental condition of the animals. One rat from the intermediate group was removed from the analysis because of a technical issue with the video recording.

Maternal behavior

Analyses of maternal behavior were made between p1 and p5 in dams from F7, the offspring of which were used in endocrinology experiments described below. See supplementary information for further details.

Assessment of endocrine consequences of line selection

Sampling of HPA axis activity

At adulthood, animals from the F8 generation were exposed to restraint stress. Rats were first wrapped

in a cloth and then restrained in wire mesh restrainers (26 × 26 cm^2) for 30 minutes. Restrained rats were kept in a clean cage, in a quiet, low-lit room during this period. Blood samples were taken via tail-nick at the onset of restraint (basal), and from the same nick at 15, 30, 60, and 90 minutes after the onset of restraint. The initial sample gave a measure of corticosterone level at circadian nadir. One week later, to obtain a measure of corticosterone at circadian peak, the same rats' blood was sampled via a new tail nick at lights off (1900h ± 10 minutes). All blood samples were chilled, centrifuged and the plasma stored at -20^{o}C for subsequent analysis. One week later, rats were sacrificed by decapitation and brain, pituitary gland, and adrenal glands were collected. Adrenal glands were cleaned of fat and weighed. Brains were flash frozen in isopentane chilled to -45°C. Pituitary gland and adrenals were placed in separate RNAse-free tubes and flash frozen using liquid nitrogen. All tissue samples were subsequently frozen at -80^{o}C until further analysis. To obtain tissue from central nucleus of the amygdala (CeA) and paraventricular nucleus of hypothalamus (PVN), brains were sectioned using a cryostat. 200µm slices were mounted on Superfrost lides, and the region of interest sampled via bilateral 1mm punches or a single, medial 1mm punch, respectively. This tissue was collected into separate RNAse-free tubes and stored at -80^{o}C until RNA extraction.

Corticosterone measurement

Measurements of free corticosterone were obtained from all blood plasma samples, via use of an enzymatic immunoassay kit performed according to manufacturer's instructions (Enzo Life Sciences, Switzerland). Levels were calculated using a standard curve method.

Gene expression analysis

Total RNA from the CeA, PVN, pituitary and adrenal glands was isolated using RNAqueous Micro kits (Ambion, USA), and complementary DNA was synthesized using the Superscript VILO kit (Invitrogen, USA) according to the manufacturer's instructions. For quantitative polymerase chain reaction (qPCR), PCR reactions were performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, USA) in an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Singapore). Two genes were used as internal controls: TATA box binding protein (*Tbp*), and eukaryotic elongation factor-1 (*Eef1a1*). Primers for the genes of interest were designed using the Assay Design Center software from Roche Applied Science. A list of genes investigated, and their respective primer sequences, are detailed in Supplementary table 1. Gene expression was analyzed with the qBase 1.3.5 software using the comparative cycle threshold method (Schmittgen & Livak, 2008).

Statistics

Data were analyzed using SPSS 17.0 (Chicago, USA). Results are presented as the mean ± SEM. Group comparisons were performed using ANOVA, with further analyses of main effects performed via Bonferroni post-tests. In general, corticosterone measurements and latencies violated the assumptions

required to perform analysis using ANOVA. Such analyses were therefore performed using Kruskal-Wallis tests, with Mann-Whitney post-hoc tests used to explore significant results. Statistical significance level was set at $p < 0.05$. A p-value was considered as tending toward significance when $0.05 \leq p \leq 0.1$.

Results

Individual differences in corticosterone response to repeated juvenile stress in the Wistar rat strain and characterization of the response to selection for the trait in the genetically-selected lines

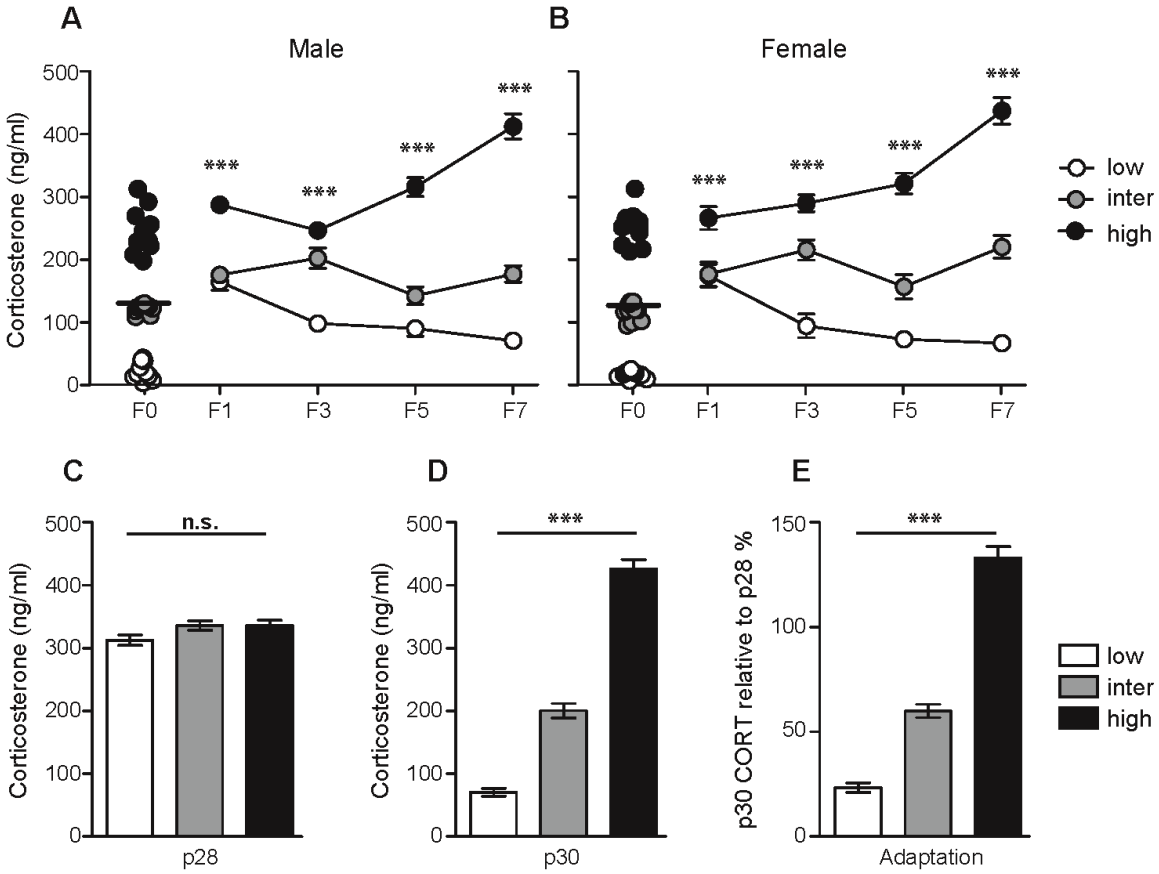

Figure 1 Degree of habituation to repeated stress is heritable and diverges across selection. Plasma corticosterone measures obtained at offset of stress on the final day (p30) of the stress adaptation test (SAT) in male (A) and female (B) Wistar rats in F0, and following generations, of the differential habituation selection lines. Data are shown as individual values for the rats selected as breeders from the F0 generation, as well as the mean of the entire F0 population (not shown in its entirety). Measuring corticosterone in all samples obtained following the SAT in rats from F7, we observed that the selection is specific to habituation to stress. Corticosterone response does not differ between the lines upon first exposure to stress (C) but is highly different following the final exposure (D). This is reflected in the relative habituation between first and third stress exposures (E). Significant differences between lines are indicated by asterisks (Kruskal-Wallis tests; *** = p<0.001; n.s. = non-significant; see text for details of post-hoc differences).

The corticosterone response at offset of stress on the final day (p30) of the three day stress exposure protocol varied widely between individuals of the F0, parental generation. Values from F0 males ranged from 7.1-316.3 ng/ml (n=98; mean: 124.7 ng/ml ± 7.11) and females from 6.3-312.9 ng/ml (n=102; mean: 112.3 ng/ml ± 7.75). Only those F0 individuals selected to breed the next generation, that is, the individuals with the most extreme values, are represented in Figs. 1A and 1B. Though male and female p30 corticosterone levels did not differ, selection response data for each sex are represented separately.

Figure 1 shows the response to selection over the first seven generations of breeding. From the first generation, the lines differed in p30 corticosterone response to stress (F1 males: $H(2) = 45.4$, $p<0.001$; F1 females: $H(2) = 14.2$, $p<0.001$). However, though low- and intermediate-lines differed from the high-line from F1 (F1 males: $U=287$, $p<0.001$; $U=269$, $p<0.001$; F1 females: $U=373$, $p<0.001$; $U=446.5$, $p<0.001$), they did not significantly differ from one another at the origin of the selection procedure (F1 males: $U=864$, n.s; F1 females: $U=643.5$, n.s). In subsequent generations, in both male and female rats, lines diverged further (F3, F5, F7 males: $H(2) = 56.2$, 60.8, 86.1, all $p<0.001$; F3, F5, F7 females: $H(2) = 43.6$, 67.9, 75.9, all $p<0.001$). The values of each line differed from the other, with the low-line having the lowest corticosterone values and the high-line the highest (F3, F5, F7 males: $U=19$-565, all $p<0.001$; F3, F5, F7 females: $U=5$-832, all $p<0.001$). Divergence occurred across generations in both directions, with low-line values getting lower (F1 vs. F7 – male: -57%; female: -60%) and high-line values getting higher (F1 vs. F7 – male: +43%; female: +63%). Estimations of narrow-sense heritability (h^2), using the formula: R (response to selection) / S (strength of selection), were in agreement with the divergence data; an h^2 of 0.29 was found for the selection trait.

Importantly, differences in corticosterone response to stress between the lines are specific to adaptation to repeated stress exposure. For example, as represented in Fig. 1C, in rats from the F7 generation, measurements of corticosterone responsiveness to a first stress episode (i.e. following stressors on p28), did not differ between the lines (Figure 1C. $H(2) = 5.85$, n.s). In contrast, p30 corticosterone measures for the three lines differed greatly (Figure 1D. $H(2) = 163.37$, $p<0.001$; post hoc comparisons: $U=38$-665.5, all $p<0.001$; Fig. 1E). Furthermore, when p30 corticosterone level was normalized to p28 corticosterone level for each individual, counteracting the potential influence of within-group individual variation, the strong distinction between each of the lines held (Figure 1E. $H(2) = 158.66$, $p<0.001$; post hoc comparisons: $U=86$-737, all $p<0.001$). Put in other terms, within F7 generation, 93% of low-line rats and 95% of high-line rats had p30 corticosterone concentrations concordant with the original selection criteria (<100ng/ml corticosterone for low-line, >200ng/ml for high-line).

Behavioral phenotype of the selected lines

We examined the socio-affective behavior of animals from generation F4. Male rats of F4 generation, born from F3 parents but not themselves exposed to the stress adaptation test, underwent a series of behavioral tests at adulthood (Figure 2: EPM, RI, FST).

No significant effects of line selection were found in anxiety-like behavior, nor in locomotion. Specifically, when exposed to the elevated plus maze, rats from the lines did not differ in the proportion of time spent in any zone of the maze (Figure 2A - Closed arm: $F_{(2,33)} = 2.53$, n.s.; Centre square: $F_{(2,33)} = 1.604$, n.s.; Open arm: $F_{(2,33)} = 1.918$, n.s.). There was a non-significant trend for high-line rats to spend more time in the protected, closed arms of the maze than the other lines (Closed arm %: $F_{(2,33)} = 2.53$, $p<0.1$). In addition, lines did not differ in distance travelled whilst on the maze, indicating no decrement in locomotor activity in any particular line (Figure 2B - Distance: $F_{(2,33)} = 0.063$, n.s.).

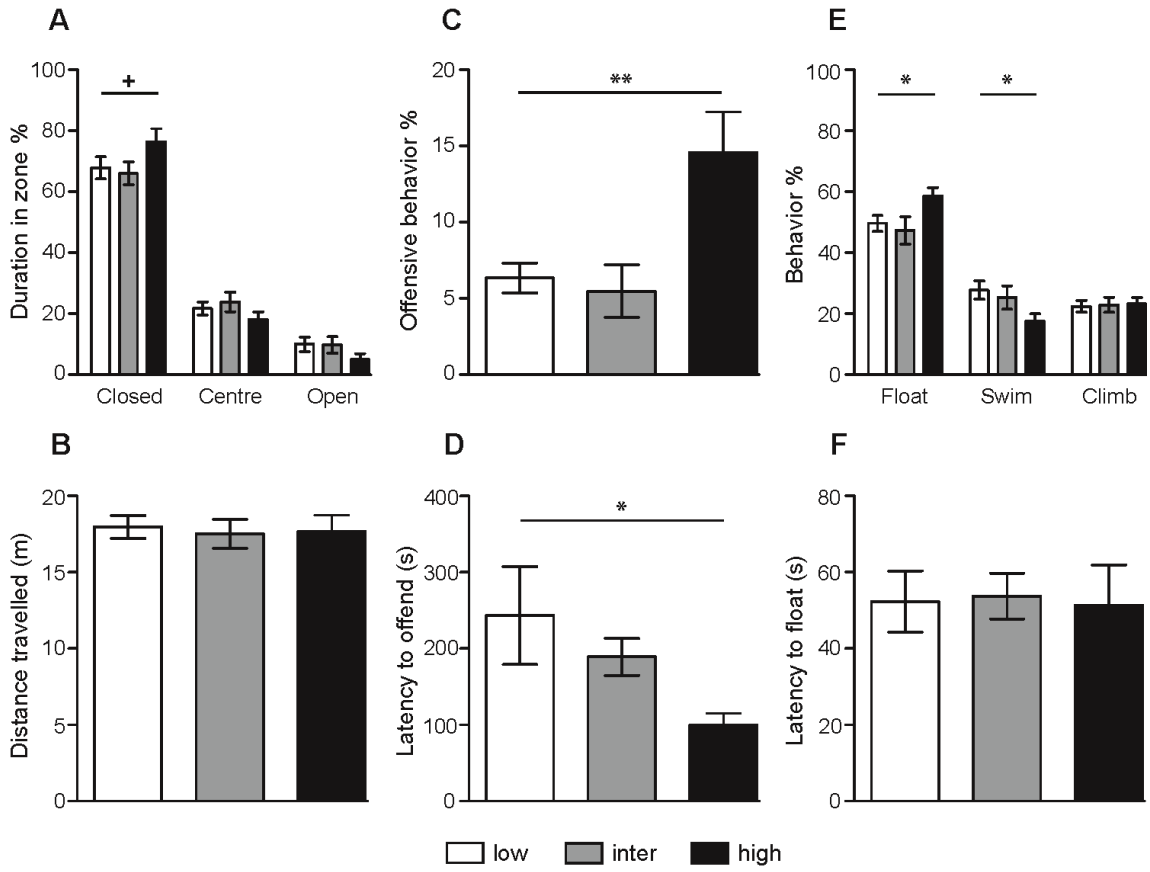

Figure 2 Constitutively different habituation to repeated stress gives rise to differences in psychopathology-like behavior in the absence of any stress experience. High-line rats from the F4 generation displayed a tendency to spend more time in the protected arms of the elevated plus maze (A), an effect not related to differences in locomotion (B). When exposed to a smaller intruder rat, high-line rats emitted significantly more aggression (C) and were quicker to aggress (D) than rats from the low- and intermediate-line. This pattern was repeated following exposure to forced-swimming, to which high-line rats responded by floating more, and swimming less, than rats from the low- and intermediate-line (E). No difference in latency to float was evident (F). Significant differences between lines are indicated by asterisks (ANOVA & Kruskal-Wallis (latency to offend) tests; + = $p<0.1$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; see text for further details).

Exposure to a resident-intruder test revealed large differences in territorial aggression between rats of different lines. In the first instance, lines differed in the time taken to initiate offensive behavior towards the intruder (Figure 2D - $H(2) = 8.402$, $p<0.05$). Specifically, high-line rats were faster than low- and intermediate-line rats to offend; low and intermediate lines did not differ in this respect (L vs I: U=69, n.s.; L vs H: U=33, $p<0.05$; I vs H: U=25, $p<0.01$). The propensity to differ in aggression was also observable in percentage of time spent performing offensive behavior (Figure 2C - $F_{(2,33)} = 6.608$, $p<0.01$). Again, high-line rats were more aggressive than low- and intermediate-line rats (L vs I: $t_{(22)}=0.925$, n.s.; L vs H: $t_{(22)}=-2.707$, $p<0.05$; I vs H: $t_{(22)}=-3.014$, $p<0.01$ – Bonferroni corrected). No differences in social exploration were evident between the lines ($F_{(2,33)} = 0.95$, n.s.).

Upon being challenged by a first session of forced-swimming, lines spent equal time floating, swimming and climbing during 15 minutes (Float: ($F_{(2,32)} = 0.252$, n.s; Swim: $F_{(2,32)} = 0.187$, n.s.; Climb: $F_{(2,32)} = 0.194$, n.s.). When exposed to a second session 24 hours later, lines did differ in percentage of time spent floating and swimming (Figure 2E - Float: $F_{(2,32)} = 3.413$, $p<0.05$; Swim: $F_{(2,32)} = 3.528$, $p<0.05$). The significance of the differences between lines did not survive multiple post hoc comparisons. However, the high-line displayed a strong tendency to float more and swim less than other lines (Float: L vs H: $t_{(22)}=-2.657$, $p<0.1$; Swim: $t_{(22)}=2.89$, $p<0.1$).

Next, in order to check the stability of behavioral traits observed in F4, a sample of male rats from the F6 generation were subjected to testing in EPM and RI experiments. In contrast to rats from F4 generation, rats from F6 did differ in anxiety-like behavior (Figure 3). Differences were evident both in terms of time spent in the closed arms (Figure 3A: $F_{(2,27)} = 4.544$, $p<0.05$) and time spent in the open arms ($F_{(2,27)} = 5.675$, $p<0.01$). Specifically, high-line rats spent more time in the closed arms and less time exploring the open arms of the maze than low-line counterparts (Closed: $t_{(21)}=-2.821$, $p<0.05$; Open: $t_{(21)}=2.767$, $p<0.05$). This was not accompanied by differences in distance travelled on the maze between the lines (Figure 3B: $F_{(2,27)} = 1.08$, n.s.).

The pattern of aggressive behavior observed in F4 generation rats was recapitulated in rats from F6 generation. Again, lines differed in time taken to initiate offensive behavior towards the intruder (Figure 3D - $H(2) = 6.659$, $p<0.05$), with high-line rats being faster than low-line rats to offend (L vs H: U=24, $p<0.05$). Lines also differed in percentage of time spent performing offensive behavior (Figure 3C - $F_{(2,29)} = 5.456$, $p<0.01$), with high-line rats more aggressive than low-line rats (L vs H: $t_{(22)}=-3.211$, $p<0.05$). No differences in overall social exploration were evident between the lines ($F_{(2,29)} = 2.005$, n.s.).

In addition to behavioral measurements described above, we also performed observations of maternal behavior of dams as they cared for the pups used in endocrine investigations. Assessment of several aspects of maternal behavior revealed a lack of difference between the lines in terms of caregiving (see supplementary information for full results).

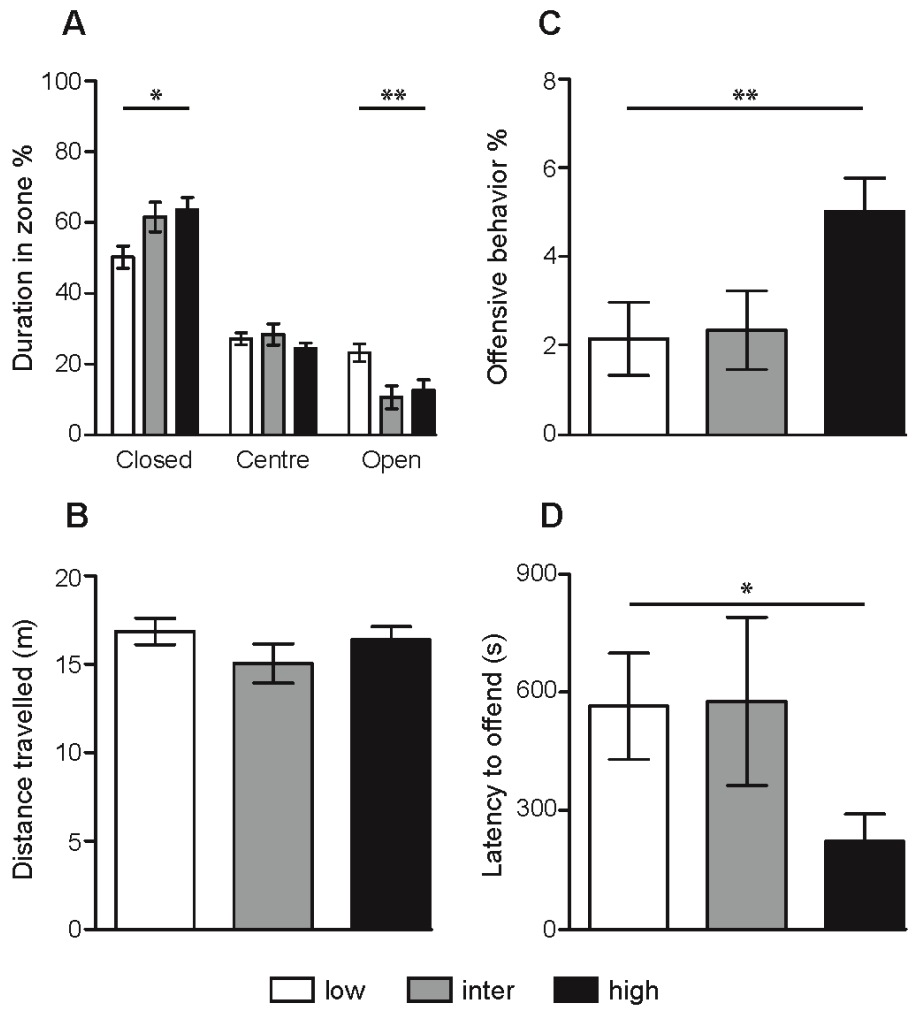

Figure 3 Phenotypic differences between low- and high-line rats in anxiety-like and aggressive behavior were stable across generations. In common with the F4 generation, high-line rats from the F6 generation displayed increased anxiety-like behavior, spending more time in the protected arms, and less time in the open arms, of the elevated plus maze than low-line rats (A). This effect was not related to gross differences in locomotion (B). When exposed to a smaller intruder rat, high-line rats emitted significantly more aggression (C) and were quicker to aggress (D) than rats from the low-line. Significant differences between lines are indicated by asterisks (ANOVA & Kruskal-Wallis tests; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; see text for further details).

Endocrine phenotype of the selected lines

Several measures of HPA axis activity were obtained from adult rats of the F8 generation of the lines (Figure 4). Interestingly, no differences were observed between the lines in corticosterone level at circadian nadir (Figure 4C: $H(2) = 1.602$, n.s.), nor at circadian peak (Figure 4D: $H(2) = 1.628$, n.s.). Lack of evidence for differential basal HPA axis function in any particular line was supported by the finding of similar bodyweight-adjusted adrenal gland measurements (Figure 4E: $F_{(2,31)} = 1.69$, n.s.).

As expected, lines differed in their corticosterone responses to restraint stress (Figures 4A and 4B). Though starting from an equal baseline, after 15 minutes of exposure to restraint stress, the rise in corticosterone levels in plasma differed between groups (Timepoint 2: H(2) = 12.801, p<0.01), with high-line rats having higher corticosterone levels than low and intermediate lines (L vs I: U=68, n.s.; L vs H: U=23, p<0.001; I vs H: U=27, p<0.01). The difference between lines was sustained at 30 and 60 minutes after the onset of restraint (Timepoint 3: H(2) = 10.825, p<0.01); Timepoint 4: H(2) = 9.263, p<0.01) with high-line rats again having higher corticosterone values than low-line rats (Timepoint 3: L vs I: U=37.5, n.s.; L vs H: U=24, p<0.001; I vs H: U=53, n.s.; Timepoint 4: L vs I: U=35, n.s.; L vs H: U=27, p<0.01; I vs H: U=69.5, n.s.). At 90 minutes after the onset of stress, i.e. 60 minutes after the offset of stress, corticosterone levels had recovered and no longer differed between the lines (Timepoint 5: H(2) = 2.001, n.s.). These differences are further reflected by the difference in area under the curve (AUC) between lines (Figure 4B: H(2) = 9.882, p<0.01), with the high-line having a significantly greater response across the whole experiment than the low-line, and the intermediate-line not differing from either (L vs I: U=38, n.s.; L vs H: U=27, p<0.01; I vs H: U=55, n.s.).

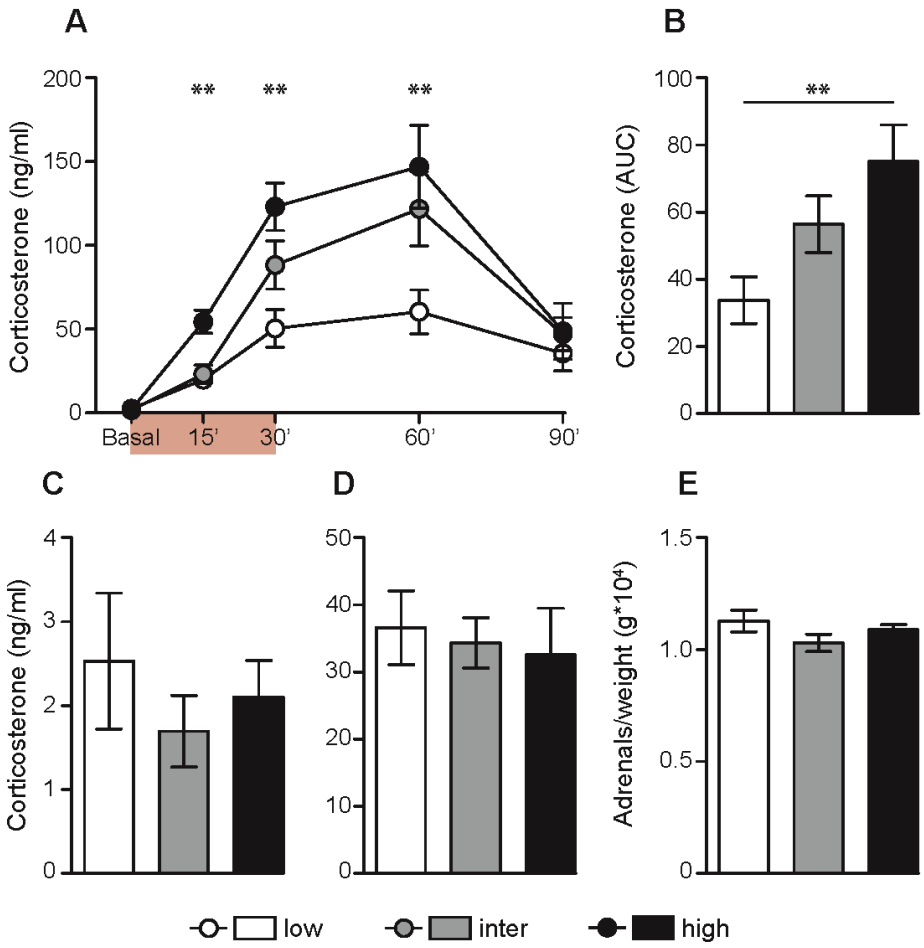

Figure 4 Constitutive differences in stress habituation are associated with altered physiological stress-reactivity in adulthood, though basal HPA axis activity did not differ, in rats from F8 generation. In the initial stages of restraint stress exposure, low and intermediate lines had a lower corticosterone response

compared to high-line rats (A). At the end of exposure, and 30 minutes into recovery, the difference in response between low- and high-line was maintained. These differences in corticosterone across the time-course are reflected in differences in area under the curve (AUC) between low and high lines (B). Lines did not differ in basal corticosterone level at diurnal nadir (C), nor at diurnal peak (D). Weight of adrenal glands, normalized to body weight, was similar between lines (E). Significant differences between lines are indicated by asterisks (Kruskal-Wallis tests; ** = $p<0.01$; see text for further details).

Expression of genes involved in regulation of the HPA axis

Gene expression analyses were performed upon tissue sampled from multiple nodes of the HPA axis. The results are summarized and detailed in Figure 5 and Table 1, respectively.

At the level of the brain, in the central nucleus of the amygdala, we tested the expression of genes related to excitability as well as genes more typically implicated in HPA axis function. In this region we found differences between the lines with regard to the expression of *Gad67*. *Gad67* encodes the 67kDA isoform of glutamic acid decarboxylase, an enzyme critically implicated in the synthesis of GABA. Higher expression of *Gad67* was evident in intermediate- and high-line rats as compared to low-line rats. In the PVN, a region considered to be the effector of a stress response, differences between lines were observed in the expression of several genes. Specifically, lines differed in expression of *Crhr1*, the gene encoding subtype 1 receptor of corticotropin-releasing hormone (CRH), such that the low-line displayed lower expression of the gene relative to the intermediate line. Lines also expressed variable levels of *Fkbp5* mRNA. Both intermediate- and high-line rats expressed higher levels of *Fkbp5* than low-line rats, though post hoc testing revealed this increase to be only marginally significant with regard to the low- versus high-line group comparison. Low-line rats additionally expressed higher levels of *Avp,* encoding arginine vasopressin, relative to both other lines. In contrast, high-line rats expressed higher levels of the gene encoding subtype 1a receptor of AVP, *Avpr1a,* than the low-line group.

In peripheral tissues further differences in gene expression were found. In the pituitary, high-line rats expressed higher levels of *Avpr1b* in comparison to both low- and intermediate-lines. A U-shaped function was observed in terms of expression of *Pomc*. Post hoc comparison revealed that high-line rats expressed significantly higher levels of the gene compared to intermediate-line rats, whereas the comparison between low- and intermediate-lines revealed only a trend towards a difference. Additionally, within adrenal tissue high-line rats expressed significantly lower levels of *Mrap*, which encodes melanocortin 2 receptor accessory protein, when compared to low-line rats. No further significant differences were found.

Table 1 Candidate gene expression under basal conditions in the central nucleus of the amygdala (CeA), paraventricular nucleus of hypothalamus (PVN), pituitary gland, and adrenals of low-, intermediate-, and high-line rats. Expression given is relative to two housekeeping genes (*Eef1a1* & *Tbp*). For further details regarding each of the genes, see supplementary table 1. Where a significant difference was found between lines, gene names are shown in bold (ANOVA, followed by post hoc comparisons; + = p<0.1; * = p<0.05; ** = p<0.01).

	Gene	Low			Intermediate			High			Line comparison		Post-hoc comparison		
		mean	SEM	N	mean	SEM	N	mean	SEM	N	F	Sig.	L : I	L : H	I : H
CeA	*Crh*	1.92	0.22	12	1.96	0.19	12	1.81	0.16	11	0.17	ns			
	Crhr1	1.41	0.08	12	1.70	0.11	12	1.51	0.11	11	2.26	ns			
	Gr	1.24	0.05	12	1.33	0.05	12	1.20	0.04	11	1.89	ns			
	Mr	1.60	0.10	12	1.77	0.14	12	1.90	0.15	11	1.21	ns			
	Fkbp5	1.62	0.11	12	1.43	0.08	12	1.53	0.09	11	1.02	ns			
	Avpr1a	4.08	0.55	12	4.02	0.48	11	3.40	0.36	11	0.62	ns			
	Gad67	1.26	0.04	12	1.49	0.05	12	1.46	0.07	11	5.36	**	*	*	ns
	Vgat	1.49	0.10	12	1.68	0.07	12	1.51	0.05	11	1.91	ns			
	Vglut	14.09	3.68	12	18.25	3.83	12	16.64	2.52	11	0.38	ns			
PVN	*Crh*	3.00	0.24	12	3.06	0.14	11	3.55	0.22	12	2.14	ns			
	Crhr1	1.71	0.16	12	2.48	0.18	11	2.04	0.23	10	3.96	*	*	ns	ns
	Crhr2	5.60	0.62	11	7.16	0.61	12	5.37	0.61	11	2.60	+			
	Gr	1.57	0.08	12	1.72	0.05	12	1.62	0.07	11	1.37	ns			
	Mr	1.69	0.10	12	1.74	0.12	11	1.84	0.05	12	0.73	ns			
	Avp	6.48	0.85	11	4.10	0.44	12	3.27	0.61	11	6.48	**	*	**	ns
	Avpr1a	1.69	0.20	11	2.03	0.15	12	2.40	0.23	11	3.30	*	ns	*	ns
	Fkbp5	1.42	0.06	12	1.38	0.07	11	1.65	0.09	12	4.07	*	ns	+	*
	Sgk1	2.15	0.17	12	2.53	0.15	11	2.58	0.19	11	1.96	ns			
	Cbr1	3.81	0.51	11	3.42	0.36	12	4.24	0.60	11	0.69	ns			
Pituitary	*Crhr1*	1.33	0.07	11	1.33	0.04	11	1.42	0.06	12	0.90	ns			
	Crhr2	1.24	0.06	11	1.30	0.06	11	1.27	0.08	12	0.21	ns			
	Gr	1.22	0.06	11	1.30	0.05	11	1.26	0.04	12	0.64	ns			
	Fkbp5	1.42	0.08	11	1.46	0.09	11	1.37	0.05	12	0.36	ns			
	Avpr1b	1.94	0.18	11	2.04	0.18	11	2.82	0.22	11	6.06	**	ns	**	*
	Pomc	1.99	0.11	10	1.60	0.09	11	2.23	0.14	12	7.52	**	+	ns	**
Adrenals	*Mc2r*	1.30	0.05	12	1.24	0.04	12	1.25	0.05	11	0.46	ns			
	Mrap	1.65	0.11	12	1.41	0.10	12	1.29	0.06	11	4.00	*	ns	*	ns
	Cyp11a1	1.17	0.04	12	1.20	0.03	12	1.14	0.03	11	0.81	ns			
	Star	2.13	0.18	12	2.10	0.12	12	2.04	0.12	11	0.11	ns			
	Th	2.68	0.18	11	2.34	0.16	12	2.20	0.17	11	2.10	ns			
	Pnmt	1.91	0.18	12	1.86	0.14	12	1.64	0.14	11	0.83	ns			

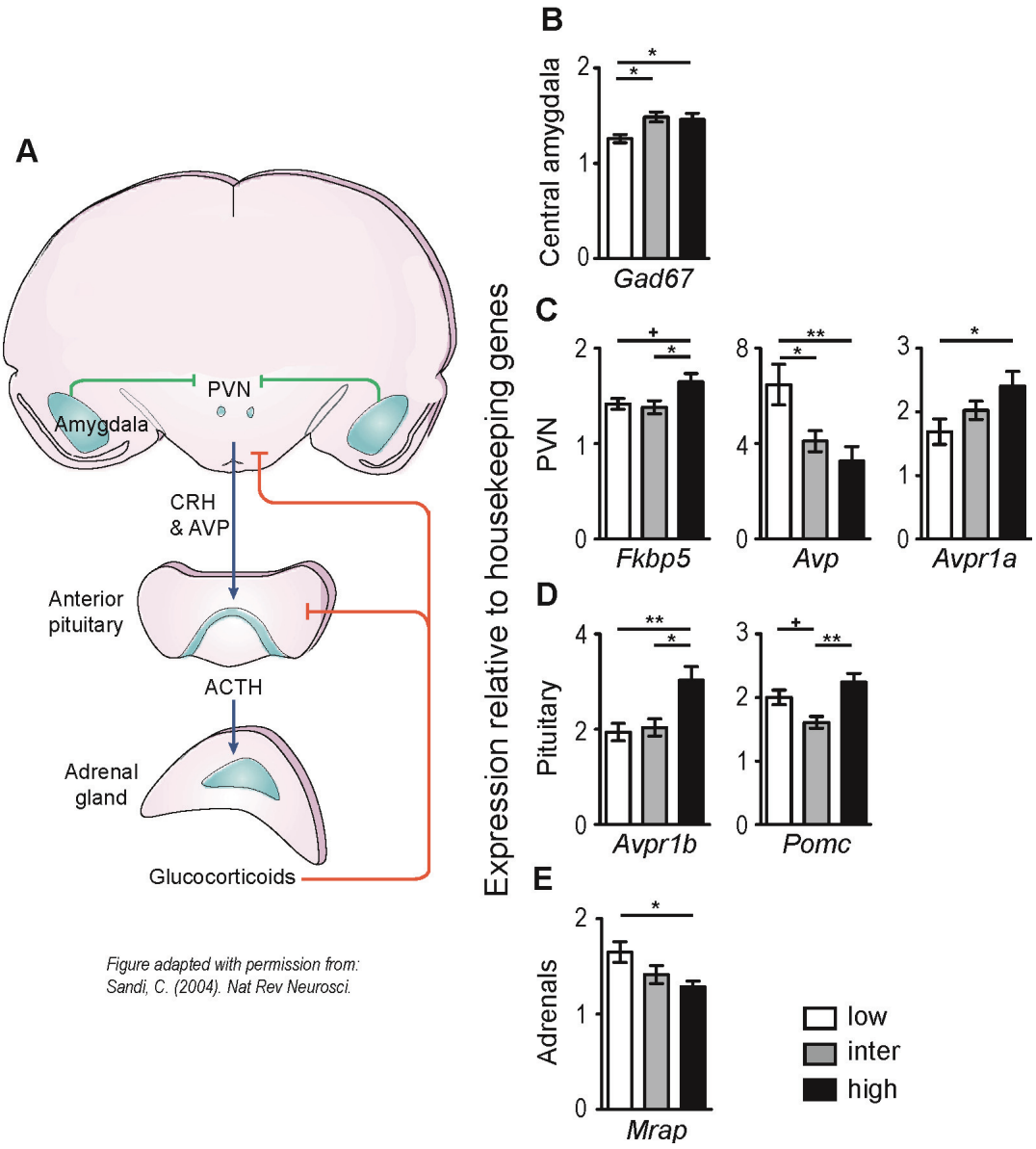

Figure 5 Constitutive differences in stress habituation are associated with variation in gene expression under basal conditions. Differences between the lines were found at multiple levels of the HPA axis (A), both centrally (B; C) and peripherally (D; E). Expression given is relative to two housekeeping genes (*Eef1a1* & *Tbp*). Bonferroni-corrected post hoc comparisons between lines are shown (+ = p<0.1; * = p<0.05; ** = p<0.01; see text and Table 1 for additional details).

Discussion

Converging lines of evidence have suggested that impairment in adaptation of HPA axis activity in the face of repeated exposures to stress may increase the propensity to accumulate allostatic load, thereby leading to an increased vulnerability to psychopathology (de Kloet et al., 2005; Flak et al., 2012; Kirschbaum et al., 1995; Kudielka et al., 2006; McEwen, 1998; Preussner et al., 1997). The mechanisms underlying this susceptibility are not easily studied and progress in this field has been hampered by the lack of a reliable and specific animal model within which to test hypotheses. Here, we report the development of two selectively bred rat lines that show habituation and non-habituation of the corticosterone response to repeated stress exposure, as well as a control line intermediate for this trait. In the absence of the application of stress, constitutive differences in stress habituation alone gave rise to phenotypic variation in psychopathology-like behaviors, specifically anxiety-like, depression-like, and aggressive behavior. Differences in stress habituation were observed alongside a distinct neuroendocrine phenotype. The different lines displayed altered corticosterone reactivity to restraint stress but parity in measures of basal HPA axis activation. Variation in the expression of several HPA axis related genes was found at multiple levels of the axis in these same animals.

Selective breeding has proved a useful approach in behavioral genetics research (Scharf & Schmidt, 2012). To date, researchers applying this approach in rats in relation to affective disorders have primarily selected for variation in behavioral traits, thought to reflect specific symptomatic aspects of psychopathology (anxiety-like: Liebsch et al., 1998; depression-like: Bignami, 1965; aggression: Naumenko et al., 1989). To our knowledge, despite frequent association of HPA axis dysregulation with psychopathological disorders (Ehlert et al., 2001; McEwen, 1998; Tarullo & Gunnar, 2006; Walker et al., 2008; Yehuda et al., 2009), only one other group has used an aspect of HPA axis activity to selectively breed rodents (Touma et al., 2008). Our study is the first to use stress habituation as a selection criterion, and also the first to do so in rats.

The response to genetic selection for low and high corticosterone responses to repeated stress (i.e. the propensity to habituate or not) was strong, specific, and equally evident in both sexes. By the third generation of selection each line's p30 corticosterone response had diverged from the others', and these differences increased further across generations of selection. In common with findings in humans, and taken together with our narrow sense heritability estimate (h^2 of 0.29), this indicates a robust genetic basis for the phenotype (Federenko et al., 2004). As we have used repeated stress in phenotyping for our trait of interest, the purely genetic basis for inheritance of the trait cannot be assumed. There are examples from both the human and rodent literature that have provided evidence for epigenetic inheritance of HPA axis dysregulation, notably in cases where the parents were exposed to traumatic stress (Bierer et al., 2014; Cordero et al., 2012; Franklin et al., 2010; Gapp et al., 2014; Yehuda et al., 2014, 2016). The epigenetic transmission of traits may occur through a number of routes, one of which is via changes in the quality of caregiving delivered by the mother (Champagne et al., 2003). Here,

however, we observed no differences between the lines across several aspects of maternal behavior, making this an unlikely source of phenotypic variation. In studies where this factor was controlled for (Cordero et al., 2012) or where there was no contact between trauma-exposed individuals and the dams, nor the offspring (Franklin et al., 2010; Gapp et al., 2014), intergenerational transmission of traits has still been observed. In the latter cases, however, the stress exposure giving rise to these effects, chronic *unpredictable maternal separation* combined with *unpredictable* maternal *stress,* could be considered somewhat extreme in nature. In contrast, the three day stress protocol used to select breeders in our study failed to elicit marked differences, neither in behavior nor in gene expression, in rats exposed to the same protocol in another study (Tzanoulinou et al., 2014) and may be therefore less likely to induce epigenetic alterations that would be passed on to the subsequent generation.

We examined the behavioral phenotype of the lines in the F4 generation, and despite the early point in selection, we observed several differences in psychopathology-like behavior. Specifically, rats from the high-line displayed higher levels of anxiety-like, depression-like and aggressive behaviors when compared to the low-line. These phenotypic differences between low- and high-line groups, in particular with regard to anxiety-like behavior and aggression, were recapitulated, or enhanced, when measured in a later generation (F6), underlining the stability of these aspects of the phenotype. Curiously, the behavioral phenotype of the intermediate-line vacillated between appearing 'low-line-like' in F4 generation and 'high-line-like' in F6. Whether this indicates the lack of a true intermediate phenotype in terms of stress habituation is unclear but suggests that future experiments could benefit from the addition of an extra non-selected group for comparative purposes.

In terms of aggression, we found that rats derived from the high-line, who also demonstrated constitutively high corticosterone reactivity to stress, were both quicker to initiate hostilities and performed more offensive behavior overall. This is contrast to findings reported from the differential stress reactivity mouse lines developed by Touma and colleagues (2008), where the line selected for low corticosterone reactivity to stress was found to be the more aggressive. There are several possible explanations for this disparity. One such explanation is that differences could be reflective of the strong deviation in several other aspects of neuroendocrine profile between our rat lines and the differential stress-reactivity mouse lines, most notably in terms of HPA axis rhythmicity and negative feedback capacity (Touma et al., 2008; Heinzmann et al., 2014). Additionally, it could be that the disparity reflects a more general species difference in the link between HPA axis function and aggression in rat and mouse selection lines. Support for this assertion comes from findings showing that in other mouse lines, in these cases selected for aggression, aggressiveness was found alongside corticosterone hypo-reactivity to stress (Caramaschi et al., 2008; Sandnabba, 1985; van Oortmerssen & Bakker, 1981; Veenema et al., 2003). Our findings are more closely in line with those from rat models showing that in lines selected for various psychopathology-like behavioral indices, but eventually differing in both stress reactivity and aggression, the line displaying enhanced reactivity to stress was also the more aggressive (Albert et al., 2008;

Bignami, 1965; Coppens et al., 2012; Díaz-Morán et al., 2012; Landgraf et al., 1999; Liebsch et al., 1998; Naumenko et al., 1989; Neumann et al., 2010; Steimer & Driscoll, 2003; Veenema et al., 2007). The finding of enhanced aggression in conjunction with enhanced anxiety-like behavior in the high-line does not fit well with the idea that animals tend to show a passive or active coping style when faced with a variety of challenges (Koolhaas, 2008). It does however course with findings in the human literature indicating that individual differences in stress habituation are associated with variation in affective indices such that individuals habituating to a lesser extent report suffering lower self-esteem, being more depressed of mood, and being more defensive (Kirschbaum et al., 1995; Preussner et al., 1997; Kudielka et al., 2006).

We did not observe differences in basal activity of the HPA axis at circadian trough, nor at circadian peak, pointing toward the absence of general dysregulation of HPA axis function in the lines. Enhanced corticosterone levels at circadian peak (i.e. awakening level) have been reported several times as a common endocrine disruption occurring in depressed individuals (de Kloet et al., 2005). Though our findings appear reliable, it would be interesting to apply a more comprehensive study of HPA axis rhythmicity, using a method allowing for more frequent sampling (Landgraf et al., 1999; Touma et al., 2008; Waite et al., 2012). However, methods that allow for frequent sampling of HPA axis activity (e.g. in-dwelling jugular vein catheters or regular collection of fecal boli from single-housed animals) may impart stress upon the animal that we wished to avoid in this case. In spite of the limited number of data points gathered, a lack of variation in adrenal weight between the lines supports our conclusion.

Interestingly, we found differences between the lines in corticosterone responsiveness to restraint stress, with high-line rats showing the strongest response and low-line rats the weakest. Lines displayed equal recovery in corticosterone levels 60 minutes after cessation of the stressor, indicative of equally efficacious negative feedback mechanisms. Variation in corticosterone levels between lines appeared most evident in the initial stages of the stress exposure. Multiple factors might underlie this variability including: differential perception with regard to the absolute stressfulness of the experience; differential capacity of fast feedback mechanisms; more functional variability in the release of and receptiveness to corticotrophs (Tasker & Herman, 2011). Recent findings from the differential stress reactivity selection lines indicate that differential corticosterone responses to stress, and associated behavioral phenotypes, are associated with alterations in functionality at multiple levels of the HPA axis (Heinzmann et al., 2014).

In addressing potential molecular-genetic correlates of the phenotypic differences between the lines we indeed found variation in gene expression within several nodes of the HPA axis. Unexpectedly, given differences in corticosterone responsiveness to stress between the lines, we did not find any differences in the expression of the gene encoding corticotropin-releasing hormone (*Crh*) in the PVN. CRH release is considered to be at the apex of HPA axis activation (Ulrich-Lai & Herman, 2009), regulating the expression of *Pomc,* which encodes proopiomelanocortin (POMC), the polypeptide pre-cursor of ACTH,

in the pituitary (Bale & Vale, 2004). Additionally, we found differential expression of *Avp*, the gene coding for vasopressin. Vasopressin is a corticotropic peptide co-released from the PVN along with CRH, and known to potentiate the actions of CRH (DeBold et al., 1984). Interestingly, the difference found did not go in the expected direction, with low-line rats showing higher expression relative to intermediate- and high-line rats. The opposite pattern of results was found with reference to *Avpr1b* expression in the pituitary gland. Activation of vasopressin receptors of subtype 1b, coded for by *Avpr1b*, stimulates the production of POMC, which is then converted in part to ACTH. In conjunction, these two results clarify the finding of increased expression of *Pomc* in both low- and high-lines relative to the intermediate-line, suggesting that both low- and high-lines have similar capacity to mount a strong ACTH response to stress. Taken together with a lack of evidence of differential expression of genes determining adrenal sensitivity to ACTH (*Mc2r*) or involved in synthesis of glucocorticoids and adrenaline (*Cyp11a1, Star, Th, Pnmt*), it would appear that differences between the lines are potentially more likely to be centrally mediated (Ulrich-Lai & Herman, 2009). This raises an interesting possibility since it is generally considered that it is limbic brain regions, and not the HPA axis itself, that mediate habituation in the face of repeated stress (Herman, 2013).

In central nucleus of the amygdala (CeA), a brain region implicated in both anxiety (Phelps & LeDoux, 2005) and aggression (Halász et al., 2002; Márquez et al., 2013; Tulogdi et al., 2010), we found increased expression of *Gad67* in intermediate- and high-line rats relative to low-line rats. *Gad67* encodes the 67kDa isoform of glutamic acid decarboxylase, an enzyme key in the activity-dependent production of GABA from glutamate. The CeA is thought to have an excitatory impact upon HPA axis activity via its output to the bed nucleus of the stria terminalis (BNST) (Ulrich-Lai & Herman, 2009). The BNST acts as a hub where stress-related signals from several limbic brain regions are integrated, determining its HPA axis inhibiting output to the PVN (Johnson et al., 2016). The projection from CeA to BNST is GABAergic and an increase in GABA activity derived from CeA could conceivably give rise to more ready activation of HPA axis via enhanced disinhibition of PVN (Ulrich-Lai & Herman, 2009). In this vein, evaluation of potential changes in gene expression in the BNST is warranted. Moreover, independent of its excitatory influence on the HPA axis, the CeA is implicated in perception of and response to threat (Fox et al., 2015; Johnson et al., 2016). Enhanced inhibitory activity from this region could potentially explain the stronger reaction of high-line rats to the threat of exposure experienced in the EPM and to a normally non-threatening intruder in the resident-intruder test.

A limitation of the current study is that we have not yet confirmed that variations in the expression of genes reported here are translated at the level of the respective proteins, and doing so represents an important next step. It is further necessary to establish whether any particular variation has a causal implication in the neuroendocrine and behavioral phenotypes reported herein, since there is the potential that some alterations may be compensatory rather than actively involved in phenotypic variation. Given the frequent association of *Fkbp5* and *Avpr1b* activity in modulating affective, aggressive, and endocrine

phenotypes in both human and animal models (Binder et al., 2004; Dempster et al., 2007; Holz et al., 2015; Luppino et al., 2014; Minelli et al., 2013; Pagani et al., 2015; Touma et al., 2011; van West et al., 2004; Wersinger et al., 2008), variation in these two genes represent particularly promising candidates.

In summary, we report the successful development of two selectively bred lines differing in propensity to habituate in terms of corticosterone response to repeated stress, as well as a control line intermediate for this trait. Constitutive stress-non-habituating rats displayed enhanced aggression, anxiety-like, and depression-like behaviors, enhanced reactivity to acute stress, and a number of alterations in the expression of genes within multiple nodes of the HPA axis. Together, these findings add weight to the link between constitutive differences in stress adaptability and vulnerability to develop psychopathology-like alterations. The lines represent a promising model with which to further examine the relationship between stress adaptability and stress-related pathophysiology more generally, especially with respect to underlying mechanisms. Moreover, the potential of treatments enhancing stress habituation to enhance resilience to psychopathology could be assessed using this model.

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Author contributions

S.W.: performed and organised line selection and all experiments, scored videos, analysed data, wrote the initial version of the manuscript

O.Z.: performed the qPCR work

I. GdS.: scored FST videos and helped in many selection protocols

C.S.: provided the concept, ideas and feedback for the experiments, corrected the manuscript

Supplementary information

Gene	Full name	Primer sequences	
		Forward	Reverse
Avp	Arginine vasopressin	CGCTGCCAGGAGGAGAACTAC	GCTGTACCAGCCTAAGCAGCA
Avpr1a	Arginine vasopressin receptor, subtype 1a	AGATGTGGTCAGTCTGGGATG	AAACTTTGGACGCAGTCTTGC
Avpr1b	Arginine vasopressin receptor, subtype 1b	TGTGTGGGATGAGAATGCCC	TGTGCACTTGGGGCTTAGAG
Cbr1	Cannabinoid receptor, type 1	GGACATGGAGTCCTTTATGATTC	GAGGGACAGTACAGCGATGG
Crh	Corticotropin-releasing hormone	GAGAAGAGAGCGCCCCTAAC	CGGGACTTCTGTTGAGGTTC
Crhr1	Corticotropin-releasing hormone receptor, type 1	TCCGCTACAACACGACAAAC	AGAATCTCCTGGCACTCAGAA
Crhr2	Corticotropin-releasing hormone receptor, type 2	TGAACCCATTTTGGATGACA	GTTGATGATGAGGGCGATTC
Cyp11a1	Cholesterol side-chain cleavage enzyme	CTCCTGCGAGGGTCCTAAC	GGCCATCACCTCTTGGTTTA
Eef1a1 (hk)	Eukaryotic translation elongation factor 1, alpha 1	TGTGGTGGAATCGACAAAAG	CCCAGGCATACTTGAAGGAG
Fkbp5	FK506 binding protein 5	GGGAACGGTGTACTTCAAGG	TTCTGACAAGCCGTACTCCA
Gad67	Glutamate decarboxylase 67	TACAACCTTTGGCTGCATGT	TGAGTTTGTGGCGATGCTT
Gr	Glucocorticoid receptor	AAAGCTTCTGGACTCCATGC	TCAATACTCATGGTCTTATCCAAAAA
Mc2r	Melanocortin receptor, type 2	CAGAAACTGGATCCTTCCGCA	GCGGTTAAGAAGGGGATGGT
Mr	Mineralocorticoid receptor	CAAGCTGGCATGAACTTAGGA	TCCTCGTGGAGGCCTTTT
Mrap	Melanocortin receptor associated protein	CTGTCCCGTTCACCAGCTAT	ACTATGCCTTACCTGTGGGGA
Oxtr	Oxytocin receptor	GAGCGTTTGGGACGTCAATG	TGCACGAGTTCGTGGAAGAG
Pnmt	Phenylethanolamine N-methyltransferase	GAGCCTTCGACTGGAGTGTG	CAATGGGCAAGACTCGCTTC
Pomc	Pro-opiomelanocortin	GCCACTGAACATCTTCGTCCT	AGCGACTGTAGCAGAATCTCG
Sgk1	Serum and glucocorticoid-regulated kinase 1	GCTCGAAGTACCCTCACCTAC	GCGTTCATAAGTTCGGGCTC
Star	Steroidogenic acute regulatory protein	ACACTTTGGGGAGATGCCTG	AGCCACCCCTTGAGGTCAATA
TBP (hk)	Tata box binding protein	CCCACCAGCAGTTCAGTAGC	CAATTCTGGGTTTGATCATTCTG
Th	Tyrosine hydroxylase	GGAACGGTACTGTGGCTACC	TGCATTGAAACACGCGGAAG
Vgat	Vesicular GABA transporter	GGGCTGGAACGTGACAAA	GGAGGATGGCGTAGGGTAG
Vglut	Vesicular glutamate transporter	GTCATGACTATCATCGTACCCATC	GTAFCTTCCATCCCGAAACC

Supplementary Table 1 List of candidate genes including full name of protein product, in addition to forward and reverse primer sequences. The initials '*hk*' denote the two housekeeping genes used.

Materials & Methods

Subjects

Measures of maternal behavior were determined in F7 dams (n=9-12), who bred the animals used in endocrinology experiments.

Maternal behavior observations

Prior to the first birth, and in line with previous breedings, dams were provided with three 3-ply tissues as nest material, in addition to normal housing conditions. Maternal care was observed using a protocol adapted from Cordero and colleagues (2012). For the first five whole days postpartum (i.e. p1-p5) each dam was observed daily for a minimum of five seconds every three minutes, during 60 minute observation periods (20 observations/period x four periods per day = 80 observations/dam/day). Observations occurred at regular times (0800, 1200, 1700, 2000h). Dams were observed in their homecage and minimum disturbance was ensured during the five-day observation period. Briefly, behaviors scored were: i) Dam nursing pups, including the positions a) arched-back nursing, b) 'blanket' posture (mother lays over the pups), and c) passive posture (mother lays either on her back or side while the pups nurse; ii) mother licking/grooming any pup, in either a) arched-back posture, and b) any other

posture; iii) mother off pups (dam away from the pups, e.g., while resting or exploring, eating or drinking). As faithful recording of the behavior of the dam under red light conditions was difficult, the coding scheme was simplified for dark cycle observations. If the dam was on the nest they were considered to be nursing, and if off nest then not nursing. The percentage of all samples during which the dam was engaged in nursing was calculated per session and per day. Results are presented as percentage per day. Owing to the well-described importance of individual differences in arched-back nursing, and licking and grooming behavior, in pups' behavior and HPA axis reactivity (for review see Weaver, 2007), the relative representation of these behaviors within the dams' repertoire was also determined.

As described, pups were counted and, if necessary, litters reduced to a maximum of 12 pups on p2. In doing this, dams were removed from the cage, as were pups. Dams and pups were returned simultaneously to the homecage, off nest. The latencies for the dam to approach the first pup, pick it up, place it back in the nest, as well as the latency to return six pups, were recorded (respectively termed: contact; take; return; finish in Figure S1D). To cause minimum disturbance to other measurements, this test took place after the second maternal behavior measurement of the day (1200h).

Statistics

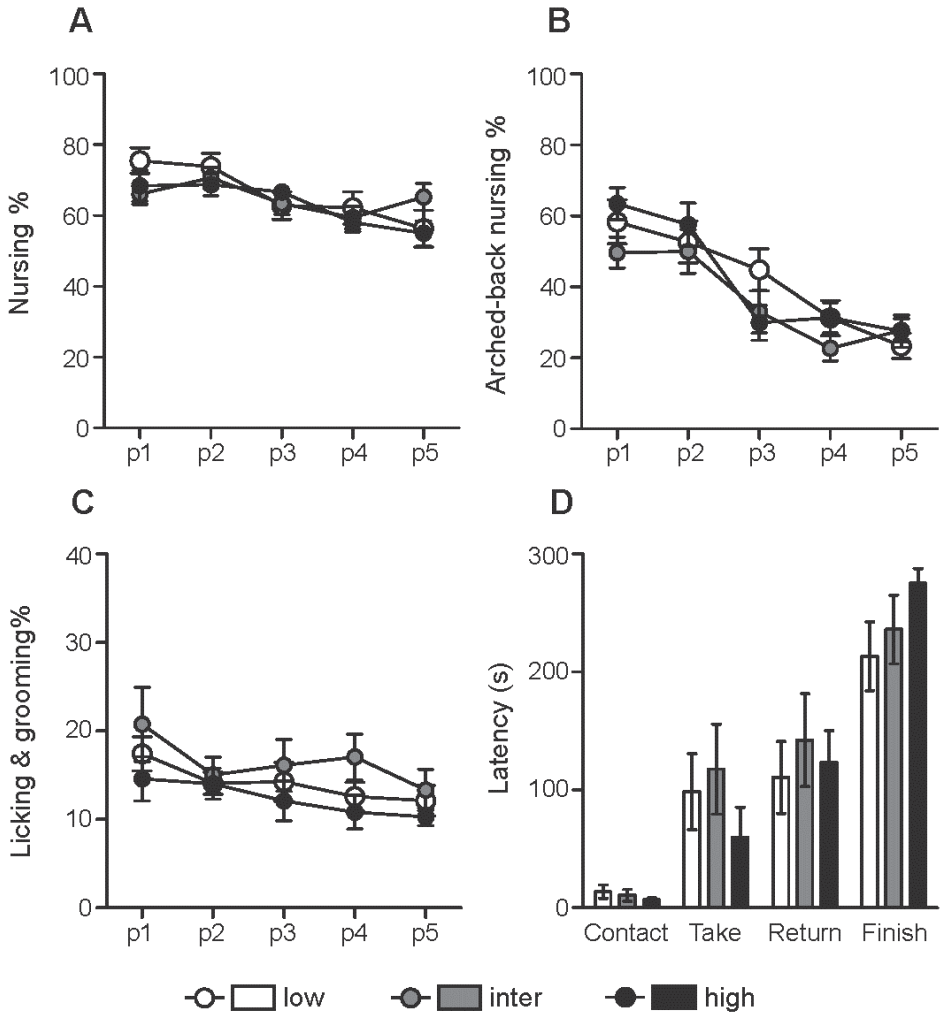
Maternal behavior measurements were taken over multiple time points and thus analyzed using 2-way repeated measures ANOVA, with selection line as the between-subjects factor and day as the within-subjects factor. For within-subjects ANOVA, where sphericity assumptions were violated, the Greenhouse-Geisser correction was applied. Latencies to reach certain milestones in the pup retrieval test were non-normally distributed and therefore these data were analyzed using Kruskal-Wallis tests for each milestone. Statistical significance level was set at $p < 0.05$.

Results

Examination of maternal behavior over the first five full days of life indicated that though the intensity of pup-directed behavior declined across days (proportion of time spent nursing, main effect of day: $F_{(4,116)} = 8.884$, $p<0.001$, $\varepsilon = 0.72$; proportion of all nursing delivered in arched-back posture: $F_{(4,116)} = 31.438$, $p<0.001$; proportion of all pup-directed behavior consisting of licking and grooming: $F_{(4,116)} = 3.228$, $p<0.05$, $\varepsilon = 0.70$), each of the lines demonstrated these declines equally (day*line interaction: nursing, $F_{(8,116)} = 1.385$, n.s.; arched-back nursing, $F_{(8,116)} = 1.438$, n.s.; licking and grooming, $F_{(8,116)} = 0.324$, n.s.).

When the nest was disturbed in order to count the pups on p2, and pups were returned to the homecage off nest, dams from each line were equally reactive in retrieving them. The time taken for the dam to make the first contact after being replaced to the cage, pick up the first pup, place that first pup

back in the nest, and go on to retrieve a further five pups, did not differ between groups (contact: H(2) = 0.664, n.s.; take: H(2) = 2.275, n.s.; return one: H(2) = 0.228, n.s.; return six: H(2) = 3.041, n.s.).



Supplementary Figure 1 Constitutive differences in stress adaptation are not associated with differences in maternal behavior. Examination of maternal behavior over the first five days of life indicated that though the intensity of pup-directed behavior (proportion of time spent nursing (A); proportion of all nursing delivered in arched-back posture (B); proportion of all pup-directed behavior consisting of licking and grooming (C) declined across days, each of the lines demonstrated these declines equally. Lines performed equally in the pup retrieval test performed on p2 (D).

Chapter 4

Constitutive differences in glucocorticoid habituation to repeated stress relate to psychopathological impact of peripubertal stress

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Abstract

Experience of adversity early in life and dysregulation of hypothalamus-pituitary-adrenocortical (HPA) axis activity are risk factors often independently associated with the development of psychopathological disorders, including depression, PTSD and pathological aggression. Additional evidence suggests that in combination these factors may interact to shape the development and expression of psychopathology differentially, though little is known about underlying mechanisms. Here, we studied the long-term consequences of early life stress exposure on individuals with differential constitutive adaptability to repeated stress, assessing both socio-affective behaviors and brain activity in regions frequently found to be sensitive to pathological alterations following stress. Two rat lines, genetically selected for either low or high glucocorticoid responsiveness to repeated stress (i.e. strong or impaired corticosterone habituation, respectively) were exposed to a series of unpredictable, fear-inducing stressors on intermittent days during the peripuberty period. Results indicated that being both constitutively impaired in terms of stress habituation and having experience of peripuberty stress independently enhanced levels of psychopathology-like behaviors, as well as increasing basal activity in several subregions of the prefrontal cortex in a manner that was associated with increased behavioral inhibition. Interestingly, peripuberty stress had a differential impact on aggression in the two rat lines, enhancing aggression in the stress-habituating low-line rats but not in the already high-aggressive, high-line rats. Taken together, these findings indicate that aberrant HPA axis activity around puberty, a key developmental period in the social repertoire of both rats and humans, may alter behavior such that it becomes anti-social in nature.

Introduction

Experience of adversity in childhood and adolescence is recognized as a major risk factor in the development of psychopathological disorders later in life (Caspi & Moffitt, 2006; Heim & Binder, 2012; Nugent et al., 2011; Widom & Maxfield, 1996). However, not all individuals experiencing early adversity go on to develop psychopathology and increased understanding of the factors underlying this variability would assist in the identification and treatment of vulnerable individuals. A growing number of findings indicate that having a constitution conferring differential physiological sensitivity to stress may be a factor that modulates the outcome of early life stress exposure (Bevilacqua et al., 2012; Binder et al., 2004; Binder et al., 2008; Luppino et al., 2014; Szczepankiewicz et al., 2013; van West et al., 2004), implicating this as a key arena within which to focus research.

Exposure of an individual to a stressful challenge is characterized by a response that includes metabolic, behavioral and physiological components, and involves the activation and interaction of several neuro-physiological systems (McEwen, 2001). The consequences of the stress response are generally adaptive in the short term, restoring homeostasis and mediating adaptation following cessation of the stressor, a process termed allostasis. However, repeated, prolonged or inadequate stress responses may eventually lead to physiological damage, termed allostatic load, as well as leading to additional dysregulation of the systems mediating stress responses (McEwen, 1998). Accumulation of allostatic load is thought to form the pathological basis of stress-related disorders (McEwen, 2007).

Following activation of the sympathetic nervous system (SNS) in response to challenge, the hypothalamus -pituitary-adrenal (HPA) axis coordinates actions that enable an individual to cope with the challenge (Shirazi et al., 2015). Activation of the HPA axis involves a cascade of responses that starts with the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) by the paraventricular nucleus of the hypothalamus (PVN). The release of CRH and AVP activates adrenocorticotrophic hormone (ACTH) secretion from the pituitary which, in turn, triggers the production and release of glucocorticoids (primarily cortisol in humans; corticosterone in most rodents) from the adrenal glands into the circulation (Ulrich-Lai & Herman, 2009). Circulating glucocorticoids exert a multitude of effects, both genomic and non-genomic, via their actions upon mineralocorticoid (MR) and glucocorticoid (GR) receptors (de Kloet, 2014; de Kloet et al., 2008; Joëls et al., 2013). Activation of GR in several brain areas, notably including the PVN, acts to inhibit continuation of the stress response, with this negative feedback acting via both fast and slow mechanisms (Tasker & Herman, 2011). A common allostatic adaptation of the HPA axis in response to repeated exposure to the same stressor is reduction of the glucocorticoid response across stress exposures (Deinzer et al., 1997; Federenko et al., 2004; Gerra et al., 2001; Pruessner et al., 1997). Adaptation of this kind minimizes the impact of frequently experienced stressors, and both under- or over -efficacious engagement of this process could leave an individual open to the accumulation of allostatic load (McEwen, 1998).

Here we aimed to assess in rats the influence of constitutive differences in capacity to the habituate the glucocorticoid response to repeated stress on the neurobehavioral outcome following exposure to stress during the peripuberty period. In outbred rats, previous studies have found peripuberty stress to increase the expression of anxiety-like, depression-like and aggressive behaviors, in concert with behaviorally-consequent shifts in activation of brain regions important in socio-emotional function (Márquez et al., 2013; Tzanoulinou et al., 2014a; Tzanoulinou et al., 2014b). After exposing genetically-selected stress-habituating and non-stress-habituating rats to peripuberty stress, or control conditions, we measured the impact of both factors upon the development of psychopathology-like behaviors (specifically, anxiety-like, depression-like, and aggressive behaviors) in the long-term. We additionally sought to examine whether basal activity of the brain differed between the lines, and in function of peripuberty stress exposure. We focused our investigation on subregions of prefrontal cortex, amygdala, and hippocampus, brain regions that are: i) subject to ongoing development during adolescence (Spear, 2000; Casey et al., 2008); ii) previously shown to be affected by peripubertal stress (Marquez et al., 2013; iii) found to show altered activation at rest in stress-related psychopathologies (New et al., 2009; Koch et al., 2016; Sripada et al., 2012; Wang et al., 2016).

Materials & Methods

Subjects

Experimental animals were male offspring of rats from the F6 generation of differential stress-habituation selection lines recently developed by our laboratory (see Chapter 3 of this thesis). A full description of the procedure used to generate these lines can be found in the supplementary materials. Male and female Wistar Han rats, acting as intruders and as cohabitating partners, respectively, were purchased from a commercial breeder (Janvier, France).

Experimental animals were derived from 10-12 litters. At weaning on postnatal day (p) 21, pairs of male rats from different litters were matched according to weight and mixed among home cages. Additional care was taken to ensure that weight was well matched between treatment groups within each line. Animals assigned to the same experimental group were housed together. Peripuberty stress was applied over seven, intermittent days between p28 and p42. Control rats were handled briefly on the days that stress took place. After this point rats remained undisturbed, except for weekly cage changes, until experimental procedures began at adulthood (designated as p90). Before the first behavioral test, all rats were handled on three occasions for two minutes per occasion.

Rats were maintained on a 12-h light–dark cycle (lights on at 0700h), in a temperature- and humidity-controlled environment (21±1 °C; 55% humidity ±5%), with *ad libitum* access to laboratory chow and water. Experiments were performed between 0800 and 1200, except where otherwise stated, with at least one week between each test. All procedures were conducted in accordance with the Swiss National

Institutional Guidelines on Animal Experimentation and approved by a license from the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

Experimental design

A 2 x 2 factorial design was used: two genetically-selected breeding lines (low and high corticosterone in response to repeated stress, i.e. stress habituating and stress non-habituating, respectively) were exposed to one of two treatment conditions (peripuberty stress (PPS) or control-handling (control)), resulting in four experimental groups. Starting from n=22 rats in each group, following assessment of anxiety-like behavior on the elevated plus maze (EPM), n=8/group went forward for measurement of depression-like behavior, followed by measurement of brain energy metabolism using 2-deoxyglucose autoradiography. An additional n=12/group were assessed for aggression in the resident-intruder test.

Peripuberty Stress protocol

The stress protocol was performed as previously described (Marquez et al., 2013). Following exposure to an open field (50 x 50 x 30cm) for five minutes on p28, the stress protocol consisted of the presentation of two different stressors, each one lasting 25 minutes (see Figure 1 for stress schema). These were either; exposure to the synthetic fox odor trimethylthiazoline (TMT; Phero Tech Inc., Canada) or to an elevated platform (EP). TMT exposure was administered in a plastic box (38 x 27.5 x 31 cm) via a scent-charged cloth. The box was placed under bright light (210–250 lx). The elevated platform (12 x 12cm, elevated 95cm from the ground) was also under direct bright light (470–500 lx). Following each stress session, animals were returned to neutral cages for 15 minutes. A transparent Plexiglas wall perforated with holes separated pairs of cagemates during this time. Following the holding period, animals were returned to their home cage. The stressors were applied during peripuberty, on seven intermittent days between p28–p42, following a variable schedule. Tail blood samples were taken on p28, p30 and p42, once at the offset of stress and again 30 minutes later. All blood samples were chilled, centrifuged and the plasma stored at -20^{o}C until subsequent analysis.

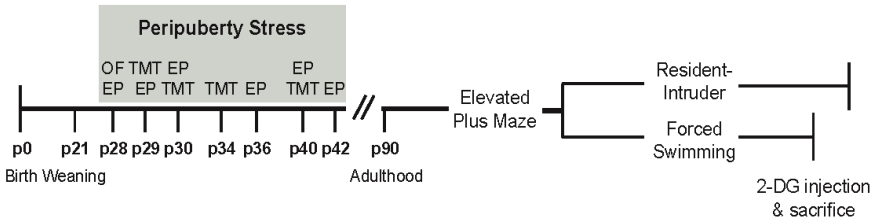


Figure 1 Experimental design. Animals were weaned at p21 and assigned to Control or Peripuberty Stress (PPS) groups. The stress protocol consisted of exposure to an open field (OF) on p28, followed by intermittent, variable exposure to an elevated platform (EP) and predator odor (trimethylthiazoline; TMT). Control animals were handled briefly on the days on which their experimental counterparts were exposed to stress. Behavioral testing started at p90, with a minimum delay of one week imposed between tests.

Behavioral procedures

Elevated Plus Maze

Anxiety-like behavior was evaluated using the EPM test (Pellow & File, 1986). The apparatus consists of two opposing open arms (50 x 10cm) perpendicular to two enclosed arms (50 x 10 x 50cm) that extend from a central platform (10 x 10cm), elevated 65 cm above the floor. Light levels were maintained at 14-16 lx on the open arms and 5-7 lx on the closed arms. At the start of the test, the rat was placed on the central platform facing a closed arm and allowed to explore the maze for five minutes. In between animals, the apparatus was cleaned with 5% ethanol solution and dried. Behavior was monitored using a ceiling-mounted video camera and analyzed with a computerized tracking system (Ethovision 9; Noldus IT, Netherlands). The time spent in the open and closed arms, and distance moved, were recorded. Two rats were excluded from the analysis as they fell from the maze before the end of the five minute test period (one each from the 'Low PPS' and 'High Control' groups).

Forced swimming test

Rats were submitted to a forced-swimming test (FST) to evaluate depression-like behavior (Porsolt et al., 1978). Animals were placed in a plastic beaker (25 cm diameter x 46 cm) containing 30 cm of water (25° C) for 15 minutes. A second session was performed 24h later for 5 minutes. Both sessions were recorded using a ceiling mounted video camera, and the time spent immobile (making only those movements necessary to keep the snout above the water), swimming or climbing was quantified manually with the aid of in-house software (Clicker; EPFL, Switzerland) by an experimenter blind to the experimental condition.

Resident-intruder test

Prior to the night of the resident-intruder (RI) test, experimental rats cohabited with a female partner for 10 days in order to encourage territoriality. The female was removed 30 minutes before the onset of the test, and replaced afterwards. The test was performed during the beginning of the dark cycle (between 1900 and 2200h). The resident was exposed in its home cage to a smaller (5-10% lighter), unfamiliar male intruder of the same strain for 30 minutes. Each intruder was used only once. Encounters were video-recorded and scored offline by an experimenter blind to the experimental group, assisted by Observer software (Noldus IT, Netherlands). The following parameters were quantified in terms of frequency and duration: offensive upright, lateral threat, keeping down, attack, biting, social investigation, non-social investigation and auto-grooming. The cumulative duration of the first four behaviors were summed to provide a measure of total offensive behavior. Latency to the first offensive event initiated by the resident was also recorded. To determine holistically the aggressiveness of each rat aggression *z* scores were calculated from raw scores of variables described above. Specifically, total offensive behavior, frequency of attacks, and latency to offend were taken into account. The *z* scores for

these variables were integrated to derive a single aggression score.

Rats are a highly social species and, as such, have developed stereotyped patterns of social interaction, such as intention signaling and submissive posturing, that allow for the resolution of territorial and hierarchical conflicts with minimal injury to both parties (Koolhaas et al., 2013). Here, forms of attack performed by the resident which fell outside of species-specific norms of interaction were noted and collated. Specifically, the targeting of bites to vulnerable body parts (i.e. the head, genitals or underbelly), absence of signaling of intent to attack, and persistence of an attack in the face of submission by the intruder were all considered to engender "abnormal" forms of aggression (Haller, 2013).

Brain energy metabolism

One week after testing in the forced swimming test animals were injected intraperitoneally with ^{14}C-2-deoxy-D-glucose (165µCi/kg; Hartmann Analytic, Germany) 45 min before being sacrificed via decapitation in order to evaluate brain glucose metabolism under basal conditions. Brains were removed and flash frozen in isopentane chilled to -45°C, and subsequently stored at −80 °C until further processing.

Coronal sections (20µm thick) were cut on a cryostat. One out of six sections within several pre-determined regions of interest were collected on Superfrost slides. The selected ROIs fulfilled the following criteria: i) subject to ongoing development during adolescence (Spear, 2000; Casey et al., 2008); ii) previously demonstrated to be affected by peripubertal stress (Marquez et al., 2013); iii) found to show altered activation in stress-related psychopathologies such as PTSD (New et al., 2009; Koch et al, 2016; Sripada et al., 2012; Wang et al., 2016). Prefrontal cortex, hippocampus, and amygdala all met these criteria. Collected slides were processed for autoradiography along with a calibrated ^{14}C-microscale on XAR-5 Kodak Biomax MR autoradiography film (Sigma-Aldrich, Switzerland) for 3 days. After developing the films, the slides were counterstained with 0.2% cresyl violet acetate (Sigma-Aldrich, Switzerland), dehydrated through increasing concentrations of ethanol, cleared in xylene, and coverslipped with DPX (Sigma, Switzerland) to provide a histological control.

Images from the 2-DG XAR films were obtained with a digital camera and aligned with the corresponding Nissl-stained images to allow for structure identification using MCID Core TM 7.0 software (MCID, UK). ^{14}C -2-deoxy-glucose uptake was measured by densitometric analysis of the XAR films. Briefly, the images were calibrated with ^{14}C standard curves, the regions of interest were delineated manually by an experimenter blind to the experimental group and the optical densities were obtained for these regions. The 2-DG uptake was normalized to that of the whole slice, which did not vary among the experimental groups, in order to control for any differences in film exposure. Data shown are averaged from three sections, including both hemispheres, per region of interest. Analyses of 2-DG expression in the various regions of interest were conducted by another researcher, also blind to the experimental condition.

Corticosterone measurement

Measurements of free corticosterone were obtained from all blood plasma samples, via use of an enzymatic immunoassay kit performed according to manufacturer's instructions (Enzo Life Sciences, Switzerland). Levels were calculated using a standard curve method.

Statistics

Data were analyzed using SPSS 17.0 (Chicago, USA). Results are presented as the mean ± SEM. A mixed 3-way repeated measures ANOVA was used to analyze measurements of corticosterone concentration obtained from plasma taken across the stress protocol, with selection line as the between-subjects factor and postnatal day and sample timepoint as the within-subjects factors. For within-subjects ANOVA, where sphericity assumptions were violated, the Greenhouse-Geisser correction was applied. All other variables were analyzed using 2-way ANOVA, with selection line and treatment as between-subjects factors. To permit this data transformation, shown in brackets, was performed on the following behavioral parameters to produce a normal distribution of data: offensive behavior % (arcsine); attack frequency and latency to offend (log10). Significant interactions were broken down by line and explored using independent samples t-tests, with treatment group as the between-subjects factor. Behavioral variables were correlated with basal brain activity using Pearson's correlation. Statistical significance level was set at $p<0.05$. A p-value was considered as tending toward significance when $0.05 \leq p \leq 0.1$.

Results

Constitutive differences in stress habituation are reflected by differential corticosterone response across repeated exposures to stress during the peripubertal period

Analysis of plasma corticosterone concentration from samples obtained at various timepoints across the peripubertal stress protocol revealed a significant interaction between selection line, postnatal day and sampling timepoint (3-way RM ANOVA: $F_{(2,82)} = 22.38$, $p<0.001$). When broken down by sampling timepoint, analysis revealed that corticosterone response to stress differed between lines across the duration of the protocol not only in the response sample (Fig 2A: 2-way RM ANOVA: $F_{(2,84)} = 17.61$, $p<0.001$) but also in the recovery sample (Fig 2B: 2-way RM ANOVA: $F_{(2, 82)} = 3.76$, $p<0.05$, $\varepsilon = 0.83$). As expected, and in line with previous findings (see chapter 3), low- and high-line rats did not differ in corticosterone response to stress upon first exposure (p28: $t_{(42)} = -1.58$, ns) but did differ when sampled at the offset of stress on the third (p30: $t_{(42)} = -9.31$, $p<0.001$) and final days of the protocol (p42: $t_{(42)} = -5.35$, $p<0.001$). Low-line rats demonstrated significantly lower corticosterone levels than high-line rats in response to both of these later stress exposures. Similar differences between lines were observed in the recovery plasma sample, though in this case low-line rats also had significantly lower corticosterone levels (i.e. quicker recovery) at this timepoint on the first day of stress exposure (p28: $t_{(41)} = -5.31$, $p<0.001$), as well as on the other days sampled (p30: $t_{(42)} = -3.78$, $p<0.01$; p42: $t_{(42)} = -4.11$, $p<0.001$).

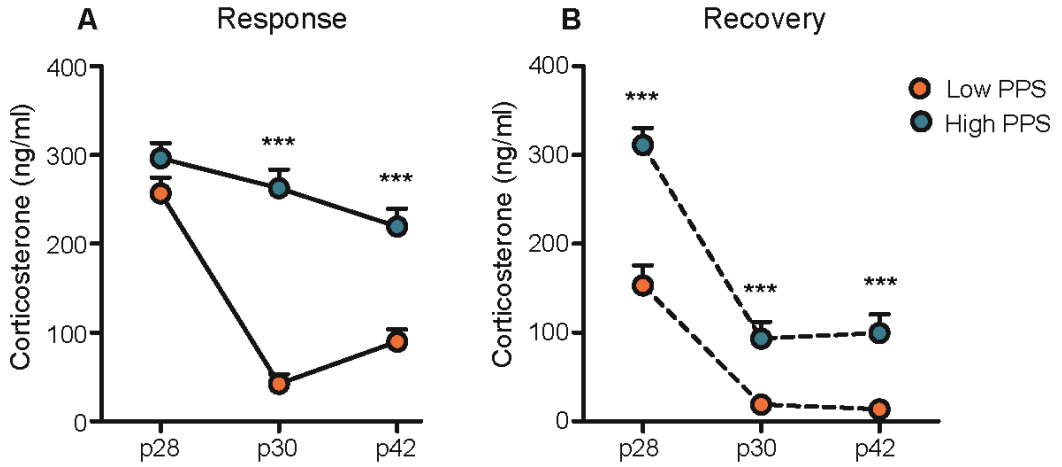


Figure 2 Corticosterone response to repeated peripuberty stress (PPS) differed in accordance with selection line. Low- and high-line rats did not differ in their response to a first exposure to stress. Thereafter, low-line rats showed marked habituation of the corticosterone response to following stressors which was attenuated in high-line rats (A). These differences were reflected by corticosterone concentration in a second plasma sample, taken 30 minutes after the offset of stress (B). In addition to strong habituation across stressors, low-line rats showed accelerated recovery of the HPA axis following the first stress session on postnatal (p) day 28 (Independent sample t-tests: *** = p<0.001; see text for further details).

Exposure to peripuberty stress gave rise to dissociable alterations in psychopathology-like behaviors between the differential stress habituation lines

Elevated plus maze

We first evaluated whether rats exposed to peripuberty stress demonstrated differential behavioral responses to the elevated plus maze when compared with their respective control groups. In chapter 3 of this thesis, we reported enhanced anxiety-like behavior in the high-line relative to the low-line in the absence of stress exposure, and that finding was recapitulated here. Specifically, irrespective of stress experience, high-line rats spent more time in the protected, closed arm of the maze than low-line rats (Figure 3A: m.e. of line, closed arm: $F_{(1,82)} = 11.83$, $p<0.001$). Accordingly, they also spent less time on the open arm (m.e. of line: $F_{(1,82)} = 9.48$, $p<0.01$) and less time in the centre square (m.e. of line, centre: $F_{(1,82)} = 3.96$, $p<0.01$) when compared to low-line rats. Exposure to peripuberty stress acted to enhance anxiety-like behavior, irrespective of line. Specifically stressed rats spent more time in the closed arm (m.e. of treatment: $F_{(1,82)} = 4.88$, $p<0.05$), and less time on the open arm of the maze (m.e. of treatment: $F_{(1,82)} = 4.52$, $p<0.01$). The interaction between line and peripuberty stress exposure did not reach statistical siginificance (line*treatment: $F_{(1,82)} = 2.71$, n.s.), preventing further examination of the relative prominence of differences between lines. In addition to influence on the time spent exploring certain zones of the maze, peripubertally stressed rats showed a reduction in distance travelled on the maze (m.e. of treatment: $F_{(1,82)} = 7.66$, $p<0.01$).

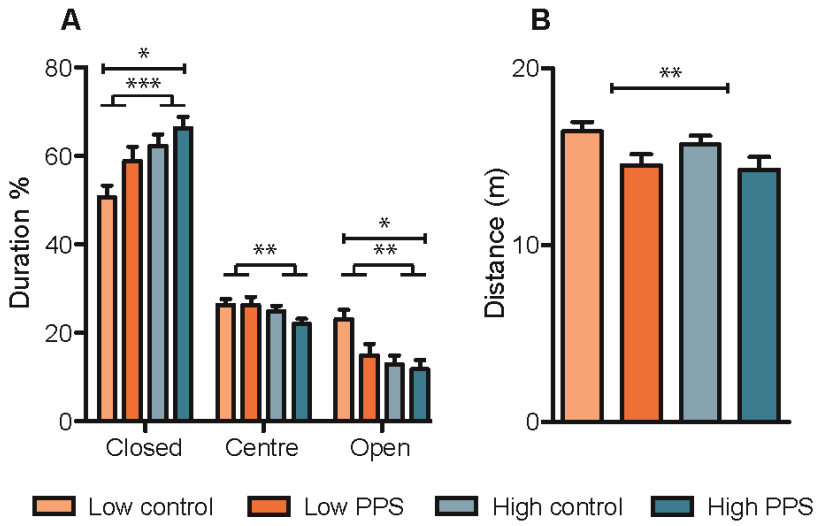


Figure 3 Anxiety-like behavior on the elevated plus maze. Low-line rats spent less time in the closed arm of the maze, and more time on the open arm when compared to high-line rats (A). Following exposure to peripuberty stress (PPS), rats spent more time in the closed arm and less time in the open arm of the maze. Additionally stressed rats showed a decrement in locomotion in terms of distance covered on the maze (B) in comparison to non-stressed controls. (2-way ANOVA: main effect of treatment is represented by the uppermost capped line, main effect line is represented by the lower line; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; see text for further details).

Forced-swimming test

To assess animals' depression-like behavior, we exposed a subset of the group (n=8/group) to a two-day forced-swimming procedure. In chapter 3 of this thesis we reported enhanced depression-like behavior in the high-line relative to the low-line rats in the absence of peripuberty stress exposure, and that finding is also recapitulated here. Specifically, irrespective of stress experience, high-line rats spent more time floating on the second exposure to the water than low-line rats (Fig 4B: m.e. of line: $F_{(1,28)} = 4.27$, $p<0.05$), a finding which the animal behavior literature would typically take to engender an increase in behavioral despair. No corresponding differences between lines were found in amount of time swimming or struggling (m.e. of line, swimming: $F_{(1,28)} = 1.60$, n.s.; m.e. of line, struggling: $F_{(1,28)} = 0.51$, n.s.). Lines did not differ in behavioral response to the first episode of forced swimming (Fig 4A). Moreover, exposure to peripuberty stress did not influence any aspect of behavioral response, neither on first exposure, nor on second exposure to forced swimming.

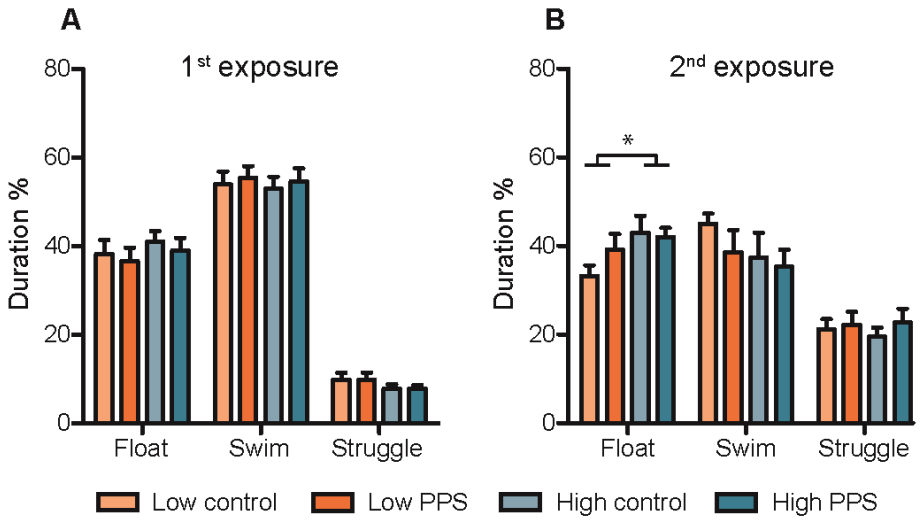


Figure 4 Depression-like behavior in the forced-swimming test. Neither selection line, nor prior exposure to peripuberty stress (PPS), influenced behavioral coping response to the first exposure to forced -swimming (A). When re-exposed to this stressor on the following day, compared with low-line rats, high-line rats spent significantly more time engaged in passive coping, as indexed by time spent floating (B). (2-way ANOVA: main effect of line; * = p<0.05; see text for further details).

Resident-intruder test

In a second subset of animals (n=12/group) we evaluated the influence of line and peripuberty stress on aggression in the resident-intruder test. In terms of total duration of the test spent engaged in offensive behavior, we found higher levels of aggression in high-line rats relative to low-line rats (Fig 5A: m.e. of line: $F_{(1,44)} = 8.90$, $p<0.01$). A similar effect was found in the relative frequency of attacks, whereby high-line rats attacked the intruder more times than low-line rats (Fig 5C: m.e. of line: $F_{(1,44)} = 4.98$, $p<0.05$). In terms of readiness to perform a first offensive action, we found a variable influence of selection line and stress exposure on behavior (Fig 5B: line*treatment: $F_{(1,44)} = 4.39$, $p<0.05$). Experience of peripuberty stress did not alter the already short latency of high-line rats to initiate hostilities (t-test: $t_{(22)} = -1.00$, n.s.), whereas it tended to shorten the time taken for low-line rats to do so (t-test: $t_{(22)} = 1.84$, $p<0.1$).

In order to take a more holistic view of the aggressiveness of each rat, we derived a composite z score from the variables outlined above. This approach has previously proved useful in other studies aimed at the differentiation of individual differences in the response to traumatic stress exposure (Anacker et al., 2016). In accordance with our previous findings (see chapter 3), analysis of the aggression score showed that, irrespective of early life experience, high-line rats were more aggressive than low-line rats (Fig 5D: m.e. of line: $F_{(1,44)} = 9.13$, $p<0.01$). Interestingly, exposure to peripuberty stress had differential effects on the aggressiveness of the different selection lines (line*treatment: $F_{(1,44)} = 4.10$, $p<0.05$). Peripuberty stress

did not alter the already pronounced levels of aggression in high-line rats (t-test: $t_{(22)} = 0.14$, n.s.) but significantly enhanced the aggressiveness of the typically non-aggressive low-line rats (t-test: $t_{(22)} = -2.57$, $p<0.05$).

Additionally, we assessed the propensity of animals from each experimental group to perform abnormal forms of aggression including: targeting of bites to vulnerable body parts, failure to signal intent to attack, and continued attack despite clear signaling of submission by the intruder. Taking into account only rats that performed at least one attack (Low control: n=7; Low PPS: n=9; High control: n=10; High PPS: n=10), the percentage of rats from each group performing aberrant forms of attack were as follows: Low control, 14%; Low PPS, 56%; High control, 10%; High PPS, 0%. It thus appeared that low-line rats exposed to peripuberty stress were much more likely to engage in this atypical form of behavior than animals from any other experimental group.

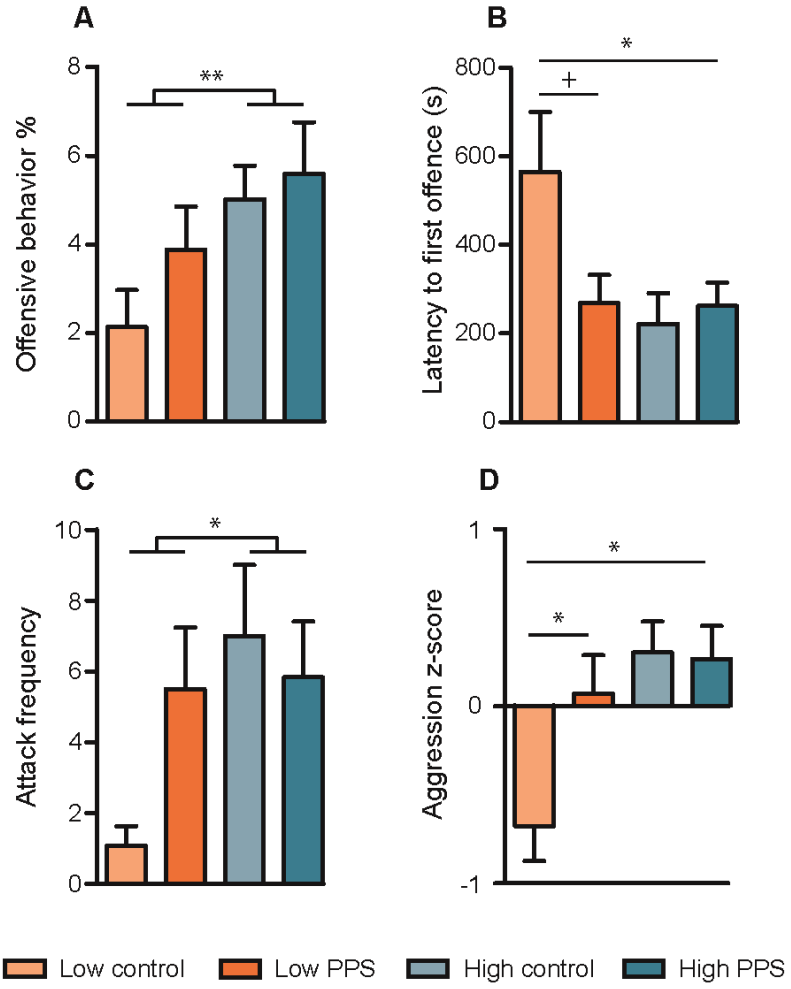


Figure 5 Aggression in the resident-intruder test. Compared to low-line rats, high-line rats spent a greater duration of the test engaged in offensive behaviors (A) and attacked more frequently (C). There was an interaction between selection line and experience of peripuberty stress (PPS) in terms of readiness to engage in offensive behavior (C), with low-line PPS rats tending to be quicker to attack than low-line controls. When these three measures were combined (D), an interaction between selection line and treatment revealed that PPS exposure increased the overall aggressiveness of low-line rats, whilst not altering relative aggressiveness of high-line rats. (2-way ANOVA: interaction is represented by a wide, uncapped line; main effect of line is represented by a line with feet; post hoc t-tests by a narrow, uncapped line; + = $p<0.1$; * = $p<0.05$; ** = $p<0.01$; see text for further details).

Selection line and peripuberty stress exposure are associated with differences in brain metabolism under basal conditions

To interrogate potential neurobiological correlates of variation in psychopathology-like behaviors relating to both constitutive stress habituation and peripuberty stress exposure, basal brain energy metabolism was studied via ^{14}C 2-deoxy-glucose (2-DG) autoradiography (Figure 6). We focused our analyses on several predefined regions of interest, specifically: prefrontal cortex, dorsal hippocampus and amygdala.

In the ventral subdivision of orbitofrontal cortex analyses revealed both an effect of selection line and of stress experience in brain activity under basal conditions. Specifically, in comparison to low-line rats, high-line rats showed greater uptake of 2-DG (Fig 6A: m.e. of line: $F_{(1,28)} = 5.36$, $p<0.05$). Additionally, groups exposed to stress during peripuberty showed increased uptake of 2-DG relative to control groups (m.e. of treatment: $F_{(1,28)} = 4.86$, $p<0.05$). A similar pattern of findings was observed in the medial subdivision of orbitofrontal cortex (m.e. of line: $F_{(1,28)} = 2.93$, $p<0.1$; m.e. of treatment: $F_{(1,28)} = 5.76$, $p<0.05$) but not in the lateral subdivision. Caudally, in medial prefrontal subregions, the same pattern of differential 2-DG uptake was repeated in the prelimbic cortex (Fig 6B: m.e. of line: $F_{(1,28)} = 6.64$, $p<0.05$; m.e. of treatment: $F_{(1,28)} = 6.83$, $p<0.05$). No additional effects were found either dorsally, in anterior cingulate cortex, or ventrally, in infralimbic cortex.

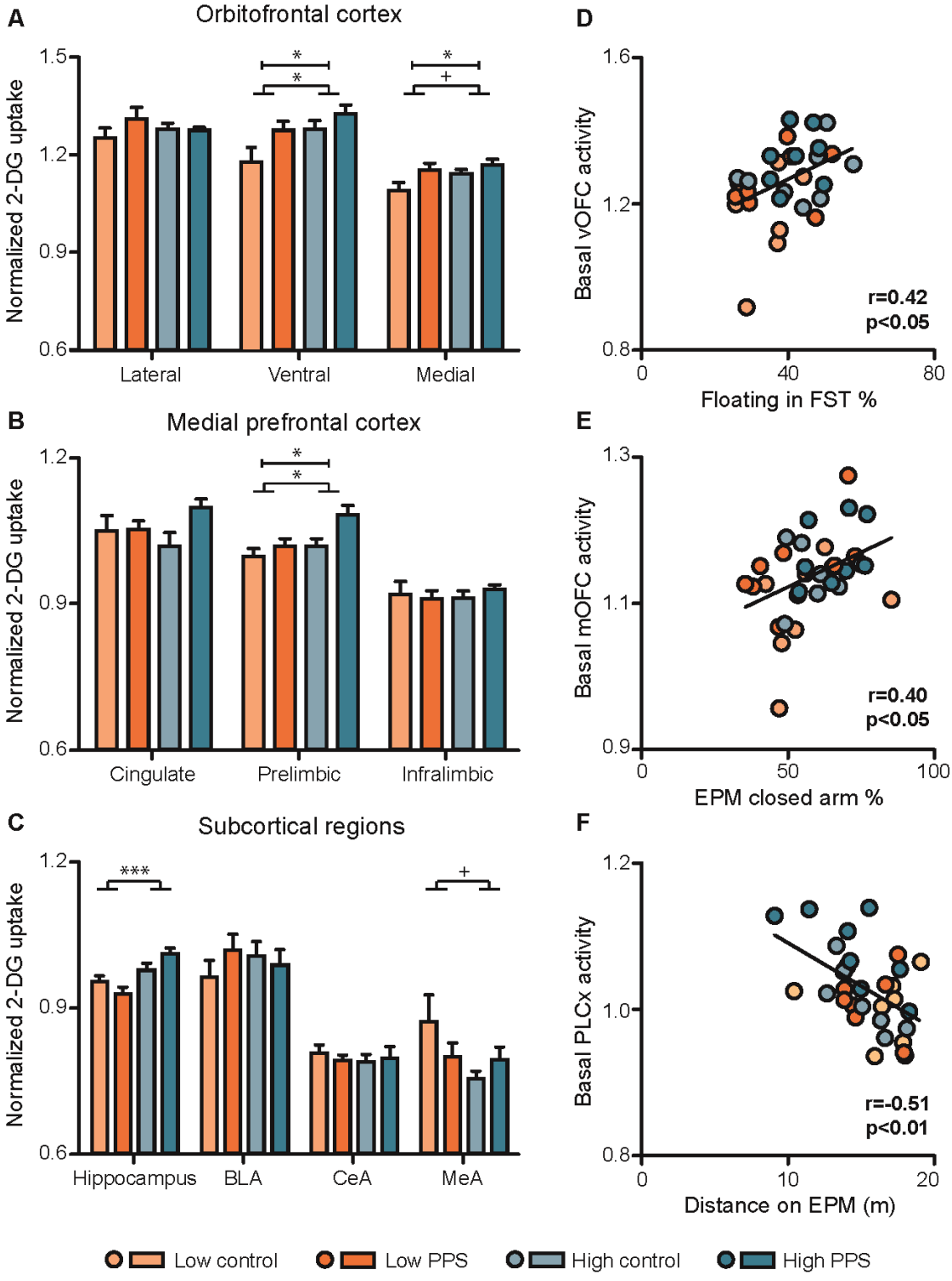
In subcortical regions (Fig 6C), analyses revealed additional variation in basal energy metabolism according to selection line in dorsal hippocampus. Specifically, relative to low-line rats, high-line rats had enhanced 2-DG uptake in dorsal hippocampus (m.e. of line: $F_{(1,27)} = 15.82$, $p<0.001$). Interestingly, there was evidence indicating differential modulation of basal metabolism in dorsal hippocampus in the lines following experience of peripuberty stress (line*treatment: $F_{(1,27)} = 5.46$, $p<0.05$). However, further analyses revealed the differences between control and stress groups to be non-significant (t-test: low-line: $t_{(14)} = 1.52$, n.s.; high-line: $t_{(13)} = -1.76$, n.s). Somewhat surprisingly, no differences were evident in basolateral amygdala or in central nucleus of the amygdala. However, a non-significant trend towards reduced 2-DG uptake in medial amygdala of high-line rats relative to low-line rats was found (m.e. of line: $F_{(1,27)} =$

5.36, p<0.05).

Basal activity within stress-sensitive brain regions was associated with prior psychopathology-like behavioral tendencies.

In brain regions where both constitutive differences in habituation and stress experience were found to influence resting activity, as indexed by 2-DG uptake, we performed correlations with the primary outcome measures derived from the elevated plus maze and forced-swimming tests. We found floating behavior upon second exposure to forced-swimming, typically thought to reflect behavioral despair in the face of an uncontrollable challenge, correlated positively with basal activity in the ventral subdivision of the orbitofrontal cortex (Fig 6D: r=0.42, p<0.05). Similarly, time spent in the closed arm of the elevated plus maze, often conceptualized as anxiety-like behavior, correlated positively with basal activity in the medial subdivision of the orbitofrontal cortex (Fig 6E: r=0.40, p<0.05). In contrast, basal activation of the prelimbic portion of the medial prefrontal cortex was in strong negative correlation with distance travelled on the elevated plus maze (Fig 6F: r=-0.51, p<0.01). We note that these findings are not corrected for multiple comparisons, and that results may not retain statistical significance if corrections were performed.

Figure 6 Quantification of brain activity under basal conditions, as indexed by uptake of 2-deoxyglucose (2-DG), in stress-sensitive limbic brain regions. In ventral and medial subdivisions of the orbitofrontal cortex (A), relative to low-line rats, high-line rats had enhanced 2-DG uptake. Moreover, PPS exposed rats showed enhanced 2-DG uptake relative to controls in those same regions. This pattern was mirrored in the prelimbic division of the medial prefrontal cortex (B) but not in other subregions. In subcortical brain areas (C), high-line rats again had higher 2-DG uptake than low-line rats. There was a tendency toward the opposite pattern in medial amygdala. Levels of brain activity under basal conditions were associated with several key indices of behavioral performance in tests of psychopathology-like behavior. (2-way ANOVA: main effect of treatment is represented by the uppermost capped line, main effect line is represented by the lower line; + = p<0.1; * = p<0.05; *** = p<0.001; see text for further details). Abbreviations: FST = forced-swimming test; EPM = elevated plus maze; vOFC = ventral orbitofrontal cortex; mOFC = medial orbitofrontal cortex; PLCx = prelimbic cortex; BLA = basolateral amygdala; CeA = central nucleus of the amygdala; MeA = medial nucleus of the amygdala.


A
Orbitofrontal cortex
Normalized 2-DG uptake
1.5
1.2
0.9
0.6
*
*
*
+
Lateral
Ventral
Medial
B
Medial prefrontal cortex
Normalized 2-DG uptake
1.2
0.9
0.6
*
*
Cingulate
Prelimbic
Infralimbic
C
Subcortical regions
Normalized 2-DG uptake
1.2
0.9
0.6
***
+
Hippocampus
BLA
CeA
MeA
D
Basal vOFC activity
1.6
1.2
0.8
r=0.42
p<0.05
0
40
80
Floating in FST %
E
Basal mOFC activity
1.3
1.1
0.9
r=0.40
p<0.05
0
50
100
EPM closed arm %
F
Basal PLCx activity
1.2
1.0
0.8
r=-0.51
p<0.01
0
10
20
Distance on EPM (m)
Low control
Low PPS
High control
High PPS

Discussion

Here we explored the interaction of two key risk factors for psychiatric disorders, constitutive stress sensitivity and early life stress exposure, in the development and expression of psychopathology-like behaviors in adult rats. Confirming previous findings, both risk factors were found to influence the expression of psychopathology-like behavior. Specifically, in the absence of early stress exposure, high-line rats (i.e. constitutive non-habituaters) demonstrated increased anxiety-like, depression-like and aggressive behaviors relative to low-line rats (see chapter 3). Moreover, in accordance with earlier studies, exposure to peripuberty stress enhanced anxiety-like and aggressive behavior in its own right (Marquez et al., 2013; Tzanoulinou et al., 2014a; Tzanoulinou et al., 2014b; Cordero et al., 2016).

Surprisingly, we did not observe an unequivocal synergy between the two factors, as might have been expected from the findings of studies which employed a similar approach (Clinton et al., 2014; McIlwrick et al., 2016; Stedenfeld et al., 2011). In the case of aggression, and to a lesser extent anxiety-like behavior, exposure to peripuberty stress did enhance psychopathology-like behavior. However, for the most part, these stress-induced alterations were expressed by the stress-habituating low-line, in accordance with similar studies (Cohen et al., 2006). Whether this indicates that the high-line were truly insensitive to peripuberty stress cannot be clarified without further investigation. However, it appears that a ceiling may have been reached in the high-line in terms of aggressive and anxiety-like behavior, such that it could not be further enhanced by peripuberty stress exposure. Interestingly, a similar lack of enhancement in psychopathology-like outcomes was found following application of adolescent stress to the high-anxious, stress-sensitive, “low-responder” rat line (Rana et al., 2016), adding weight to this speculation.

Observation of the corticosterone response to stress at several timepoints across the peripuberty stress protocol indicated that the rats used in this experiment responded in accordance with their selection line. From a similar initial corticosterone response, low-line rats showed marked habituation of the response over stress sessions, an effect strongly attenuated in high-line rats, in line with previous findings (see chapter 3). In addition, we found that low-line rats demonstrated robust negative feedback inhibition of the corticosterone response, as indexed by the recovery sample taken 30 minutes after the cessation of stress, an effect particularly prominent following the first stress exposure. Enhanced negative feedback of the HPA axis, via increased glucocorticoid sensitivity, is one of the key neuroendocrine alterations found in individuals with PTSD (Yehuda et al., 1993; Yehuda et al., 1995). Interestingly, in addition to trauma specific symptoms, PTSD sufferers frequently present with other comorbidities including affective perturbations and enhanced anger (Contractor et al., 2015; Durham et al., 2016). Previous studies (see chapter 3) found a lack of difference in the expression of the gene coding glucocorticoid receptors, *Nr3c1*, in the PVN and pituitary gland of the low- and high-line rats, indicating that this factor is unlikely to be responsible for the differential negative feedback. However, reduced expression of *Fkbp5* was found in the PVN of low-line rats. FKBP5 regulates the sensitivity of the glucocorticoid receptor to its ligand by

altering its binding affinity (Binder, 2009), and a reduction in its expression in the low-line could conceivably lead to an enhancement of negative feedback efficacy in the group relative to others. Indeed, the impact of early life adversity on development of psychopathology has been found to be modulated by variation in the expression of FKBP5 in a number of studies (Bevilacqua et al., 2012; Binder et al., 2008; Bryushkova et al., 2016; Klengel et al., 2013; Kohrt et al., 2015).

Low stress reactivity, potentially occurring as a result of high glucocorticoid sensitivity, has been described in psychopathological disorders characterized by pathological aggression including conduct disorder, anti-social personality disorder, and psychopathy (O'Leary et al., 2007; O'Leary et al., 2010; Loney et al., 2005; McBurnett et al., 2000; Raine, 1996). Moreover, early life stress has also been implicated as a risk factor for the development of pathological aggression (Caspi et al., 2002; Weder et al., 2009; Beach et al., 2010; Viding & McCrory, 2012; Fanning et al., 2014; Lee et al., 2014; Widom & Maxfield, 1996). Our finding here that peripuberty stress selectively enhances aggression in the low-line, especially in light of increased incidence of abnormal forms of aggression in this group, suggests that the low-line may provide a useful animal model within which to explore the mechanisms underlying development of pathological aggression following adverse early life experience.

We additionally sought to investigate the interaction between constitutive stress-sensitivity and peripuberty stress experience on the activity of brain regions regularly found to show altered function in psychopathological conditions (New et al., 2009; Koch et al., 2016; Sripada et al., 2012; Wang et al., 2016). We found enhanced basal activity in several subregions of the prefrontal cortex in high-line rats relative to low-line. Moreover, peripuberty stress experience was found to increase activity in the same regions. Similar to the behavioral measures described previously, no synergy between the two factors was found in any brain region. In relating brain activity measures to behavioral findings, we found that basal activity in ventral and medial subdivisions of orbitofrontal cortex were positively associated with floating behavior in the FST and time spent in the closed arms of the EPM, respectively. We additionally found activity in the prelimbic cortex to be negatively associated with locomotion on the EPM. Taken together, these findings suggest that propensity toward higher activity in the prefrontal cortex at rest is associated with the strength of behavioral inhibition under test conditions.

A limitation of the study relates to the quality of the information provided by the 2-deoxyglucose method itself. The method provides an index of the metabolic demand for glucose within the cells of each region but does not provide any information regarding the nature of those cells, nor the signals they propagate (Ackermann et al., 1984; Nudo & Masterton, 1986). Previous studies that employed the same peripuberty stress protocol reported reductions of GAD protein, an enzyme involved in the activity-dependent synthesis of GABA, in the same regions that we report enhanced 2-deoxyglucose uptake, namely: ventral and medial orbitofrontal cortex; and medial prefrontal cortex (Tzanoulinou et al., 2016). This leads us to speculate that the alterations in 2-DG uptake found here under resting conditions may reflect enhanced excitatory drive via reduced inhibition. In agreement with this idea, increases in anxiety-like behavior and

alterations in affective and social motivation have been found under conditions of tonically-increased activation of prefrontal cortex (Ferenczi et al., 2016; Yizhar et al., 2011).

In summary, in contrast to expectations, we report a general lack of synergism between the two psychopathology-related risk factors studied here, constitutive stress sensitivity and early life stress exposure. Rats with attenuated ability to habituate their glucocorticoid response to repeated stressors had enhanced levels of psychopathology-like behaviors which were unaltered by experience of peripuberty stress. In contrast, rats with constitutively strong habituation of HPA axis responses to stress appeared more vulnerable to the effects of peripuberty stress, particularly with regard to aggression. The behavioral findings reported here, considered in conjunction with neuroendocrine alterations, implicate the stress-habituating low-line as a potentially useful model with which to further explore mechanisms related to the development of disorders of pathological aggression.

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Author contributions

S.W.: performed all experiments, organised and performed the stress protocol, scored videos, analysed data, wrote the initial version of the manuscript

C.S.: provided the concept, ideas and feedback for the experiments, corrected the manuscript

Supplementary information

Selective breeding procedure

Wistar Han rats were obtained from a commercial breeder (Charles River, France: 30 male & 30 female; parental generation; PG) and bred in our animal facility. The entire offspring of these pairings (F0) was subject to a 'stress adaptation test' (SAT). The SAT is a truncated version of the peripubertal stress protocol developed in our laboratory (Toledo-Rodriguez & Sandi, 2011) which, though clearly stressful, has been shown to be insufficient in begetting behavioral alterations associated with the longer protocol (Toledo-Rodriguez & Sandi, 2007; Tzanoulinou et al., 2014). Tail blood samples were taken at two timepoints on two separate days of the protocol; immediately after, and 30 minutes after, cessation of exposure to the stressors. Three breeding lines were established according to the outcome of the SAT. Rats with extremely low or extremely high secretion of corticosterone on the final day of the SAT, i.e. animals expressing habituation or non-habituation of the HPA axis response to repeated stress, were selected for the 'low' and the 'high' breeding line, respectively. A third breeding line, 'inter', was established consisting of animals with intermediate corticosterone values in the SAT.

Ten males and ten females from F0 were selected as founder pairs for each breeding line. Their offspring (F1) and the majority of animals from each subsequent generation were also tested in the SAT and selected for breeding based on their corticosterone response on post-natal day (p) 30. Selection was strictly within line, i.e. an animal from the low-line could only ever be selected to be a breeder within the low-line. To minimize effects of genetic drift, animals were mated within a system that strictly excluded sibling matings. Moreover, in order to balance the potential contribution of each litter to the next generation, litter size was reduced to a maximum of 12 pups at p2. Care was taken to ensure as much variability in pairings as possible; for example, if two animals from the same litter went forward to breed the next generation then they were not paired with animals coming from a single, alternate litter.

Stress Adaptation Test (SAT)

The protocol was based on multiple exposures to fear-induction procedures. Measures of acute stress reactivity, stress recovery (within session), and stress adaptation (across sessions) could be obtained whilst minimizing the stress exposure required to do so. Following exposure to an open field (50 x 50 x 30cm) for five minutes on p28, the stress protocol consisted of the presentation of two different stressors, each one lasting 25 minutes. These were either; exposure to the synthetic fox odor trimethylthiazoline (TMT) or to an elevated platform (EP). TMT exposure was administered in a plastic box (38 x 27.5 x 31 cm) via a scent-charged cloth. The box was placed under a bright light (210–250 lx). The elevated platform (12 x 12cm, elevated 95cm from the ground) was also under direct bright light (470–500 lx). Following each stress session, animals were returned to neutral cages for 15 minutes. A transparent Plexiglas wall perforated with holes separated pairs of cagemates during this time. Following the holding period,

animals were returned to their home cage. The stressors were applied during juvenility, on three consecutive days across p28–p30, during the light phase and following an unpredictable schedule. Tail blood samples were taken on p28 and p30, once at the offset of stress and again 30 minutes later.

General Discussion

General discussion and future perspectives

Experience of adversity early in life may have lifelong consequences for the individual. Understanding the mechanisms that lead from adversity to psychopathology is therefore important but so too is an appreciation of how stress effects are translated differently from individual to individual. Gaining such an understanding would represent a clear benefit in the development of intervention strategies that could be applied, appropriately and with greater chance of being successful, to an increased number of individuals. We hope that the work presented in this thesis will add to the already growing number of voices calling for explicit consideration of this additional complexity in research using animal models.

Here, using rats as a model system, we aimed to address the role of individual differences in hypothalamic -pituitary-adrenal (HPA) axis responsiveness to repeated stress in the programming of differential neurobehavioral outcomes both in response to peripuberty stress, and in its absence. These pages summarize our findings, as well as discussing outstanding questions and the future research directions that might arise from those questions.

Peripuberty stress gives rise to an aggressive profile, in association with neuroanatomical alterations in stress-sensitive limbic regions, but only in some individuals

Peripubertal stress has been found to lead to alterations in several forms of psychopathology-like behavior in adulthood including increased anxiety- and depression-like behavior, increased aggression and reduced sociability (Cordero et al. 2012; Márquez et al., 2013; Poirier et al., 2014; Tzanoulinou et al., 2014a; Tzanoulinou et al., 2014b; Veenit et al., 2014; Cordero et al., 2016). In addition, behaviorally-consequent shifts in activation in brain regions important in socio-affective functions have been found using this model (Marquez et al., 2013). Intriguingly, there have been hints in some of these findings that, even though at the group level peripubertally stressed rats differed from control rats, there may be individual differences in response to peripuberty stress, particularly with regard to aggressive behavior (Tzanoulinou et al., 2014b; Cordero et al., 2016).

Thus, using a behavioral profiling approach, in combination with *ex vivo* structural neuroimaging, we sought to examine explicitly the existence of individual differences in neurodevelopmental trajectory following exposure to peripuberty stress. In so doing we found that exposure to peripuberty stress led to reductions in mean diffusivity in infralimbic cortex, amygdala, hippocampus, and subiculum *only* in those individuals that developed an aggressive phenotype. Peripubertally stressed rats that did not display aggressive behavior were affected in terms of other non-aggression related behaviors, specifically they demonstrated increased anxiety-like behavior and reduced sociability. This phenotype was not associated with any observable neuroanatomical alterations in the brain regions examined, however. If considered at the group level, the behavioral alterations found here in terms of increased aggression and anxiety, and reduced sociability, were well aligned to previous findings from our laboratory (Cordero et al., 2012; Marquez et al., 2013; Tzanoulinou et al., 2014b). However, the use of the profiling approach allowed us to

show critical individual differences in the long-term response to peripubertal stress in terms of both behavior, and associated neuroanatomy.

Though we found alterations in brain tissue microstructure in the majority of regions we examined, we did not find any differences in regional volume. This is in contrast to studies in humans affected by aggression -related psychopathologies, which have demonstrated reductions in the volume of prefrontal cortex (Raine et al., 2000; Sala et al., 2011), hippocampus (Barkataki et al., 2006; Coccaro et al., 2015; Dolan et al., 2002; Morandotti et al., 2013; Sala et al., 2011; Zetzsche et al., 2007) and amygdala (Coccaro et al., 2015) relative to controls. Findings in rats regarding the effects of stress on neuronal morphology in the abovementioned structures have indicated heterogeneity in structural alterations between subregions within the same region (amygdala: Padival et al., 2015; hippocampus: Pinto et al., 2015). This could potentially account for the lack of volume differences found within these regions in the present study, since the regions were only considered as a whole. Moreover, rats exposed to chronic stress leading to alterations in neuronal morphology in prefrontal cortex, hippocampus, and amygdala did not show concomitant changes in regional volume, indicating that changes in tissue microstructure are not always reflected in macrostructural properties (Henckens et al., 2015).

A critical question that we were unable to address here was whether differences in neurodevelopment associated with aggression were indeed caused by peripubertal stress or whether the individuals who demonstrated aggressive phenotypes were developing along a different trajectory irrespective of stress exposure. A study of mice, in which MRI scans were performed before and after exposure to chronic social defeat stress, indicated that pre-existing differences in hippocampal structure, as well as magnitude of stress-induced volume change, predicted susceptibility to the behavioral effects of the stress (Tse et al., 2014). In order to draw stronger conclusions regarding the causal implication of peripuberty stress in aggression-associated alterations in brain development, a similar, longitudinal study ought to be performed.

Individual differences in responsiveness to repeated stress are implicated in different developmental trajectories, even in the absence of adverse experiences early in life

Converging lines of evidence have suggested that impairment in adaptation of HPA axis activity in the face of repeated exposures to stress may increase the propensity to accumulate allostatic load, thereby leading to an increased vulnerability to develop psychopathology (de Kloet et al, 2005; Flak et al., 2012; Kirschbaum et al., 1995; Kudielka et al., 2006; McEwen, 1998; Pruessner et al., 1997). Though this concept is well-accepted, the complexity of performing such experiments, especially using human participants, has meant that it is not particularly well-supported experimentally. In the study reported in chapter 2, we therefore additionally asked whether individual differences in glucocorticoid responsivity to stress during peripuberty might have been associated with the development of an aggressive phenotype, and indeed we found that to be the case. Specifically, rats showing impaired initial adaptation of corticosterone

response across stressors were those found to be more aggressive at adulthood. Whether impairment in habituation to corticosterone responses to peripuberty stress is causally implicated in the development of structural and behavioral phenotypes could be addressed by studies directly manipulating capacity for corticosterone release on the latter days of the stress protocol. Artificially blocking the synthesis of corticosterone with metyrapone, or imposing an artificial clamp on HPA axis reactivity with dexamethasone, could represent useful strategies in this aim.

It is likely that individual differences in corticosterone habituation across peripuberty stress reflect constitutive differences between rats. Indeed, in studies of HPA axis habituation to repeated stress in humans, roughly 35% of individuals presented a reduced propensity to habituate (Kirschbaum et al., 1995; Wüst et al., 2005), and, via twin studies, this quality was found to be highly heritable (Federenko et al., 2004). In order to further examine the specific influence of constitutive differences in HPA axis responsiveness upon vulnerability to develop psychopathology, we were obliged to first generate a model. We have reported here the development of two selectively bred rat lines that came to show ready and impaired habituation of the corticosterone response to repeated stress exposure (named the "low-" and "high-line", respectively), as well as a control line intermediate for this trait. In so doing we found that the response to selection for propensity to habituate was strong, specific, and equally evident in both sexes, indicative of a firm genetic basis for this trait. In rats drawn from the F4 and F6 generations of these lines, in the absence of any stress exposure, constitutive differences in stress habituation alone gave rise to phenotypic variation in psychopathology-like behaviors, specifically anxiety-like, depression-like, and aggressive behavior. High-line rats showed higher levels of psychopathology-like behavior across all behaviors measured.

In the selected lines, constitutive differences in stress habituation were observed alongside a distinct neuroendocrine phenotype. The high-line rats displayed elevated corticosterone reactivity to restraint stress but parity in measures of basal HPA axis activity, measured at diurnal peak and trough. Considered together, the aggressive behavior and neuroendocrine phenotypes observed were in contrast to those reported from the differential stress reactivity mouse lines developed by Touma and colleagues (2008), the only other rodent model of genetic selection for an HPA axis activity trait. In that case, the line selected for low corticosterone reactivity to stress was found to be the more aggressive. The behavioral differences found in Touma's mouse lines were observed in the context of a highly different, broad effect neuroendocrine phenotype to that observed in the rat lines described in this thesis, with comparative differences most notable in terms of HPA axis rhythmicity and negative feedback capacity (Heinzmann et al., 2014; Touma et al., 2008). This contrast may be taken as an indication that habituation to stress is regulated such that there is but limited overlap with the regulation of general stress reactivity, which is in line with the generally adaptive nature of stress habituation (Herman, 2013).

In addressing potential molecular-genetic correlates of phenotypic differences between the lines we found variation in gene expression within several nodes of the HPA axis. The most notable findings were

that high-line rats had increased expression of *Avpr1b* in pituitary and *Fkbp5* in PVN. Given the role of the products of these genes in activation, and negative feedback regulation of the HPA axis, respectively, differences in both may partially explain the heightened corticosterone responsiveness to acute stress that was observed. We additionally found high-line rats to have higher expression of inhibition-related gene, *Gad67,* in the central nucleus of the amygdala (CeA). Inhibitory output from the CeA is thought to have a disinhibitory effect on HPA axis activity via its actions upon the limbic system-HPA axis relay, the bed nucleus of the stria terminalis (Johnson et al., 2016; Ulrich-Lai & Herman, 2009). Enhanced CeA inhibitory drive in the high-line could therefore also be associated with their high HPA axis responsiveness to stress. Interestingly, no significant differences between the lines in *Crh* expression, nor in the genes encoding its receptors, *Crhr1* and *Crhr2,* were found. This finding was surprising since CRH release is considered to be at the apex of HPA axis activation (Ulrich-Lai & Herman, 2009), and alterations in this peptide and its receptors have been reported alongside psychopathology-like behavior in several model systems (Coplan et al., 1996; Holsboer, 2001; Labermaier et al., 2014; Lu et al., 2008; Reul & Holsboer, 2002; Veenit et al., 2014). There was also a lack of evidence for differential regulation of genes involved in adrenal sensitivity to ACTH and synthesis of glucocorticoids, and thus it would appear that differences in HPA axis activity between the lines may be centrally mediated (Ulrich-Lai & Herman, 2009). This raises an interesting possibility since it is generally considered that it is limbic brain regions, and not the HPA axis itself, that mediate habituation in the face of repeated stress (Herman, 2013).

An important next step would be to confirm whether variations in the expression of genes we found translate to similarly altered levels of the respective proteins. It would then be necessary to establish whether variation in the level of a particular protein might be causally implicated in the neuroendocrine and behavioral phenotypes reported herein. Given the frequent association of *Fkbp5* and *Avpr1b* activity in modulating affective, aggressive, and endocrine phenotypes in both human and animal models (Binder et al., 2004; Dempster et al., 2007; Holz et al., 2015; Luppino et al., 2014; Minelli et al., 2013; Pagani et al., 2015; Touma et al., 2011; van West et al., 2004; Wersinger et al., 2008), these two genes represent promising candidates. In addition, gaining an appreciation of the neural basis of differential neuroendocrine and behavioral phenotypes observed between the lines could lead to important insights, as well as pointing toward particular regions within which to focus further investigations. Studies probing the neural basis of HPA axis habituation to stress have suggested that the proper functioning of medial prefrontal cortex, basolateral amygdala, and the posterior part of the paraventricular thalamus, is key in allowing habituation to repeated stress (Bhatnagar et al., 2002; Grissom & Bhatnagar, 2011; Jaferi & Bhatnagar, 2006; Jaferi et al., 2003; Weinberg et al., 2010). Moreover, evidence suggests that these regions are functionally interconnected (Li & Kirouac, 2012; Vertes et al., 2015) and all are considered to additionally play a role in socio-affective functioning (Blair, 2010; Coccaro et al., 2007; Davidson, 2002; Koenigs & Grafman, 2009; Price & Drevets, 2012).

It is important to note that all experiments using the differential stress habituation lines were carried out

in relatively young rats, between three and five months of age. Though we did not observe differences between the lines in terms of the diurnal rhythmicity of HPA axis function or general health, this does not mean that such differences would not express themselves over time. Given the well-documented role of HPA axis function in determining physical health across the lifespan (de Kloet et al., 2005; Lupien et al., 2009; McEwen, 2007), it would be important to determine whether having a constitution potentially causing differential exposure to glucocorticoids over the lifespan leads to altered trajectories of aging.

Constitutive differences in responsiveness to repeated stress hold differential risks for the development of psychopathology following peripuberty stress

As a final step we combined both approaches to ask whether peripuberty stress would have the same impact on individuals differing in their propensity to habituate to that stress. In line with previous findings from this laboratory, we found peripuberty stress to increase the expression of anxiety-like behavior and aggression (Marquez et al., 2013; Tzanoulinou et al., 2014a; Tzanoulinou et al., 2014b; Cordero et al., 2016). However, vulnerability to the programming effects of stress were differentially expressed between low- and high-line rats. Specifically, increases in anxiety-like and aggressive behavior were shown primarily by the stress-habituating low-line. The high-anxious, high-aggressive phenotype of the high-line rats was not further altered. These findings are in accordance with similar studies (Cohen et al., 2007; Rana et al., 2016). Our findings, and those of Rana and colleagues (2016), would appear to suggest that there may be a limit upon how much influence constitutive HPA axis responsivity can have over behavioral outcomes, at least in instances when stress is applied during adolescence. Future studies could focus on assessing the impact of stress applied at other points in development on the neurobehavioral trajectory of the lines. In general, findings from other laboratories suggest that stress exposure in early postnatal life, or in adulthood, may have increased impact in selectively bred lines (Clinton et al., 2014; McIlwrick et al., 2016). Should it be found that the lines were more sensitive to stress outside of the peri-adolescent period, this would be an interesting finding in its own right. This is because such a finding would suggest that there is some unique factor at play during adolescence that somehow limits the impact of exposure to high levels of glucocorticoids.

We additionally sought to investigate the interaction between constitutive stress-sensitivity and peripuberty stress experience on the activity of brain regions often found to show altered function in psychopathological conditions (Koch et al., 2016; New et al., 2009; Sripada et al., 2012; Wang et al., 2016). We found enhanced basal activity in several subregions of the prefrontal cortex in high-line rats relative to low-line. Moreover, peripuberty stress experience was found to increase activity in the same regions. Similar to our behavioral findings, no synergistic effect was found between the two factors in any brain region studied. In associating brain activity measures to behavioral findings, we found a propensity toward higher activity in the prefrontal cortex at rest to be associated with the strength of behavioral inhibition under challenge. Taken into consideration with findings from chapter 1, and the abovementioned importance of prefrontal cortex in stress habituation, these findings implicate prefrontal cortex as a region

of particular interest in the interaction between HPA axis responsiveness and the development of psychopathology-like behavioral alterations, in common with a growing number of studies (Kim & Haller, 2007; Kumar et al., 2014; Larrieu et al., 2014; McCreary et al., 2016; McKlveen et al., 2013; Tzanoulinou et al., 2016; Yuen et al., 2012).

Evidently, there is some level of incongruence between the behavioral findings reported in each of the studies presented in this thesis. In outbred rats exposed to peripuberty stress, we reported that development of an aggressive phenotype following stress was associated with impaired habituation of the corticosterone response to that stress, whereas development of an anxious phenotype was associated with stronger habituation. This is in contrast to behavioral observations made from rats selectively bred to have either constitutively impaired or strong corticosterone habituation to repeated stress. Indeed, in these selection lines enhanced levels of anxiety-like behavior and aggression were found exclusively in the high-line, bred for impaired stress habituation. Why this should be the case is not clear. Indeed, whether we should expect congruence between such different models is questionable. When the lines were exposed to peripuberty stress, only the stress-habituating low-line rats showed alteration of their behavioral phenotype. Investigations of whether there were differential trajectories following stress independently in each line did not uncover any clear impact of variation in habituation greater than that already observed between lines.

One important outstanding question relates to what may mediate the specific sensitivity of low-line rats to the programming effects of peripuberty stress. Given that these rats responded in accordance with their line and showed strong habituation of corticosterone across peripuberty stress it is unlikely to have resulted from the actions of glucocorticoids. The finding of relatively low *Fkbp5* expression in the PVN of low-line rats may potentially provide a mechanistic explanation for the apparent enhancement in glucocorticoid receptor sensitivity of low-line rats. Glucocorticoids ordinarily have an immunosuppressive action but in absence of a sufficient response to stress, as may be the case in low-line rats, corticosterone might fail to buffer the harmful effects of released cytokines (Elenkov & Chrousos, 2002; McEwen et al., 1997). Examining the immune function of the lines therefore represents a potential future line of investigation. Indeed, enhanced inflammation associated with early life stress has been linked to the subsequent development of pathological aggression (Fanning et al., 2015), making this possibility important to consider. Moreover, low reactivity of the HPA axis is associated with a range of disorders in humans, including: PTSD, conduct disorder, anti-social personality disorder, and psychopathy (Loney et al., 2005; McBurnett et al., 2000; O'Leary et al., 2007; O'Leary et al., 2010; Raine, 1996; Yehuda & LeDoux, 2007). Interestingly the low HPA axis reactivity found in those diagnosed with PTSD is often found in the context of heightened sympathetic nervous system (SNS) activity (Mason et al., 1986; Yehuda et al., 2006; Yehuda et al., 1998), whereas in disorders of anti-sociality evidence suggests low HPA axis activity is found alongside blunted SNS activity (El-Sheikh et al., 2008; Lorber, 2004). Determining the combination of SNS and HPA axis activity within the low-line may help to determine next steps.

General conclusion

We have established the incidence of individual differences in behavioral trajectory following peripuberty stress, and found development of an aggressive phenotype to be associated with alterations in brain structure in regions commonly associated with both regulation of the HPA axis and in socio-affective functioning. Development of an aggressive phenotype was associated additionally with differential patterns of glucocorticoid responsiveness to the stress, such that impairment in habituation of corticosterone responses across stress exposure was associated with aggression. Upon development and testing of genetically-selected lines differing in propensity to habituate to repeated stress, we again found that constitutive impairment in stress habituation was associated with increased psychopathology-like behavior in its own right, a trajectory which did not become more pronounced with exposure to peripuberty stress. In contrast, constitutively strong habituation of HPA axis response to stress led to enhanced sensitivity to the programming effects of peripuberty stress. These findings were most pronounced with regard to aggressive behavior. In conclusion, we suggest that our findings support the implication of aberrant HPA axis activity during development in producing an aggressive phenotype later in life.

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Addendum

Curriculum Vitae

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Personal details

Birthdate: 08.05.1982
Nationality: British
Languages: English (native speaker); French (fair)

Education

Ecole Polytechnique Fédérale de Lausanne, Switzerland 2012-2016
PhD candidate in the Neuroscience program
Thesis title: Implication of individual differences in glucocorticoid responsiveness to stress in the development of psychopathology-like behaviour and underlying neurobiology

University of Sussex, Brighton, UK 2008-2010
Master of Science, Substance Misuse
Grade: Distinction
Thesis title: Effects of chronic ethanol treatment on impulsive action in mice: deficits in learning to control impulsivity within a vulnerability period.

University of Sussex, Brighton, UK 2000-2003
Bachelor of Science, Experimental Psychology
Grade: Second Class, with honours

Work experience

Doctoral Assistant 2012-2016
Laboratory of Behavioral Genetics, EPFL

Research Technician 2007-2012
Behavioural Neuroscience Group, University of Sussex

Data Analyst 2003-2007
Lloyds Bank Plc., Brighton

Technical skills

Behavioural tests and procedures in rodents
Appetitive and aversive instrumental and Pavlovian conditioning models, using both operant chambers and other apparatus
Fine-grained analysis of spontaneous exploratory and social behaviours
Intracranial surgery with recovery
Subcutaneous and intraperitoneal injections
Perfusion fixation

Histological and molecular skills

Brain sectioning & fresh tissue microdissection
Immunofluorescence
Histological staining
qPCR & PCR
Respirometry
Bright field & confocal microscopy

Software skills

SPSS, Graphpad Prism, SAS, SQL
Noldus Ethovision & Observer
Adobe Illustrator
MCID & Image J
Microsoft Office

Publications

Walker SE, Peña-Oliver Y, Stephens DN (2011). Learning not to be impulsive: disruption by experience of alcohol withdrawal. *Psychopharmacology*, 217 (3), 433-442.

Dixon CI, **Walker SE,** King SL, Stephens DN (2012). Deletion of the *gabra2* gene encoding $GABA_A$ α2-subunit results in hypersensitivity to the acute effects of ethanol but does not alter ethanol self-administration. *PLoS One*, 7(10):e47135.

Knapp S, Anstee QM, Maguire E, Hosie AM, Thomas P, Mortensen M, Bhome R, Matthews GAC, Martinez A, **Walker SE**, …., Stephens DN, Belelli D, Lambert J, Smart TG, Thomas HC (2013). Mutations in the *Gabrb1* gene promote alcohol consumption through increased tonic inhibition. *Nat. Commun*, 4, 2816.

Walker SE, Papilloud A, Huzard D, Sandi C (2016). The link between aberrant hypothalamic-pituitary-adrenal axis activity during development and the emergence of aggression – Animal studies. *Neurosci Biobehav Rev*, doi: 10.1016/j.neubiorev.2016.10.008.

Kukalev A, Ng YM, Ju L, Saidi A, Lane S, …., **Walker SE**, …., Stephens DN, Carr AM, Lamsa K, Tse E, Yu V (2016). Deficiency of *Cks1* leads to memory consolidation defects and p27 dependent formation of neuronal cofilin aggregates. *Cereb Cortex*, (in press).

Walker SE, Wood TC, Bernanos M, Williams SCR, Cash D, Sandi C. Not all rats have an equal response to peripuberty stress exposure: implication of individual differences in glucocorticoid responsiveness to repeated stress in the development of an aggressive phenotype and associated neuroanatomical alterations. (in preparation)

Walker SE, Zanoletti O, Guillot de Suduiraut I, Sandi C. Rats selected for adaptation versus non-adaptation of glucocorticoid response to repeated juvenile stress differ in behavioral and endocrine profile: a new model for HPA axis activity related psychopathology. (in preparation)

Walker SE, Sandi C. Constitutively strong adaptation of glucocorticoid response to repeated adolescent stress enhances the psychopathology-inducing effect of exposure to peripuberty stress. (in preparation)

Selected posters

Walker SE, Knapp S, Thomas HC, Stephens DN. A role for $GABA_A$-receptors containing β1 subunits in the control of ethanol-motivated behaviour. EBPS, 13th Biennial Meeting, Rome, Italy, 2009

Walker SE, Dixon CI, King SL, Stephens DN. Deletion of the *gabra2* gene encoding $GABA_A$ α2-subunits increases acute ataxic effects of ethanol but does not alter ethanol self administration. BNA, 21st Biennial Meeting, Harrogate, UK, 2011.

Dixon CI, **Walker SE**, Lambert JJ, Belelli D, King SL, Stephens DN. Early life stress alters responsivity to cocaine. EBPS, 15th Biennial Meeting, La Rochelle, France, 2013.

Walker SE, Fournier C, Guillot de Suduiraut MI, Sandi C. Constitutional differences in glucocorticoid responsiveness to peripuberty stress are associated with differences in psychopathology-like behaviors in rats. FENS, 9th Forum of Neuroscience, Milan, Italy, 2014.

Walker SE, Fournier C, Sandi C. Constitutional differences in glucocorticoid responsiveness to peripuberty stress are associated with differences in psychopathology-like behaviors in rats: further findings. EBBS-EBPS, 2nd joint meeting, Verona, Italy, 2015.