The molecular and behavioral function of SLC6A15, a novel candidate gene for depression

Dissertation

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Sara Santarelli

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1. Gutachter: PD Dr. Mathias V. Schmidt

2. Gutachter: Prof. Dr. Wolfgang Enard

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"It is imperfection - not perfection - that is the end result of the program written into that formidably complex engine that is the human brain, and of the influences exerted upon us by the environment and whoever takes care of us during the long years of our physical, psychological and intellectual development."

Rita Levi-Montalcini

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Abstract

Early experiences profoundly affect the adult coping response to stress and, consequently, adult vulnerability to psychopathologies, such as major depression. The prevailing view that stress effects are cumulative has been recently challenged. The recently formulated match/mismatch hypothesis of psychiatric disorders proposes that individuals experiencing high levels of psychosocial stress early in life are programmed for dealing with high psychosocial stress later in life and therefore become resilient to high stress levels at adulthood. The first aim of this doctoral thesis was to test this hypothesis by comparing the developmental effects of two different rearing conditions, in response to two opposite adult environments. First, we investigated which are the characteristics of the rearing environment that provide adequate coping skills to adult social chronic stressors (Chapter 1). Afterwards, we extensively characterized the phenotype of mice reared in positive or negative environment following different adult environmental conditions. Moreover, we could elucidate gender-specific factors in stress adaptation and coping skills development, one of the most underestimated issues in preclinical studies (Chapter 2-3). Not only environmental, but also genetic factors play a key role in vulnerability to stress and psychiatric disorders. Recently, a genome-wide association study proposed SLC6A15, a gene coding for neuron-specific neutral amino acid transporter, as a candidate for vulnerability to stress and major depression. In humans, risk allele carriers for a SNP in the SLC6A15 regulatory region display altered hippocampal glutamate levels, hippocampal volume and HPA axis activity. The second aim of this doctoral work was to better understand the role of SLC6A15 in brain function, especially in relation to stress and the glutamatergic system. We investigated the effects of SLC6A15 manipulation on behavior and neurochemistry in mice. We profiled two mouse models, SLC6A15 total knockout (KO) and virus-mediated hippocampal SLC6A15 overexpression (OE), under basal conditions and following chronic social stress. We performed an extensive characterization of the two models, focusing our attention to emotional behavior, hippocampal neurochemistry and gene expression. SLC6A15 expression was directly correlated to glutamate and proline content in the hippocampus under basal condition (Chapter 4). Following chronic stress, KO animals showed lower levels of anxiety- and depressive-like behavior, along with an altered HPA axis responsiveness. In support of those findings, OE animals displayed, already under basal condition, an increase in anxiety- and depressive- like behavior, together with an increase of GluR1 levels (Chapter 5). Taken together, those results will improve our understanding of the developmental and molecular mechanism underlying stress resilience and depression vulnerability.

List of abbreviations

5HTT	serotonin transporter
AAV	adeno-associated virus
ACTH	adrenocorticotropic hormone
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
ANOVA	analysis of variance
ATB^{0+}	neutral and cationic amino acid transporter
AVP	arginine vasopressin
B^0AT	neutral amino acid transporters
BDNF	brain-derived neutrophic factor
BG	background
BGH	bovine growth hormone
BGT1	betaine transporter
BSA	bovine serum albumin
CA1	cornu ammonis region 1
CA2	cornu ammonis region 2
CA3	cornu ammonis region 3
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CN	communal nesting
CNS	central nervous system
CORT	corticosterone
CRH	corticotropin-releasing hormone
CSDS	chronic social defeat stress
CT1	creatine transporter
CUMS	chronic unpredictable mild stress
Da	Dalton
DAT	dopamine transporter
DEX	dexamethasone
DG	dentate gyrus
DMS V	Diagnostic and Statistical Manual of Mental Disorders
DNA	deoxyribonucleic acid
EAAT	excitatory amino acid transporter

EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EH	early handling
ELS	early life stress
EPM	elevated plus maze
FH	female housing
FKBP5	FK506 binding protein 51
FST	forced swim test
GABA	γ-Aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAT	GABA transporter
GCPR	G-protein coupled receptors
GCs	glucocorticoids
GH	group housing
Glu	glutamate
Glx	glutamate+glutamine
GLYT	glycine transporter
GR	glucocorticoid receptor
GRE	glucocorticoids responsive elements
GWAS	genome wide association analysis
GxE	gene by environment
HC	hippocampus
HPA axis	hypothalamic-pituitary-adrenal axis
HRE	hormone responsive elements
HSD	honestly significant difference test
HSP	heat shock protein
IMINO	imino acids transporter
ITI	Inter trial interval
KO	knockout
LC	liquid chromatography
LG-ABN	licking/ grooming and arched-back nursing
LTD	long-term depression
LTP	long-term potentiation
MAOI	monoamine oxidase inhibitors

MAP	mitogen-activated protein List of abbreviations
MC2R	melanocortin 2 receptors
MEMRI	manganese-enhanced magnetic resonance imaging
mGluR	metabotropic glutamate receptor
MR	mineralocorticoid receptor
mRNA	messenger RNA
MS	mass spectrometry
MWU	Mann-Whitney-U
NAA	N-acetylaspartate
Nac	nucleus accumbens
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NDRI	norepinephrine-dopamine reuptake inhibitor
NET	norepinephrine transporter
NMDA	N-methyl-D-aspartate
OE	overexpression
OF	open field
p300/CBP	binding protein 300/CREB-binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFC	prefrontal cortex
PMDD	premenstrual dysphoric disorder
PND	postnatal day
POMC	pro-opiomelanocortin
PP	prepulse
PPI/PPF	prepulse inhibition/facilitation paradigm
Pro	proline
PROT	proline transporter
PSD	postsynaptic density
PSD-95	postsynaptic density protein 95
PTSD	post-traumatic stress disorder
PVC	polyvinyl chloride
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative reverse transcription PCR

RNA	ribonucleic acid
RT-qPCR	real-time polymerase chain reaction
SA	social avoidance
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SH	single housing
SHRP	stress hyporesponsive period
SLC	solute carrier family
SN	single nesting
SNP	single nucleotide polymorphism
SRC-1	nuclear receptor coactivator 1
SSC	saline sodium citrate
SSRI	selective serotonin reuptake inhibitor
TAUT	taurine transporter
TCA	tricyclic antidepressant
UTP	uridine triphosphate
UV	ultraviolet
VTA	ventral tegmental area
WPRE	woodchuck post-transcriptional regulatory element
WT	wild type

General introduction

1. Stress

1.1 Stress definition and historical perspective

Stress has always been part of the human experience, although the medical definition of stress itself is a relatively new construct. Only in the beginning of the 20th century, the word "stress" started to be used by physicians and in medical books as "mental constraints, restrictions" leading to diseases. In the same period, while investigating physiological responses to threats, Walter Cannon introduced the first definition of stress as "the ensemble of the external stimuli that disrupted homeostasis" (Cannon, 1929). Such a definition is rather vague and a few years later it was already reformulated by Hans Selye, who conceptualized stress in his famous "general adaptation syndrome". This was the first attempt to separate the agents responsible for a disruption of the body equilibrium from the responses that are taking place in the body itself in order to return the system to homeostasis (Selye, 1946). Since then, many researchers have postulated a number of definitions of stress and tried to describe its nature, but there is still no consensus on a suitable definition. However, stress is experienced on a daily basis, and there is a strong need for innovative, meaningful approaches to advance our understanding of stress, how our body reacts to it and the role it plays in shaping our individuality.

1.2 Physiological response to stress

Stress response is the result of a coordinated action of many organs and systems. Centrally, three main networks are responsible for the stress response, distinguishable for their temporal and spatial pattern of activation. Monoaminergic systems constitute the faster network. Monoamines such as noradrenaline, dopamine and serotonin are synthetized and released within minutes after the onset of the stressor and are responsible for the immediate behavioral response to the stressor. Neuropeptides, whose classification as neurotransmitters rather than neuromodulators is still debated (Leng & Ludwig, 2008), are released after or concurrently with neurotransmitters. Among neuropeptides, corticotropin-releasing hormone (CRH) and arginine vasopressin peptide (AVP) are secreted in response to stress by the paraventricular nucleus (PVN) of the hypothalamus, with the aim to coordinate the central stress response with the periphery. Corticosteroids, steroid hormones that are produced from the adrenal glands, represent the third group of stress responders, which activate the peripheral organs and ultimately send feedback to the brain. The study of the integration of these effectors into a coordinated stress response has been one of the main aims of research in the stress field.

One of the most studied stress response system is the hypothalamic-pituitary-adrenal system (HPA) axis, which comprises components of the three aforementioned networks. The HPA axis registers modifications in the environment, assesses the harmfulness of the changes and organizes the body adjustments to adapt to the new situation (Joëls & Baram, 2009). Therefore, several studies investigating the stress response and adaptation have focused on the HPA axis. When an organism experiences stress, CRH and AVP are released from the hypothalamus into the portal circulation, quickly reaching the anterior pituitary gland, a neuroendocrine interface between the brain and the rest of the body. In response to CRH, the corticotropes of the anterior pituitary gland release adrenocorticotropin hormone (ACTH) into the general circulatory system, through which ACTH reaches the adrenal glands. Here, adrenal cortex cells express ACTH receptors, melanocortin 2 receptors (MC2R), which upon activation by ACTH initiate the synthesis and release of steroid hormones, namely glucocorticoids (GCs).

Glucocorticoids, cortisol in humans and corticosterone in rodents, are produced over the course of minutes after the stress exposure and are released in the blood stream. Mineralocorticoid and glucocorticoid receptors (MR and GR) are nuclear receptors activated by GCs, which trigger genomic and non-genomic modifications in target organs. MRs are expressed throughout the body, for example in the kidney, colon, heart, adipose tissue, sweat glands and in the central nervous system. In the latter, MRs are predominantly found in the hippocampus, an important area involved in the control of the HPA axis. Upon activation by GCs, MRs dissociate from heat shock protein 90 (HSP90), which holds them inactive in the cytoplasm, homodimerize and translocate into the nucleus where them bind to hormone responsive elements (HRE). Coactivators, such as SRC-1 and p300/CBP, are sequentially recruited to the MR complex to allow histone acetylation and target gene transcription to occur. In addition, the MR also induces non-genomic effects, activating protein kinase C and NA^+/H^+ exchange and increasing intracellular calcium. The other GCs receptors, GRs, are ubiquitously express in the body, maintained in an inactive form by a complex of co-chaperone proteins comprising HSP90, HSP70 and FKBP5. When GCs diffuse through the cell membrane into the cytoplasm and bind to GRs, the co-chaperones are released, allowing GR to homodimerize, translocate into the nucleus bind specific glucocorticoids responsive elements (GRE), to ultimately result in transactivation or transrepression. Like MRs, activated GRs also have non-genomic effects. For example Src kinase binds to inactive GR and is released when a glucocorticoid binds to GR, triggering an intracellular signal cascade resulting in reduced synthesis of arachidonic acid, a key proinflammatory molecule.

Although both GRs and MRs can bind GCs, MRs bind corticosterone with approximately a 10-fold higher affinity than GRs. This means that under basal condition, in regions where GR and

MR colocalize, such as in the hippocampus, corticosterone will occupy mainly MRs, whereas GRs are occupied only under higher levels of circulating corticosterone. Therefore, MRs are associated with the maintenance of the basal tone of the HPA axis, while GRs mediate the stress response feedback loop directed to restore disturbances of homeostasis. In conclusion, the balance between MR and GR regulates the HPA axis tone, according to the levels of GCs. Glucocorticoid receptors are not only responsible for regulating the HPA axis: after acute stress, GR activation suppresses the immune system, increases blood circulation and gluconeogenesis in order to promote the redistribution of energy to meet the immediate energy demand required to respond and adapt to the stressor (Figure 1).

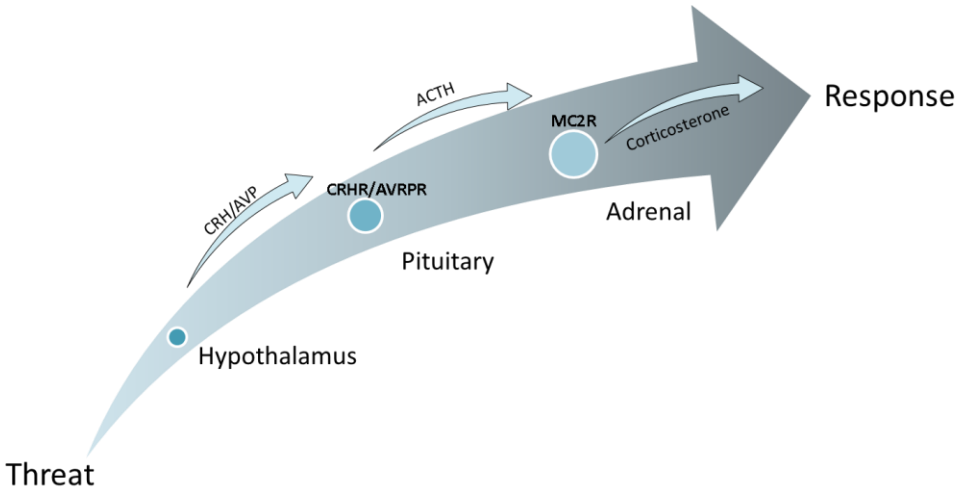

Figure 1: Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis. When a stimulus is perceived, the hypothalamus initiates a cascade of signals that leads to the activation of the pituitary, where the signal is further amplified and transmitted to the periphery. Adrenal glands initiate the production of corticosterone that will initiate the physiological response to the threat (ACTH: adrenocorticotropin hormone, AVP: arginine vasopressin peptides, AVRPR: AVP receptors, CRH: corticotropin-releasing hormone, CRHR: CRH receptors, MC2R: ACTH receptors).

The HPA axis response is vital to avoid or to buffer the negative effects of a threat. However, when the stress is terminated, it is as essential to shut down the activation of the HPA axis. Upon activation by GCs, GRs provide negative feedback to the HPA axis. Particularly in the hypothalamus, activated GR dimers translocate into the nucleus and transrepress protein synthesis, terminating the production of CRH. This triggers a negative feedback loop that inhibits ACTH production from the pituitary and finally reduces the release of GCs from the adrenals. In such a way, the HPA axis returns to basal levels of activity (Figure 2).

In adulthood the HPA axis is fully developed and functional, and tightly regulated by feedback interactions between the hypothalamus, the pituitary and the adrenals. Higher limbic structures, such as the hippocampus, are further controlling the tone and responsivity of the HPA axis. Acute physical, psychological or immunological challenges, all activate the HPA axis:

circulating GCs increase rapidly while the sympathetic nervous system is activated and a cascade of signals triggers both behavioral and somatic reactions. The heart rate and blood pressure increase, breathing rhythm accelerates and temporary storage sites of fat and glucose are mobilized. These changes prepare the whole body to adapt to the challenge. Once the threat is removed or the situation returns to a normal condition, the HPA axis is shut down and the normal functions will be slowly restored. However, prolonged activation periods, intense bursts of activity of the axis or both could disrupt the negative feedback loop leading to pathological changes, especially affecting the brain. The brain is also very sensitive to environmental adversities: in the hypothalamus, chronic stress diminishes GABAergic tone of parvocellular PVN neurons, along with an increase of CRH, AVP and c-FOS expression (Joels 2004, Cole and Sawchenko, 2002). Modifications following chronic stress have been also reported in the hippocampus: total volumes of the various hippocampal sub-regions and the total number of granule cells in the dentate gyrus are decreased even after weeks of recovery (Joels 2004). The molecular alterations observed are also reflected in increased despair and anxiety-like behaviors, anhedonia and memory impairment (Chrapusta, Wyatt, & Masserano, 2002; Garcia-Marquez & Armario, 1987; Miller & O'Callaghan, 2002; R. M. Sapolsky, Krey, & McEwen, 1986).

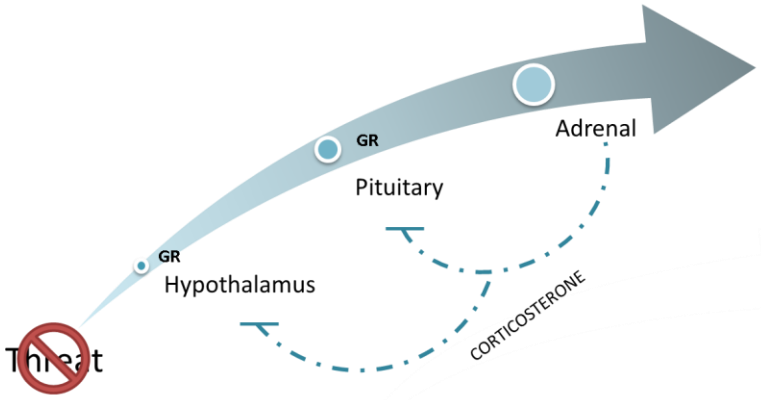

Figure 2: When the threat is removed or after sustained stimulation, circulating corticosteroids reach the brain promoting negative feedback and interrupting the cascade of signals of the HPA axis (GR: glucocorticoid receptors).

1.3 Stress as vulnerability factor for diseases

Chronic or repeatedly elevated GC levels following (prolonged) exposure to stress, cause a shift in the usage of resources from basal metabolic functions to defensive strategies, thereby promoting adaptation to the threat. For example, high GC levels promote energy mobilization over energy storage, suppress functions such as growth and reproduction, in order to improve the response to the threat and repress any auxiliary functions not directly related to coping with the stressor. However, upon repeated exposure to mild stressors, the HPA axis shows habituation,

reducing the levels of circulating GCs. The habituation is likely due to reduced central response to the stressor. Local inactivation of the prefrontal cortex (PFC) prior to restraint stress blocks the development of habituation to subsequent exposure (M. S. Weinberg, Johnson, Bhatt, & Spencer, 2010). In the PVN, blockade of MRs before the stress prevents stress habituation (Cole et al., 2000). Increased inhibitory activity of the amygdala on the PVN is believed to be the central mechanism behind habituation. It has been shown that blockade of β– adrenergic receptors in the amygdala prevents behavioral habituation to repeated stress, blocking the stress-induced increase of AVP in the PVN (Grissom & Bhatnagar, 2009).

Nonetheless, under specific conditions, like unpredictable severe stress experiences, this habituation is not observed, and rather continuous sustained HPA axis activity is registered. There are a number of known physiological, structural and molecular adaptations that usually occur following chronic stress exposure. In central structures controlling the HPA axis, such as the hippocampus and prefrontal cortex, spines and dendrites of pyramidal cells show retraction and reduced spine density (Qin, Karst, & Joëls, 2004; R. Sapolsky, Uno, Rebert, & Finch, 1990), whereas in the amygdala, stress increases spine density. Chronic stress also results in a different gene expression profile in many different brain regions. In the PVN, chronic stress induces changes CRH and AVP expression along with reduced GR expression (Ulrich-Lai & Herman, 2009; Ulrich-Lai et al., 2006). The effects of chronic stress are also visible in the periphery. For example, animals exposed to unpredictable chronic stress show hypertrophy of the adrenal glands and atrophy of the thymus due to the sustained production of GCs (Wagner et al., 2012). Hyperglycemia and insulin resistance due to increased gluconeogenesis are furthermore observed and correlate to chronic stress exposure (Dallman et al., 2006; Low, Salomon, & Matthews, 2009; Paternain et al., 2011; Shaker & Lukert, 2005; Sterlemann et al., 2008). Ultimately, the behavioral profile is dramatically changed by chronic stress, especially emotional behavior and memory (Park, Campbell, & Diamond, 2001). Altogether, such modifications are often stable over time and can be detected long after the end of the stressor (Mangiavacchi et al., 2002; Maslova, Bulygina, & Markel, 2002).

1.3.1 Early life experiences

In mammals, HPA axis development takes place during the early stages of life, in which environmental challenges, such as negative life events, have a dramatic impact on shaping the HPA axis and therefore vulnerability to psychiatric diseases. Epidemiological studies in children reveal a strong association between early negative experiences and increased incidence of psychopathology (Gilbert et al., 2009; Kaufman, Plotsky, Nemeroff, & Charney, 2000). It has been

reported that not only disruptive early life events like abuse or violence are strong predictors of later mental disorders, but also milder events with higher prevalence in the population, such as parental divorce and mild maltreatment, are triggers for later vulnerability to psychiatric diseases (Carroll et al., 2007). To identify the mechanisms of the long-term effects of early life experiences on mental health, animal models have been developed to investigate the long-term effects of negative early life events on the molecular and neurochemical development of the HPA axis.

The postnatal development of the stress system in rats and mice is characterized by a reduced responsiveness of the HPA axis : basal levels of GCs are lower and are not elevated after a mild stress event (Gunnar & Cheatham, 2003; Schmidt, van der Mark, Levine, de Kloet, & Oitzl, 2003). In addition, ACTH is produced at higher concentrations under basal conditions, but will not be responsive to a mild stressor (Schmidt et al., 2004). In virtue of the reduced responsiveness of the HPA axis in pups, this peculiar period has been defined stress hyporesponsive period (SHRP). However, the lack of HPA axis responsiveness is limited to the periphery. In fact, CRH is highly expressed in the PVN and is sensitive to stressors (Schmidt, Oitzl, Levine, & de Kloet, 2002), and both glucocorticoid receptors (MR and GR) are highly expressed in the hippocampus. The proposed role of the SHRP is to protect the developing organism from the noxious effect of high levels of stress (i.e., elevated GCs), while the brain is still able to register and encode the signals coming from the external environment. Although the short-term effects of stress in early life may be buffered by the SHRP, the long-term consequences on brain and behavior of the pups are detrimental. In altricial species, like humans and rodents, the early environment consists mainly of the mother and the maternal care she provides to the pups. Therefore, preclinical models focus mostly on interrupting the mother-pup interaction to investigate the resulting modifications on the developing HPA axis. Disrupting the mother-pup relationship for prolonged periods of time exert a detrimental role on brain development, emotional and cognitive behavior and metabolic regulation (for review see (Schmidt, Wang, & Meijer, 2011)). On the other hand, short and predictable interruptions of the maternal care have beneficial effects on HPA axis development, resulting in a protected phenotype against adult stressors (Anisman, Zaharia, Meaney, & Merali, 1998; Macrì, Zoratto, & Laviola, 2011; Raineki, Lucion, & Weinberg, 2014).

Commonly early life stress in laboratory setting is achieved by interfering with the maternal behavior. The models mostly used are based on the disruption of the mother-pup bond, separating the dam from the nest for one to twenty-four hours daily during the first two weeks of the pups´ life. The rationale behind this paradigm, called maternal separation, is to model emotional and physical neglect. Maternal separation has persistent effects on the pups' development: HPA axis development is disrupted (Levine 1957), memory formation and learning

are impaired (Ivy 2010) and emotional behavior is altered (Wang et al 2011). However, critics have questioned whether a more salient environment should be used. In the human situation, early life stress is generally a chronic persistent condition rather than intermittent disruptive events. Therefore, models of continuous adverse rearing have been developed. For example, the limited nesting and bedding material paradigm consist in providing the dam reduced nesting and bedding material during the first two weeks of life of the pups (Rice et al 2008). On the other hand, also positive and nurturing experiences have effects on neurodevelopment. Increased maternal care is thought to trigger the development of more efficient coping strategies and increased resilience against adversities later in life (Feder et al 2009). Shorter daily separations of the mother from the pups, during three to fifteen minutes, have been used to increase maternal care behavior in rodents (Liu et al., 1997a), resulting in reduced anxiety-like behavior (Durand et al 2008). An alternative way to increase the levels of maternal care is the communal nest paradigm, in which three pregnant females are housed together and build a shared nest, where all the pups are kept and reared in a shared effort from the dams (Branchi 2006). The presence of more lactating and caring dams increased the total amount of maternal care received by each pup, resulting in a marked phenotype at adulthood. Animals reared in a communal nest display higher resilience against adult chronic social stress and increased brain plasticity (D´andrea 2007, Branchi 2009) (For extended reviews about early life animal models see (Lyons, Parker, & Schatzberg, 2010; Schmidt, Wang, et al., 2011).

1.3.2 Adult stress

Paradigms investigating the effects of a prolonged period of stress on brain and behavior can be characterized based on three important features: the controllability (escapable vs unescapable), the predictability (predictable vs unpredictable) and the duration (constant or intermittent). In many established paradigms, experimental animals are exposed to predictable, unescapable, intermittent aversive manipulations like foot shock, restraint and forced swim. Such models are very easy to perform, inducing quickly an altered phenotype, however the experimental animals quickly develop habituation to the stressor and the results obtained have poor translational value. Therefore, ethologically relevant models to mimic the social environment and the unpredictability of the stressful events have been developed to better match the human situation, like social defeat and mild unpredictable stress models (Qin et al., 2004; Wagner et al., 2012; Willner, 1997). In both cases the experimental animals are exposed to ethologically relevant threats, like another aggressive conspecific or lack of resources, for prolonged periods of time with a random pattern. The resulting neurobehavioral effects on

depression and anxiety-like behaviors are robust and long lasting, although the standardization of the methods has been questioned. The use of different animals as stressors or different sequences of mild stressors bear a large variability in the overall intensity of the chronic stressor, leading to higher variability in the observed results. Overall, these paradigms result in hyperactivation of the HPA axis, resulting in increased basal and acute levels of corticosterone. In conclusion, in addition to controllability, predictability and duration, also intensity and stress typology have been recently shown to contribute to the outcome of the stress paradigm. Interestingly, homotypic stress, even if intense, show reduced long term effects, compared to heterotypic, although milder, experiences. Overall, results obtained from preclinical studies of stressful experiences during adulthood have provided valuable insight into the underlying neurobiological and neuroendocrine alterations associated with many different mood disorders like depression, anxiety and schizophrenia (Bartolomucci & Leopardi, 2009; Cryan & Holmes, 2005).

1.3.3 Adaptive or maladaptive consequences of stress

As it was already mentioned in 1.3.1, exposure to stress during sensitive developmental time windows is thought to increase the probability to develop psychiatric disorders, namely depression, following stress exposure in adulthood. However, it is also highly debated whether early life stress is able to provide resilience against subsequent stressful experiences during adolescence or adulthood. Different theories have been formulated to understand stress reactivity. At present, two main theoretical frameworks exist. The “allostatic load model”, formulated by Bruce McEwen, states that a higher number of stressful events experienced, leads to a greater vulnerability to develop depression, and can be described as a “wear and tear” cumulative effect (McEwen & Wingfield, 2003). The model suggests that after early life stress, the stress response systems, such as the HPA axis, try to restore the homeostatic equilibrium disrupted by early life adversities. In doing so, the HPA axis pays a toll, the “allostatic load”, which grows after chronic stress exposure and may lead to psychopathology (Christine Heim, Meinlschmidt, & Nemeroff, 2003; Christine Heim, Newport, Mletzko, Miller, & Nemeroff, 2008; C. R. Pryce, Rüedi-Bettschen, Dettling, & Feldon, 2002).

In contrast to the allostatic load model, a second group of theories state that early life stress may promote the development of effective coping strategies to face adverse environments during adulthood. These evolutionary-developmental approaches originated from an attempt to explain the conservation of genetic variances throughout evolution that seem to convey strong disadvantages in the case of stress. Originally formulated under the name of “biological sensitivity

to context", it emerged from the field of metabolic disease and later extended to all non-communicable disorders, including psychopathology (Belsky & Pluess, 2009; Ellis, Boyce, Belsky, Bakermans-Kranenburg, & van Ijzendoorn, 2011; P.D. Gluckman, Hanson, & Beedle, 2007). This theory states that "exposure to acutely stressful childhood environments upregulates biological sensitivity to context, increasing the capacity and tendency of individuals to detect and respond to environmental dangers and threats" (Ellis et al., 2011). Natural selection may have thus maintained variations in developmental sensitivity due to a later higher fitness outcome, in response to different early life environments faced over evolution. Interestingly, a similar concept had evolved independently to explain resilience development. The "stress inoculation hypothesis" states that brief, intermittent stress exposures early in life promote resistance to subsequent stress exposures. In 1993 it was proposed that successful coping with a specific stressor at one time, results in resilience later in life against a similar noxious stimulus (Rutter, 1993). In non-human primates, for example, brief periods of maternal separation attenuate HPA axis responsiveness to subsequent stressors (Parker, Buckmaster, Sundlass, Schatzberg, & Lyons, 2006). Likewise, infants reared in a challenging environment show lower HPA axis activation in response to adult stress exposure, when compared to infants reared in low challenging environment (Lyons & Parker, 2007). A schematic view of the match/mismatch hypothesis is presented in Figure 3.

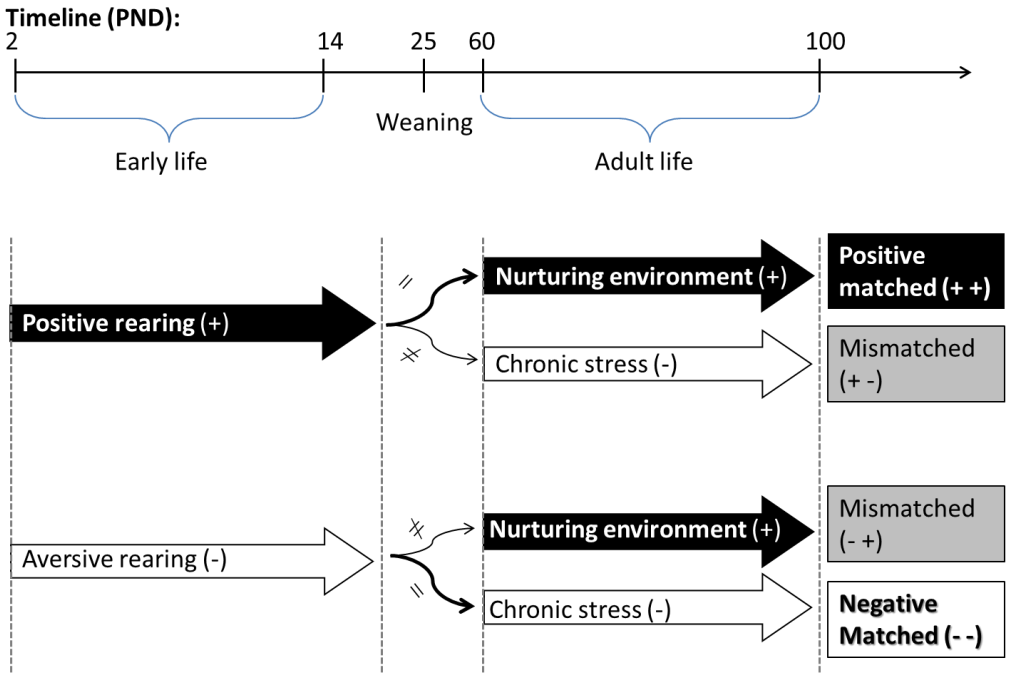

Figure 3: Schematic representation of the interaction between early life and adult life environment. Although the effects of negativities during sensitive time windows have been widely documented, reports about the long term effects and interaction between adversities during life are still sparse.

Based on such concepts, the match/mismatch hypothesis of psychiatric diseases has been formulated. Psychiatric diseases, and in particular depression, may therefore result from a mismatch between the programmed (early) and later environments in combination with genetic predisposition. Indeed, it has been reported that juvenile exposure to unpredictable chronic stress induces stress resilience rather than impairment in adulthood (Ricon, Toth, Leshem, Braun, & Richter-Levin, 2012). Furthermore, juveniles exposed to high levels of corticosteroids have shown to be better adapted to conditions associated with high corticosteroid levels in adulthood (Lyons, Parker, Katz, & Schatzberg, 2009; Oomen et al., 2010). To-date, there are already findings that support an early environment*adult environment*gene interaction. A polymorphism in the promoter region of the gene encoding the serotonin transporter (5-HTTLPR) has certainly been associated with increased vulnerability to early life stress (Caspi et al., 2003; Kumsta et al., 2010; Mirescu, Peters, & Gould, 2004). In knockout animals, in which the 5HTT gene has been ablated, the effects of early life stress are indeed much more profound (Gatt et al., 2009). However, in order to determine whether early life experiences modulate adult coping strategies in a genotype-dependent manner, more longitudinal studies are needed. Nevertheless, 5HTT knockout animals that have underwent early life stress show improved stress-coping strategies following an adult stressor (van der Doelen, Kozicz, & Homberg, 2013). The MR gene seems to be the key node of this adaptation switch: epigenetic modification of the MR, a key receptor for the regulation of HPA axis tone, is regulated by maternal stress and pharmacological MR inhibition produces behavioral changes similar to those resulting from early life stress (Gapp et al., 2014). An example of adaptive early life adversities in humans is elegantly illustrated in a study investigating the development of adulthood anxiety. Here, early life stress was associated with lower levels of implicit but not explicit anxiety, a predictor of better clinical outcome in the case of other psychiatric disorders (Edge et al., 2009).

Overall, there is preclinical, clinical and epidemiological evidence in support of each of the different frameworks. Therefore integration, rather than mutual exclusion, of the mismatch and the cumulative stress hypothesis may be the most logical action. Individuals with a highly inert adaptive capacity may suffer more under mismatched environments, while individuals with a low adaptive capacity would have the highest disadvantage following cumulative stress exposures. It is still to be debated whether genetic background alone determines individual sensitivity to a context, or whether other parameters, such as transgenerational epigenetic inheritance or gender, may also contribute to variations within individual sensitivity to a context.

2. Molecular aetiology of depression

Major depression is a multifactorial disease characterized by a pervasive degradation of the psychophysical status of an individual (American Psychiatric Association, 2013). Generalized low mood, sleep disturbances, changes in body weight and an inability to experience pleasure are the main symptoms experienced by people affected by major depression. Both environmental factors, such as adverse life experiences, and genetic variances are thought to interact, to predict the risk of developing depression. While the molecular underpinnings of depression are still not clear, a number of hypotheses have been proposed (Caspi et al., 2003; Haeffel et al., 2008; K. P. Lesch, 2004).

2.1 Monoamine hypothesis

The classical theory explaining depression etiology is the monoamine hypothesis. In 1965, Schildkraut and colleagues observed that antidepressant drugs (e.g., monoamine oxidase inhibitors, (MAOI) and tricyclic antidepressants, (TCA)) increase the availability of monoamines in brain areas controlling mood (Schildkraut, 1965). In addition, markers reflecting central levels of monoamines, namely monoamine metabolites in the cerebrospinal fluid (CSF) and in plasma, are altered in psychiatric patients (Ditzen et al., 2012). Genetic variants leading to a reduction in the efficiency of monoamine transporters, have furthermore been identified as risk factors for depression (Caspi et al., 2003). Several antidepressant drugs act to increase synaptic concentrations of monoamines; by contrast pharmacological or dietary depletion of cathecolamines induces a relapse in depressive symptoms (Delgado et al., 1990). Animal research also supports the monoamine hypothesis. Genetic ablation of monoamines transporters has been used to model chronically elevated extracellular monoamine levels, which parallel the effects of antidepressant treatment (Mathews et al., 2004; Shen et al., 2004; Xu et al., 2000). The effects of early life adversity on brain and HPA axis development are also modulated by levels of the serotonin transporter (Barr et al., 2004; Carola et al., 2008).

Therefore, drug development has mobilized efforts to synthetize new drugs that are able to control monoamines levels in the brain. However, after thirty years on the market, monoaminergic drugs have revealed some limitations of the monoamine hypothesis. Although monoaminergic drugs have a powerful effect, only 50% of patients meet remission criteria and only after six weeks of treatment (Quitkin et al., 1993). Importantly, antidepressant treatment-induced changes in the levels of monoamines occur in the range of hours after administration, whereas the clinical improvements are reported to take weeks. Such a temporal contrast has led

researchers to investigate other possible mechanisms underlying the therapeutic effects of TCA and MAOI.

2.2 Neurotrophic hypothesis

It has been suggested that the delayed onset of antidepressant effects should be ascribed to gradual changes in the synapses (e.g. remodeling of spines, weakening of synaptic connections) that slowly develop to permanently restructure neurotransmission. Only once this has occurred can clinical symptoms be improved. Interestingly, antidepressants increase plasticity whereas chronic stress reduces synaptic and dendritic remodeling, especially in the hippocampus (Duman, Malberg, & Thome, 1999; Malberg, Eisch, Nestler, & Duman, 2000). Therefore a new hypothesis of vulnerability to depression has been formulated, stating that depression results from decreased dendritic stability, which leads to spine retraction and consequently neuronal atrophy, resulting in reduced hippocampal volume (Duman & Monteggia, 2006). In support of this theory, it has been reported that levels of neurotrophins, growth factors involved in neuronal remodeling and plasticity, are elevated by antidepressants. Conversely, stress or administration of corticosterone reduces levels of neurotrophins (Castrén, Võikar, & Rantamäki, 2007; Schinder & Poo, 2000). There are a number of key findings in support of the neurotrophic theory of depression. A hallmark of depression is a reduction in hippocampal volume, which could be explained by neuronal atrophy, reduced neuron size and loss of glia (Bremner et al., 2014). Post mortem tissues from depressed patients show similar changes (Karege, Vaudan, Schwald, Perroud, & La Harpe, 2005). In animal models of chronic stress reduced BDNF levels in the hippocampus are observed, whereas antidepressants increase BDNF- mediated signaling (Duman & Monteggia, 2006; Nestler et al., 2002). In addition, BDNF signaling controls HPA axis activity, tightly regulating the expression of CRH in the PVN (Jeanneteau et al., 2012). Nevertheless, contrasting evidence exists that calls the validity of the neurotropic hypothesis into question. Inconsistent results have been reported for conditional knockouts of BDNF: forebrain deletion of BDNF receptor has no effect on behavior (Adachi, Barrot, Autry, Theobald, & Monteggia, 2008), whereas infusions of BDNF in VTA and NAc have pro-depressant effects (Berton et al., 2006).

2.3 Glutamatergic hypothesis

The first direct evidence that non-monoaminergic neurotransmitters could play a role in depression came in 1990, when N-methyl-D-aspartate (NMDA) receptor antagonists were reported to have antidepressant effects (Trullas & Skolnick, 1990). Along this line, clinical data reported that depressed individuals have increased levels of glutamate in plasma (J. S. Kim,

Schmid-Burgk, Claus, & Kornhuber, 1982), cerebral spinal fluid and brain tissue (Choudary et al., 2005; Hasler et al., 2007; Sanacora et al., 2004). Furthermore, glutamatergic transmission and volumetric changes are altered in limbic areas of patients with mood dysregulation (Manji, Drevets, & Charney, 2001).

Preclinical findings corroborate human findings: in rodent models, stress impairs the development and functioning of the glutamatergic system (Gómez-Galán, De Bundel, Van Eeckhaut, Smolders, & Lindskog, 2013), glutamate release (Sanacora, Treccani, & Popoli, 2012) and the expression of glutamatergic receptors (Schmidt et al., 2010). It is postulated that corticosterone mediates the effects of stress on glutamatergic systems. In fact, high corticosterone levels induce rapid effects on hippocampal glutamatergic transmission (Karst et al., 2005). In addition, activation of the glutamatergic system underlies synaptic plasticity and memory formation (Lamprecht & LeDoux, 2004; Rodrigues, Bauer, Farb, Schafe, & LeDoux, 2002). Overall, stress impairs glutamatergic transmission and affects synaptic plasticity, suggesting a functional link between stress experiences, glutamatergic dysfunction and vulnerability to depression. Furthermore, evidence in favor of the involvement of glutamate receptors in fast-acting neuroplasticity is observed already after a single dose of ketamine, which causes a rapid induction of the number and function of spine synapses in the medial PFC of naïve animals (Duman, 2014).

In conclusion, each of the aforementioned hypotheses describing the molecular basis of depression, have been well supported by meaningful experimental data. Therefore, depression is more likely a result of a combination of different disrupted pathways rather than an isolated impairment of one single system.

3. Glutamatergic system

3.1 Function and role of glutamate in the brain

Glutamate is the most abundant neurotransmitter in the brain, with over 70% of glutamatergic synapses present in all brain regions. Synthesis of glutamate takes place in neurons and glia via different pathways (Table 1), and is closely associated with the Krebs cycle and energy metabolism.

Table 1: Biosynthesis of glutamate in the brain.

Reactants	Products	Enzymes
Glutamine + H_2O	Glu + NH_3	glutaminase
α-ketoglutarate +NADPH+ NH_4^+	Glu + $NADP^+$ + H_2O	glutamate dehydrogenase
α-ketoglutarate + α-amino acid	Glu + α-keto acid	transaminase
1-Pyrroline-5-carboxylate + NAD^+ + H_2O	Glu + NADH	aldehyde dehydrogenase
N-Acetylaspartylglutamic acid	Glu + NAA	glutamate carboxypeptidase

Abbreviations: Glu: glutamate, NAA: N-acetylaspartate, NADP: Nicotinamide adenine dinucleotide phosphate, NAD: Nicotinamide adenine dinucleotide.

3.2 Glutamate receptors

Once synthesized, glutamate is stored in vesicles, ready to be released upon depolarization. After depolarization of the membrane at the presynaptic terminal, glutamate is released in the synaptic cleft, and diffuses to the postsynaptic density. Here, glutamate binds to two classes of receptors: ionotropic and metabotropic receptors, which in turn initiate the cellular modifications necessary for the propagation of the signal.

Ionotropic receptors are fast acting ligand-gated ion channels. Three types of ionotropic receptors have been identified: the NMDA receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and the kainate (KA) receptors. Each receptor is a heterotetramer and several subunit types have been characterized for each receptor. Ionotropic receptors play an important role in fast propagation and modulation of the synaptic signal; in particular, the subunits GluA1 and GluA2 have been associated with stress vulnerability and emotional behavior (Hubert et al 2014). The second class of glutamate receptors are metabotropic receptors, characterized as G-protein coupled receptors (GCPR) with seven transmembrane domains, which upon stimulation induce an intracellular signal cascade leading to molecular and cellular modifications. mGluRs are divided into groups I, II and III based on receptor

structure and physiological activity. Due to their distribution on both synaptic terminals and extrasynaptical sites, mGluRs are crucial for synaptic signal amplification or termination.

4. SLC6A15

4.1 SLC6A15 regulates glutamatergic system activity

In 2011, via a genome wide association analysis (GWAS), a new target gene was identified, which strongly correlated with hippocampal volume and glutamate levels in the hippocampus (Kohli et al., 2011). Specifically, Kohli and colleagues report for the first time a strong association between the SNP rs1545843 and depression, replicating their findings across seven different cohorts. This polymorphism is located on chromosome 12 in a regulatory region of the gene SLC6A15. The introduction of in vivo imaging techniques has made it possible to obtain data regarding molecular and morphological alterations in the brain of depressed patients expressing the risk allele. Indeed, the depression risk allele was associated with a reduction of glutamatergic metabolism and hippocampal volume, specifically in depressed patients. In this context, in vivo human research has revealed that glutamate levels in the hippocampus and hippocampal volume are hallmarks of major depression (Hasler et al., 2007; Videbech and Ravnkilde, 2004).

4.2 SLC6A15 as a novel candidate gene for depression

SLC6A15 belongs to the solute carrier family 6 (SLC6) and encodes a sodium-dependent transporter for neutral amino acids, B^0AT2 (Figure 4). B^0AT2 is an 82-kDa protein with 12 transmembrane domains and two large extracellular loops, rich in N-glycosylation sites. The substrate profile includes many neutral and hydrophobic amino and imino acids, such as proline and leucine, and strictly excludes aromatic and charged molecules, such as GABA and glutamate (Bröer et al., 2006; Takanaga, Mackenzie, Peng, & Hediger, 2005). Predominantly expressed in the brain, B^0AT2 is primarily localized to synaptic terminals of neurons. Recently, B^0AT2 positive astrocytes and ependymocytes have also been found close to the ventricles, suggesting that B^0AT2 regulates amino acids access to the brain (Hägglund et al., 2013). In mouse brain, SLC6A15 is predominantly expressed in hippocampus and frontal cortex, and to a minor extent also in hypothalamic nuclei, amygdala and the locus coeruleus (Hägglund et al., 2013), which are all crucial areas for mood regulation.

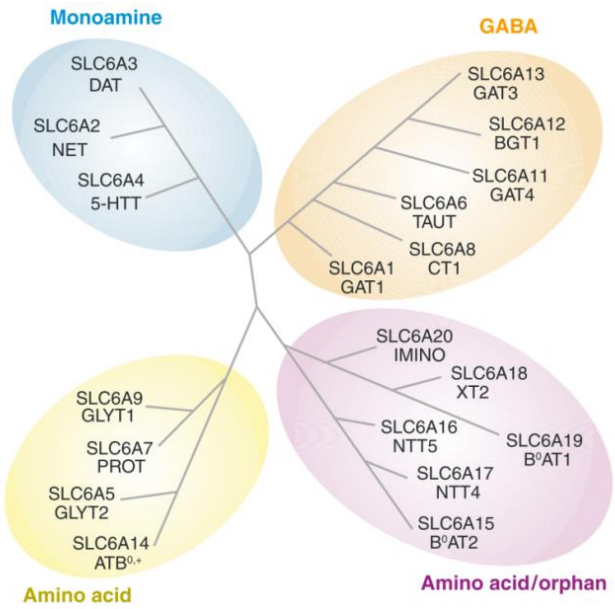

Figure 4: SLC6A gene family phylogenetic tree. Mainly localized in the brain, all the members are involved in neurotransmission. (5-HTT: serotonin transporter, ATB0+: neutral and cationic amino acid transporter, B0AT1 and 2: neutral amino acid transporters, BGT1: betaine transporter, CT1: creatine transporter, DAT: dopamine transporter, GAT1, 3, and 4: GABA transporters 1, 3, and 4, GLYT 1 and 2: glycine transporter 1 and 2, IMINO: imino acids transporter, NET: norepinephrine transporter, PROT: proline transporter, TAUT: taurine transporter, NTT4, NTT5, XT2: substrates unknown. Adapted from (Kristensen et al., 2011).

4.3 SLC6A15 and stress

Schuhmacher et al. (2013) reported that SLC6A15 risk allele carriers have enhanced HPA axis activity as well as impaired memory and attention, reinforcing the idea that SLC6A15 is linked to hippocampal function and the stress response (Schuhmacher et al., 2013). In mice, deletion of SLC6A15 did not result in an overt behavioral phenotype under basal conditions, apart from partial alteration of anxiety-like behavior after acute stress (Drgonova, Liu, Hall, Krieger, & Uhl, 2007). Taking into account its function as an amino acid transporter, it was furthermore suggested that B^0AT2 may play a role in food intake and energy metabolism. Interestingly, feeding behavior and body mass index in mice and humans are associated with the levels of SLC6A15 expression (Hägglund et al., 2013). The functional contribution and physiological significance of SLC6A15 in mood disorders is still unclear.

4.4 Effects of proline alterations in the brain

Despite a paucity of data examining the association between SLC6A15 and mood disorders, studies have previously shown that the levels of the amino acids transported by B^0AT2 are correlated with psychiatric disorders. In fact, proline, one of the main amino acids transported by SLC6A15, is involved in glutamate synthesis (Pérez-Arellano, Carmona-Alvarez, Martínez, Rodríguez-Díaz, & Cervera, 2010) (Figure 5). This is of specific interest as a number of studies have

correlated proline levels in the brain with many neurological deficits. Animal models of hyperprolinemia show glutamate-dependent alterations of sensorimotor gating and memory (Cherkin, Eckardt, & Gerbrandt, 1976; Gogos et al., 1999; Roussos, Giakoumaki, & Bitsios, 2009). Furthermore, humans with congenital abnormalities, resulting in the loss of proline dehydrogenase, the enzyme that metabolizes proline, present pathologically increased levels of proline in the blood, up to 10 times higher than control levels. As result, these patients exhibit seizures, mental retardation or other neurological problems (Illsinger, Lücke, Offner, Hartmann, & Das, 2006; Victor Nadler, Wang, & Hakim, 1988).

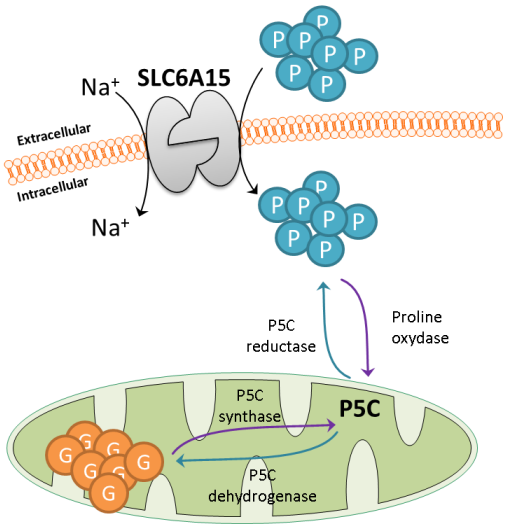

Figure 5. Glutamate synthesis from proline. Overview of the involvement of SLC6A16 in proline and glutamate levels regulation P= Proline, P5C=1-Pyrroline-5-carboxylic acid, G= Glutamate.

5. Aim of the thesis

The current thesis aims to profile individual stress vulnerability/resilience trajectories, taking into account the interaction between early life experience, adult environment and genetic background. To clarify such a complex framework, we first addressed whether socially enriched rearing affects development, and specifically coping strategies in response to chronic stressors, either environmental or psychosocial (*Chapter 1*). Afterwards, we investigated the effects of opposite rearing conditions in response to adult positive or negative environments in females (*Chapter 2*) and in males (*Chapter 3*). To address the role of genetic predisposition on brain and behavior development, we studied the role of SLC6A15 on hippocampal neurochemistry, with a special focus on the glutamatergic system (*Chapter 4*). Finally, we have explored the molecular and behavioral effects arising from the interaction between SLC6A15 and stress (*Chapter 5*).

6. Research Articles

Chapter 1	Not all stressors are equal: Early social enrichment favors resilience to social but not physical stress in male mice **Hormones and Behavior, Mar;63(3):503-9.**
Chapter 2	Evidence supporting the match/mismatch hypothesis of psychiatric disorders **European College of Neuropsychofarmacology, Jun;24(6):907-18.**
Chapter 3	The match/mismatch hypothesis of psychiatric disorders: evidence in male mice **Manuscript in preparation**
Chapter 4	The amino acid transporter SLC6A15 is a regulator of hippocampal neurochemistry and behavior **Manuscript under revision**
Chapter 5	SLC6A15, a novel stress vulnerability candidate, regulates anxiety and depressive like behaviors through glutamatergic system **Manuscript in preparation**

Not all stressors are equal: Early social enrichment favors resilience to social but not physical stress in male mice

Igor Branchi [a], **Sara Santarelli** [b], Ivana D'Andrea [c], Enrico Alleva [a]

[a] Section of Behavioral Neurosciences, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, 00161 Rome, Italy

[b] Max Planck Institute of Psychiatry, 80804 Munich, Germany

[c] Department of Neuro and Cardiovascular Pathology, Neuromed Institute — Technology Park, 86077 Pozzilli (IS), Italy

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Not all stressors are equal: Early social enrichment favors resilience to social but not physical stress in male mice

Igor Branchi [a,*], Sara Santarelli [a], Ivana D'Andrea [b], Enrico Alleva [a]

[a] Section of Behavioural Neurosciences, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, 00161 Rome, Italy
[b] Department of Neuro and Cardiovascular Pathology, Neuromed Institute – Technology Park, 86077 Pozzilli (IS), Italy

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ABSTRACT

Early experiences profoundly affect the adult coping response to stress and, consequently, adult vulnerability to psychopathologies triggered by stressing conditions, such as major depression. Though studies in animal models have demonstrated that individuals reared in different conditions are differently vulnerable to a stressor of a specific quality, no information is available as to whether such vulnerability differs when facing stressors of different qualities. To this purpose, we reared C57BL/6 male mice either in standard laboratory rearing condition (SN) or in Communal Nest (CN) condition, the latter consisting of a single nest where three mothers keep their pups together and share care-giving behavior until weaning. We scored the amount of interactions with the mother and with peers and found that CN is a form of social enrichment because both these components are significantly increased. At adulthood, we exposed SN and CN mice, for 4 weeks, to either a physical (forced swim) or a social stress (social instability). Immediately before, at week 1 and at week 4 of the stress procedure, corticosterone levels and the hedonic profile were measured. The results show that CN mice are more resilient to social stress than SN mice since they displayed no anhedonia and lower corticosterone levels. By contrast, both experimental groups were similarly vulnerable to physical stress. Overall, our results show that, in male mice, the adult vulnerability to stress changes according to the quality of the stressor, as a function of early experiences. In addition, the stressor to which CN mice are resilient is qualitatively similar to the stimuli they have experienced early on, both concerning the social domain.

Introduction

Chronic stress represents one of the most relevant risk factors for psychopathologies, including major depression (Kendler et al., 1995). This is supported by data showing that depression is often associated with an increase of corticotropin-releasing hormone (CRH) levels (Nemeroff et al., 1984) or, more in general, an altered function of the hypothalamic–pituitary–adrenal (HPA) axis (Holsboer, 2000; Ising et al., 2007). Furthermore, the exposure to stressful events has been reported to be one of the most powerful triggering factors for depressive episodes (Heim et al., 2009). However, the consequences of exposure to stress are not predictable just by knowing the magnitude of the stressor because vulnerability differs among individuals. For instance, while a serious life-threatening stress may not affect some individuals, a milder stress may trigger depression in others (Southwick and Charney, 2012). Recent studies have also demonstrated that individuality manifests itself according to the stressor-specific domain. In particular, different individuals show different degrees of HPA axis activation when exposed to qualitatively different stressors — *e.g.*, non-social *vs.* social stimuli (Kertes et al., 2009).

Inter-individual differences in stress response are determined by the genetic set up (Ising and Holsboer, 2006; Rijsdijk et al., 2003) and the environment in which the individual develops and lives (Taylor et al., 2004). With regard to the latter, several studies have illustrated its pervasive influence, especially during early postnatal phases, in determining the reactivity of the HPA axis at adulthood. For instance, a history of childhood abuse downregulates the HPA axis response, increasing vulnerability to stress, both at short- and long-term (Heim and Nemeroff, 2001; Lupien et al., 2009). In addition, children raised in families characterized by unsupportive relationships, emotional neglect or conflict are at increased risk of showing disruptions in stress-responsive biological regulatory systems, increasing the likelihood of developing psychiatric disorders (Repetti et al., 2002).

Animal models have been widely used to investigate the impact of the early social environment on the adult response to stress. For instance, seminal studies by Levine and others have shown that exposing rodent pups to handling, which consists in brief periods of separation from the mother for the first two post-natal weeks, reduces the HPA axis activation in response to stressful stimuli of the adult offspring (Cirulli et al., 2003; Levine, 1957; Macrì and Wurbel, 2006; Meaney, 2001; Oitzl et al., 2010; Pryce et al., 2005). These

* Corresponding author at: Section of Behavioural Neurosciences, Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy. Fax: +39 06 4957821.
E-mail address: branchi@iss.it (I. Branchi).

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animals also display increased exploration, reduced defecation and urination in an open field (Levine, 1957), and a reduced taste neophobia and conditioned taste aversion (Macrì et al., 2004; Weinberg et al., 1978). These studies indicate that individuals reared in different conditions are differentially vulnerable to the same stressor. However, to our knowledge, it has not been investigated whether vulnerability to stress differs when facing stressors of different qualities and whether such differential vulnerability is affected by the environment experienced during the first post-natal phase.

In the present work, we reared C57BL/6 male mice in two conditions — either the standard laboratory rearing condition (SN) or the communal nest (CN). CN consists in a single nest where three mothers keep their pups together and share care-giving behavior from birth to weaning. In this rearing condition, maternal care has been reported markedly increased (Branchi et al., 2006). Here, we scored during the first 2 weeks of life not only the amount of maternal care, but also that of peer interactions to investigate whether both these two components of early social stimulation are increased in the CN compared to the SN condition. Furthermore, as a main aim, we analyzed the coping response of SN and CN mice, in terms of adult corticosterone levels and anhedonic profile, following exposure to two qualitatively different stressors: one with a main social component and the other being predominantly physical (Fig. 1). Previous data have shown that adult CN mice display reduced anhedonia and decreased activation of the HPA axis following exposure to social stress compared to SN mice (Branchi et al., 2010; Cirulli et al., 2010). No data are available about the coping response to physical stress in CN mice and, in particular, about the coping responses to qualitatively different stressful conditions in SN and CN individuals.

Methods and materials

Animal and breeding procedure

Twenty male and 40 female C57BL/6 mice, an inbred strain were purchased from a commercial breeder (Harlan, 20050 Correzzana, MI, Italy). Upon arrival at the laboratory, the animals were housed in an air-conditioned room (temperature 21 ± 1 °C, relative humidity 60 ± 10%) with lights on from 05:00 to 17:00 h. Males and females were housed in same-sex groups of 5–6 individuals in 42 cm × 27 cm × 14 cm Plexiglas boxes with a metal top and sawdust as bedding, and with pellet food (Enriched standard diet purchased from Mucedola, Settimo Milanese, Italy) and tap water *ad libitum*. All animal handling and experimental procedures were performed according to European Communities guidelines (EC Council Directive 86/609) and Italian legislation on animal experimentation (Decreto L.vo 116/92).

Nesting condition

After 1 week of acclimatization, breeding groups (1 male and 2 females), were formed and housed in 33 cm × 13 cm × 14 cm Plexiglas boxes. Vaginal plugs were checked twice a day (at 09.00 h and 19.00 h). The male was removed on pregnancy day 12. According to the expected delivery (calculated on the basis of vaginal plug detection), mice were assigned to one of the two experimental groups: standard nesting (SN) or communal nesting (CN). For the SN group, 10 females were housed in 33 cm × 13 cm × 14 cm Plexiglas boxes.

For the CN group, 30 females were combined in trios 5 days before delivery in order to have each trio giving birth on the same day. Each trio was housed in a 42 cm × 27 cm × 14 cm Plexiglas boxes. Both in the SN and CN group, each litter was culled on the day after birth, postnatal day (PND) 1 (birth = PND 0), to six males and two females.

Pups were weaned on PND 25, and males of each litter were housed in groups of 6 animals in 42 cm × 27 cm × 14 cm Plexiglas boxes. Females were not used in this study. All behavioral studies were performed when mice were 8-month old and each behavioral test was preceded by 45–60 min of acclimatization to the experimental room. Mice were weighed at the end of the test.

Maternal behavior

Eight SN and nine CN cages were observed from PND 1 to PND 12. Maternal behavior was scored during two sessions each day. During each session, data were collected with one–zero sampling, over 30 10-s observations that were 180 s apart. The experimenter recorded whether the behavior was present or not during the 10-s observation; more than one behavior could be present and thus recorded during the same 10-s observation. The sessions started at 10:00 and 18:30. The 18:30 session was during the dark phase of the 12:12 cycle and was performed under dim red light illumination.

Maternal behavior has been analyzed according to previous work (Branchi et al., 2006). In particular, the following behaviors were scored: *arched-back nursing*: the dam is immobile and in a high upright dorsal arch posture supported by rigid fore- and hind limbs, the head is depressed, the trunk and limbs are bilaterally symmetrical, and pups are attached to the nipples; *blanket nursing*: the dam is over the pups, relatively immobile, bilaterally symmetrical, with the head not depressed, and in a low dorsal arch posture supported by rigid fore- and hind limbs or in a low dorsal arch posture supported by rigid fore limbs or rigid hind limbs or lies flat on top of the pups with little or no limb support; *passive nursing*: the dam body is lying down on her side with more than one pup usually attached to the nipples; *total nursing*: the sum of the three nursing positions; *licking/grooming*: licking and grooming of the pup body; *ano-genital licking*:

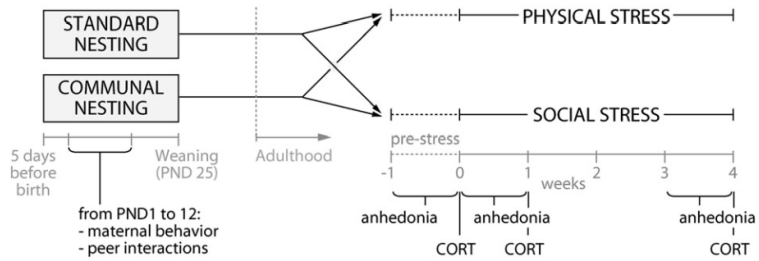

Fig. 1. Rearing conditions and experimental procedure. Scheme detailing the communal nesting manipulation and the timing of the experimental procedures. During the early post-natal phase (PNDs 1–12), the amount of maternal behavior and peer interactions have been scored. At adulthood, corticosterone levels were measured 1 day before and 1 and 4 weeks after the beginning of the chronic stress procedure. Anhedonia was assessed during the week before and the first and fourth week after the beginning of the chronic stress procedure.

licking concentrated on the ano-genital region of the pup; *licking/grooming* and *arched-back nursing* (*LG–ABN*): the sum of arched back nursing and licking/grooming; *retrieval*: the dam picks up the pup gently with the incisors by its dorsal skin and carries it to the nest; *digging*: digging in the sawdust, moving it around using the snout and/or both the forepaws and hindpaws, mostly moving around the cage and sometimes changing the arrangement of the substrate material; *rearing*: the animal stands on its hind limbs, often *sniffing*; moving: the animal moves around the cage, actively exploring; eating: *nibbling* food pellets held in its forepaws or held in the food-containing compartment of the cage; *drinking*: drinking from the water bottle; *self-grooming*: the animal licks, combs, scratches any part of own body; *resting*: the animal lies still in a sleeping mode; and *out of nest*: moving about the cage but not carrying pups or nesting material.

Peer interactions

Eight SN and nine CN cages were observed from PND 1 to PND 12. Sibling social interaction behavior was scored during two daily observation sessions using the same sample protocol used for maternal care observations. The sessions started at 11:00 and 19:30. The second session was during the dark phase of the 12:12 cycle, and was performed under dim red light illumination.

Pup to pup behavior has been analyzed according to previous work (Branchi et al., in press). Briefly the behavioral categories scored were (i) nutritive behavior: *eat* and *drink*; (ii) competitive behavior: *tenacious attachment* (pup clings to the nipple), *pushing* (pup pushing another individual to gain access to a resource), and *replacing* (pup displaces another individual from the resource); (iii) social behavior: *allogrooming* (pup is licking/grooming another individual) and *allosniffing* (pup is sniffing another individual); and (iv) general activity: *active* (any locomotor activity), *resting* (pup lying still), and *out of nest*.

Stress procedures and saccharin preference

Twelve SN and twelve CN were assigned to social stress condition. Animals were housed in the Intellicage (NewBehavior AG, Zürich, Switzerland). This is an apparatus for automatic monitoring of mouse behavior in their home-cage. This system is able to score the activity and behaviors of each individual living in a social group since every animal is identified by a subcutaneous transponder. An Intellicage consists of a large acrylic rat cage (20.5 cm high, 58 cm × 40 cm at the top and 55 cm × 37.5 cm at the base, Model Tecniplast 2000) with 4 walls separating each corner from the center so that they form 4 identical chambers to which mice have free access by entering a front hole (for a detailed description of the system see Branchi et al., 2010; Knapska et al., 2006; Mechan et al., 2009). Very briefly, Intellicages collect data about (i) number and duration of visits in the four corners (exploratory activity); (ii) number, duration and side (right or left) of nosepokes. Five days before being moved to the Intellicages, each animal was injected with a subcutaneous transponder (T-IS 8010 FDX-B; Datamars SA, Switzerland). During the entire experiment, the food was freely available. The first 6 days the animals were habituated to the Intellicage environment and to the 0.1% of saccharin solution.

Social stress consisted in modifying the composition of the social groups housed in the Intellicages. Ten male mice were housed in each Intellicage. Each day during the 4-weeks social stress procedure, 4 individuals (2 SN and 2 CN mice) were moved from Intellicage 1 to Intellicage 2 and 4 other individuals (2 SN and 2 CN mice) were moved from Intellicage 2 to Intellicage 1. This condition forced the animals to reorganize their social structure every day, imposing them a stressful condition. To assess saccharin preference, in each corner, 2 bottles were present, one containing tap water and the other containing fresh 0.1% saccharin solution; both were freely available 24/24 h. Water and saccharin solutions were substituted every 2 days. The position of water and saccharin in each corner was balanced across the 4 corners.

Twelve SN and twelve CN were assigned to the physical stress group, that consisted in daily swim sessions for 4 weeks: each experimental subject was gently placed into a cylindrical glass container (20 cm diameter, 40 cm height), filled with 25 cm of water at a temperature of 21 ± 1 °C for 10 min with a dim light illumination (1 lx). When removed from the water, the mouse was allowed to dry for 5 min under red light. Animals were single housed, for the first 6 days the animals were habituated to the new environment and to the 0.1% of saccharin solution. In each cage 2 bottles were present, one contained tap water and the other contained a daily fresh 0.1% saccharin solution; both were freely available 24/24 h. Their relative position (left *vs.* right) was random and changed every day. Fluid consumption was monitored on a daily basis by weighing the bottles. Food was freely available. Saccharin preference was determined as follows: (sucrose solution consumed / sucrose solution consumed + water consumed) × 100.

Corticosterone levels

Corticosterone levels were measured 1 day before and 1 and 4 weeks after the beginning of the chronic stress procedure. Blood was collected 1 h before lights on that is when corticosterone is at the lowest levels during the circadian profile. Blood was collected from the tail. The bleeding procedure consisted in a small and superficial cut in the tail to collect a blood sample. Blood samples were collected individually in potassium–EDTA coated 10 ml tubes (1.6 mg EDTA/ml blood; Sarstedt, Germany). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at +4 °C. Blood plasma was transferred to Eppendorf tubes for corticosterone determination and stored at −20 °C until further analysis. Corticosterone was measured using a commercially available radio-immunoassay (RIA) kit containing 125iodine labeled corticosterone (MP Biomedicals Inc., CA, USA). Vials were counted for 2 min in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000). Sensitivity of the assay was 0.125 mg/dl, inter- and intra-assay variation was less than 10% and 5%, respectively.

Statistical analysis

All data were analyzed with ANOVAs, considering rearing condition (standard *vs.* communal) and stress condition (physical vs. social) as between-subject variables and subject as a random factor nested within rearing condition; time (day) as repeated measures within subjects. *Post hoc* comparisons were performed using the Tukey's HSD test (statistical software Statview II, Abacus Concepts, CA, USA).

In all the analyses, the cage was considered as the statistical unit in order to take into account litter effects. When more individuals (*e.g.*, pups) from the same cage and experimental group were considered, the average value was used in the analysis.

Effect size was calculated by partial eta squared for ANOVA results (SPSS, IBM Corporation) and by Cohen's d index for pairwise comparisons (Cohen, 1992).

Results

Maternal behavior

In agreement with previous works (Branchi et al., 2006), higher levels of maternal care were shown in the CN condition compared to SN one. CN mothers performed higher levels of total nursing compared with SN ones [$F(1,15) = 811.68$, $p < 0.0001$; partial eta squared = 0.98]. In particular, CN mothers showed higher levels of LG–ABN [$F(1,15) = 132.35$, $p < 0.0001$; partial eta squared = 0.91], higher levels of passive nursing [$F(1,15) = 82.16$, $p < 0.0001$; partial eta squared = 0.85] and higher levels of blanket nursing [$F(1,15) = 91.26$, $p < 0.0001$; partial eta squared = 0.86]. Interaction nesting × days reached statistical significance for all maternal behaviors analyzed, respectively, for total

nursing [F(11,165) = 6.08, $p<0.0001$; partial eta squared = 0.29], for LG–ABN [F(11,165) = 11.95, $p<0.0001$; partial eta squared = 0.49], for passive nursing [F(11,165) = 2.62, $p = 0.004$; partial eta squared = 0.17], for blanket nursing [F(11,165) = 4.32, $p<0.0001$; partial eta squared = 0.22]. *Post hoc* analyses revealed that CN mothers perform higher levels of total nursing from PNDs 1 to 12 ($p<0.01$; Cohen's d > 3.8) with the exception of PND 9 ($p<0.05$; Cohen's d = 1.6), higher LG–ABN from PNDs 1 to 8 ($p<0.01$; Cohen's d > 1.2); higher passive nursing on PNDs 1, 4, 7, 8, 10, 11 and 12 ($p<0.01$; Cohen's d > 2.4), and higher blanket nursing on PND 2 ($p<0.05$; Cohen's d = 1.2) and on PNDs 3, 4, 5 10, 11, and 12 ($p<0.01$; Cohen's d > 2.8) (Fig. 2).

Peer interactions

Overall, CN pups showed significantly higher levels of social interaction with siblings. In particular, a main effect of nesting has been found for replacing behavior [F(1,15) = 13.11, $p = 0.002$; partial eta squared = 0.47] and tenacious attachment [F(1,15) = 8.04, $p = 0.01$; partial eta squared = 0.35]. In addition, significant interactions nesting × days were found for replacing, pushing and total interactions [respectively Fs(11,165) = 2.50, 6.84, and 6.36; $p = 0.007$, $p<0.0001$, and $p<0.0001$; partial eta squared = 0.31, 0.14, and 0.20]. *Post hoc* analyses revealed that CN mice perform higher total pups social interaction on PND 4 ($p<0.01$; Cohen's d = 0.4) and on PNDs 5, 6 and 7 ($p<0.05$; Cohen's d > 1.6), instead SN showed higher levels on PND 12; CN mice showed significantly higher levels of pushing behavior on PND 4 ($p<0.01$; Cohen's d = 4.5) and on PND 6 ($p<0.05$; Cohen's d = 1.9), but significantly lower levels on PND 12 ($p<0.05$; Cohen's d > 1.8); replacing behavior is significantly higher in CN mice on PND 4 ($p<0.05$; Cohen's d = 1.2) and on PNDs 5, 6 and 8 ($p<0.01$; Cohen's d > 1.2). Though the interaction nesting × days miss to reach statistical significance for tenacious attachment [F(11, 165) = 1.48, $p = 0.14$; partial eta squared = 0.09], *post hoc* analysis revealed that CN mice show higher levels of this behavior on PND 11 ($p<0.05$; Cohen's d = 1.1) (Fig. 3).

Corticosterone levels

Social stress affected HPA axis activity as shown by corticosterone levels after 1 week of stress in both experimental groups. Interestingly, after 4 weeks of stress CN mice showed reduced activation of the axis, demonstrating a better coping strategy compared to SN, as measured by the interaction of time × nest condition [F(1,15) = 6.42; $p = 0.005$; partial eta squared = 0.3]. In particular, *post hoc* analyses revealed that CN mice had significantly lower corticosterone levels after 4 weeks of social stress ($p<0.05$; Cohen's d = 1.6). Interestingly, SN and CN mice did not show any difference in corticosterone levels after physical stress, both groups showing an significant increase after 4 weeks of stress [F(1,15) = 10.28; $p = 0.0004$; partial eta squared = 0.41] (Figs. 4 and 5). Corticosterone pre-stress levels in the two groups were not overlapping because of the different pre-stress housing conditions: mice exposed to social stress were socially housed in the Intellicage, mice exposed to physical stress were housed in isolation in standard cages.

Saccharin preference

The main effect of nesting was significant for the preference for saccharin solution [F(1,14) = 5.15, $p = 0.04$; partial eta squared = 0.223], and the interaction nesting × stress was significant [F(2,36) = 3.64, $p = 0.04$; partial eta squared = 0.168]. *Post hoc* analyses revealed that CN mice showed a significant lower levels of anhedonia after 4 weeks of stress ($p<0.01$; Cohen's d = 1.0). As for corticosterone levels, the two experimental groups did not show any difference in saccharin preference during physical stress (Figs. 4 and 5).

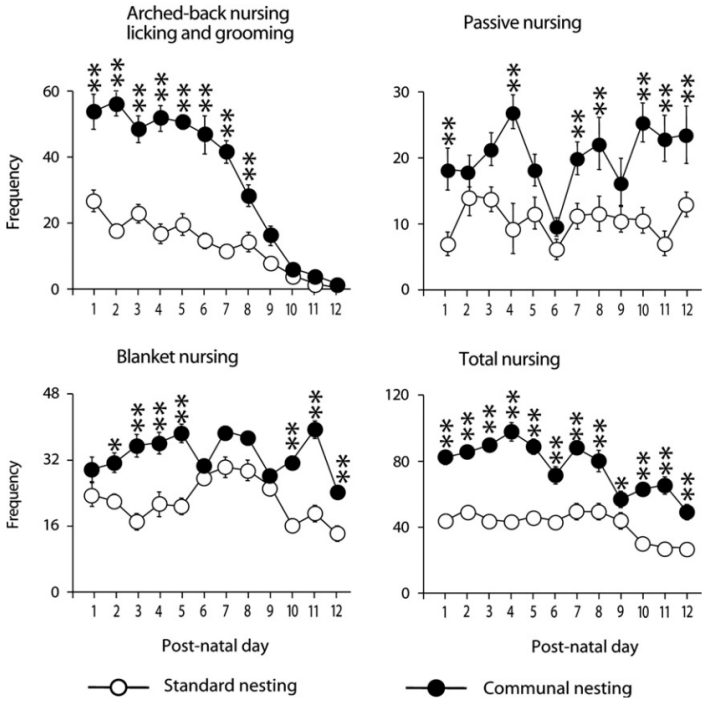

Fig. 2. Maternal care during the first 12 days of life. Levels of maternal care were higher in the communal nesting condition compared to standard nesting condition. In particular, mothers showed an overall higher level of nursing (*i.e.* total nursing). * and ** = respectively, $p<0.05$ and $p<0.01$ *vs.* standard nesting. Data shown are means ± S.E.M.

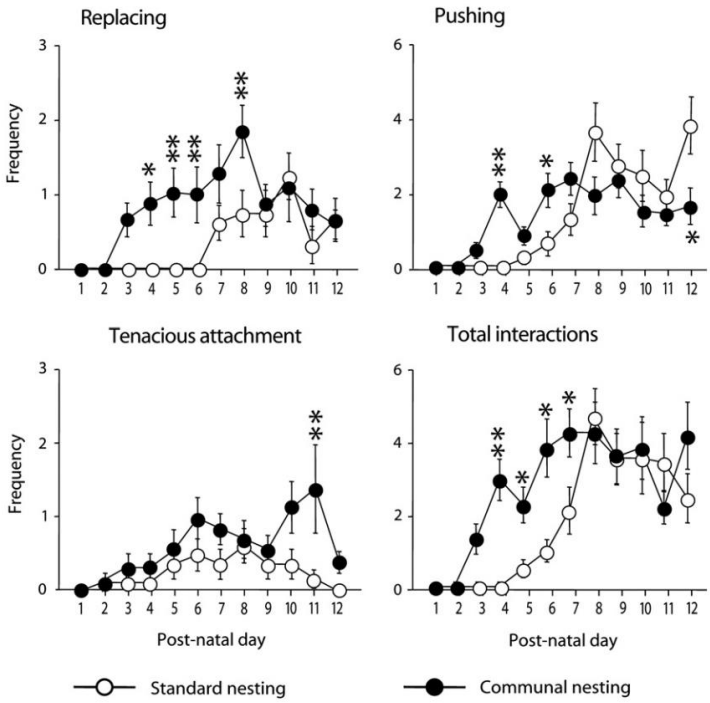

Fig. 3. Peer interactions during the first 12 days of life. Communal nesting pups showed higher levels of peer interactions particularly for those behaviors involving a direct competition (replacing and pushing). They showed an overall higher level of interactions (*i.e.* total interactions). * and ** = respectively, $p<0.05$ and $p<0.01$ *vs.* standard nesting. Data shown are means ± S.E.M.

Discussion

The present findings demonstrate that being reared in a CN provides the developing pup with a highly stimulating social enrichment. In particular, such a rearing condition leads to an increase in the levels of both main social components of the early environment — maternal care and also peer interactions. Furthermore, here we show that SN and CN rearing experiences differentially affect the adult ability to cope with different qualities of stressors, measured in terms of corticosterone levels and anhedonic response. While CN, but not SN male mice, were resilient to a social stress, both experimental groups were equally vulnerable to physical stress.

In line with previous results (Branchi et al., 2006), here we show that CN rearing entails higher levels of maternal behavior — particularly arched back-nursing, licking and grooming. The critical role played by maternal behavior in shaping the adult individual has been widely described in the literature (Champagne and Curley, 2005; Cirulli et al., 2003; Liu et al., 1997; Meaney, 2001). In particular, it has been shown that the behavior displayed by the mother affects several aspects of the offspring physiology and behavior at adulthood, including

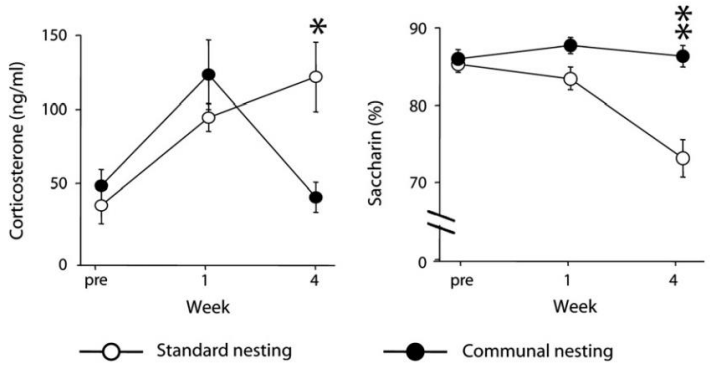

Fig. 4. Vulnerability to chronic social stress. Communal nesting mice were less vulnerable to social stress compared to standard nesting mice. In particular, at the end of the 4-week social stress procedure, communal nesting mice showed significantly lower corticosterone levels and higher saccharin preference. * = $p<0.05$ *vs.* standard nesting. Data are means ± S.E.M.

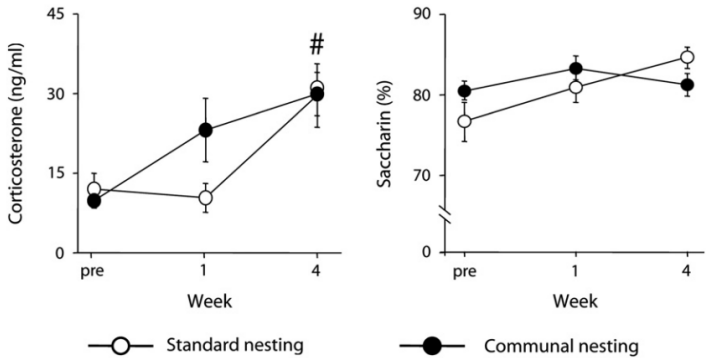

Fig. 5. Vulnerability to chronic physical stress. No difference in coping response to physical stress between communal nesting and standard nesting was found. At the end of the 4-week physical stress procedure, the two experimental groups showed similar corticosterone levels and saccharin preference. * = $p<0.05$ *vs.* standard nesting. Data are means ± S.E.M.

anxiety-like and depression-like behavior (Caldji et al., 1998; Cirulli et al., 2010; Macrì and Wurbel, 2006).

A growing literature is showing that the early social environment is characterized by the interaction not only with the mother but also with peers, and that the latter affects the developmental trajectories as well (Benus and Henkelmann, 1998; Branchi, 2009; Branchi et al., in press; Laviola and Alleva, 1995; Macrì et al., 2010; Yang et al., 2011). Consequently, the increased levels of interactions with age-mates found in CN, compared to SN, are expected contributing to shape the adult neurobehavioral profile. Indeed, modifications in early peer interactions have been shown to have long-term consequences on several neurobehavioral domains, such as social behavior and emotional response (Branchi et al., 2006, in press; Hudson, 2005; Laviola and Alleva, 1995; Terranova and Laviola, 1995). The relevance of early interaction with peers on neurobehavioral development has been investigated in Rhesus monkeys (Cirulli et al., 2009; Suomi, 2005). In this species, peer social stimulation during plastic post-natal phases leads to the development of sophisticated social behavior and emotional responses (Harlow, 1969; Harlow and Suomi, 1971; Suomi, 1979). Monkeys reared by their mothers but totally deprived of peer contact fail to develop essential social skills, such as the ability to play with peers or to cope with aggression, and traverse abnormal developmental trajectories (Harlow, 1969). In addition, infant rhesus monkeys from groups with few opportunities to interact with non-kin age-mates show less frequent play behavior, briefer in duration, and less positive in affective tone and, at adulthood, their relationships with non-kin age-mates are relatively infrequent and often hostile (Berman et al., 1997; Suomi, 2005).

The long-term effects of different early experiences on the adult coping response to a single quality of stressor (*e.g.*, either restraint or social stress) have been described and compared (Branchi et al., 2010; Caldji et al., 1998; Denenberg, 1964; Giachino et al., 2007; Macrì and Wurbel, 2006). However, no studies have so far assessed the differential adult coping response to stressors of different qualities in subjects exposed to the same experience early on.

During lifespan, individuals experience multiple stressors that may potentially lead to disease (Cohen et al., 2007). Stressors can be divided into two main categories: social ones associated with interactions with conspecifics (*e.g.*, competition and aggression) and non-social ones (*e.g.*, extreme environmental conditions). The present results show that an adult individual can show different vulnerabilities to different qualities of stressors, according to her/his early experiences. Only the SN group demonstrates to be vulnerable when exposed to a chronic social stressor – such as a continuous modification of the social group that hampers to establish a hierarchy and thus leads to social instability – showing significantly higher levels of corticosterone and increased anhedonia. By contrast, when exposed to a chronic physical stressor, both experimental groups exhibit a similar stress response, displaying increased corticosterone levels. In addition, they did not differ in their anhedonic response. In particular, none of the two showed any anhedonia. Such lack of anhedonia following physical stress may be due to the isolation condition that reportedly increases the preference for the palatable solution (Hall et al., 1998; Van den Berg et al., 2000).

It is worth noticing that the quality of the stressor to which CN male mice are resilient is similar to the quality of the stimuli they have experienced early on, *i.e.* both concern the social domain. This is in line with previous studies showing that those domains selectively affected by early experiences show differential functionality at adulthood (D'Andrea et al., 2007; Greenough et al., 1987; Rochefort et al., 2002; von Senden, 1960). For instance, CN early stimulation specifically affects the adult social behavioral domain but not learning and memory abilities (D'Andrea et al., 2007). Another study demonstrated that mice housed in an odor-enriched environment for 40 days have an enhanced survival of newly generated neurons in the subventricular zone migrating through the olfactory bulb, but not in the hippocampus, and display improved olfactory learning, but not spatial learning (Rochefort et al., 2002). Further studies are warranted to investigate whether in our model an early physical stimulation selectively affects resilience to physical stress at adulthood.

Since stress is one of the major triggers of different psychopathologies, including major depression, the identification of different vulnerabilities to stressors concerning different domains has important clinical implications. In particular, this is highly relevant in light of those theories connecting early life with later diseases such as the *match–mismatch* hypothesis (Gluckman and Hanson, 2004; Godfrey and Barker, 2001; Godfrey et al., 2007; Schmidt, 2011). This hypothesis poses that the growing individual uses environmental cues to gather information about the characteristics of its future environment and to consequently adjust its neurobehavioral profile. The efficacy of this strategy to "predict the future" is based on the assumption that the features of the environment do not change during a lifetime (Bateson et al., 2004). When a match between the predicted and actual adult environment occurs, the developmental trajectories will lead the organism to be better adapted to face the environment at adulthood. Conversely, when a mismatch occurs, predictions will result inappropriate and will be potentially pathogenic (Gluckman et al., 2005). In this framework, the present data suggest that early experiences may promote selected adaptive processes to cope later with qualitatively specific external challenges than just allowing to probe the environment as generally favorable or threatening. Thus, therapeutic interventions to counteract the consequences of stress at adulthood, including the onset of stress-precipitated diseases such as major depression, may be more effective when focused on specific domains according to the early experiences.

Overall, the present findings show that, in male mice, experiences during critical developmental periods shape not only the magnitude of the adult coping response to stress, as previously widely reported (Champagne and Meaney, 2006; Cirulli et al., 2010; de Kloet et al., 2005; Levine, 2005), but also its specificity. In our CN model, changes in the response to stress at adulthood concern stressors qualitatively overlapping with the stimulation associated to the early experiences, so that adult coping responses to social stress, but not to physical stress, are modified. Such differential vulnerability to different stressors so far has been only limitedly explored in humans (Kertes et al., 2009).

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Evidence supporting the match/mismatch hypothesis of psychiatric disorders

Sara Santarelli[1], Sylvie L Lesuis[1], Xiao-Dong Wang[2], Klaus V Wagner[1], Jakob Hartmann[1], Christiana Labermaier[1], Sebastian H Scharf[3], Marianne B Müller[1], Florian Holsboer[1], Mathias V Schmidt[1]

[1] Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, 80804 Munich, Germany

[2] Institute of Mental Health, Peking University, Beijing, China, Key Laboratory for Mental Health, Ministry of Health (Peking University), Beijing, China.

[3] F.Hoffman-La Roche AG, Pharma Research & Early Development, Discovery Neuroscience, Grenzacherstrasse 124, CH-4070 Basel, Switzerland

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Evidence supporting the match/mismatch hypothesis of psychiatric disorders

Sara Santarelli[a,*], Sylvie L. Lesuis[a], Xiao-Dong Wang[b,c], Klaus V. Wagner[a], Jakob Hartmann[a], Christiana Labermaier[a], Sebastian H. Scharf[d], Marianne B. Müller[a], Florian Holsboer[a], Mathias V. Schmidt[a]

[a]*Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany*
[b]*Institute of Mental Health, Peking University, Beijing, China*
[c]*Key Laboratory for Mental Health, Ministry for Mental Health, Peking University, Beijing, China*
[d]*F.Hoffman-La Roche AG, Pharma Research & Early Development, Discovery Neuroscience, Grenzacherstrasse 124, CH-4070 Basel, Switzerland*

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Abstract
Chronic stress is one of the predominant environmental risk factors for a number of psychiatric disorders, particularly for major depression. Different hypotheses have been formulated to address the interaction between early and adult chronic stress in psychiatric disease vulnerability. The match/mismatch hypothesis of psychiatric disease states that the early life environment shapes coping strategies in a manner that enables individuals to optimally face similar environments later in life. We tested this hypothesis in female Balb/c mice that underwent either stress or enrichment early in life and were in adulthood further subdivided in single or group housed, in order to provide aversive or positive adult environments, respectively. We studied the effects of the environmental manipulation on anxiety-like, depressive-like and sociability behaviors and gene expression profiles. We show that continuous exposure to adverse environments (matched condition) is not necessarily resulting in an opposite phenotype compared to a continuous supportive environment (matched condition). Rather, animals with mismatched environmental conditions behaved differently from animals with matched environments on anxious, social and depressive like phenotypes. These results further support the match/mismatch hypothesis and illustrate how mild or moderate aversive conditions during development can shape an individual to be optimally adapted to similar conditions later in life.

*Corresponding author. Tel.: +49 89 30622 576; fax: +49 89 30622 610.
E-mail address: sara_santarelli@mpipsykl.mpg.de (S. Santarelli).

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1. Introduction

Chronic stress is one of the predominant environmental risk factors for a number of psychiatric disorders, particularly for major depression (De Kloet et al., 2005). While it has been predicted that depression will be the leading cause for burden of disease worldwide by 2030 (WHO/Wonca, 2008), the underlying mechanisms of the disease etiology are still unclear. One important aspect, related to stress and depression vulnerability, lies in different coping strategy repertoires of each individual (Koolhaas et al., 1999), or in other words, how could similar stressors exert a very wide spectrum of effects in different individuals. These differences seem grounded in both genetic background and specific environmental conditions (Caspi et al., 2003).

The interplay between experiences during sensitive developmental periods and the later adult environment seems to be a crucial factor in shaping individual variability in stress coping strategies. There are two main hypotheses that have been formulated to address this interaction. The cumulative stress or multiple hit hypothesis states that higher numbers of stressful events during early stages of life increase vulnerability to stressors later in life (McEwen, 2003). Accordingly, the effects of a environment are additive and result in an increased allostatic load, which in turn leads to a higher risk for developing psychiatric disorders. More recently, the match/mismatch hypothesis of psychiatric disease has been formulated, which states that the early life environment shapes coping strategies in a manner that enables individuals to optimally face similar environments later in life (Belsky and Pluess, 2009; Ricon et al., 2012; Schmidt, 2011). From an ethological and evolutionary perspective, this last hypothesis is plausible: individuals raised in a stressful environment will likely also face a stressful environment in adulthood and need to be adapted to deal with this aversive situation. This conceptual framework could also help to explain many cases in which individuals that are exposed to high levels of stress show comparable behavioral performances to individuals who have never exposed to stress (Champagne et al., 2008; Oomen et al., 2010). However, it still remains unclear whether and under which conditions early programming will result in adaptive or maladaptive coping reactions during adulthood.

While the cumulative stress and the match/mismatch hypothesis seem to be conceptually different and mutually exclusive, there is substantial clinical and preclinical evidence for both. It was therefore proposed that both hypotheses may be integrated and could be applicable depending e.g. on the genetic background of the individual (Nederhof and Schmidt, 2012). In this framework, individuals with a highly inert adaptive capacity would suffer more under mismatched environments, while individuals with a low capacity would have the highest disadvantage following cumulative stress exposures. For example it could be hypothesized that females under different estrous cycles could differ in adaptive capacity, which is also supported by reports on varying stress or drug effects according to the estrous phase (Frye and Walf, 2002; Mourlon et al., 2011; Palanza et al., 2001).

This would apply for example to the important aspect of gender, as women are reported to suffer more often from depression than men (Holden, 2005; Kessler, 2003). In particular, women can be more responsive to environmental challenges, according to different phases of their menstrual cycle, as indicated by the recognition of the premenstrual dysphoric disorder (PMDD) as a disorder in the recent DMS V and its short term treatment with SSRIs (American Psychiatric Association, 2013).

A number of genetic risk factors for stress-related psychiatric disorders were described, including some polymorphisms of genes encoding for neurotrophic factors. Differences in expression levels of brain plasticity genes may lead to different behavioral responses to the same load of stress. This generates subsets of individuals that are able to effectively respond to environmental changes, while some others show no adaptation or no response at all, leading to maladaptation. In particular, *brain-derived neurotrophic factor* (BDNF), a brain plasticity marker, has been extensively investigated and correlated with stress response and major depression (for review see Duman and Monteggia, 2006). Interestingly in the context of adaptation, BDNF showed an U-shaped curve correlation with corticosterone, where moderate levels of corticosterone are inducing an increase in BDNF levels, whereas high levels of corticosterone do not induce any change in BDNF (Schaaf et al., 1997). In general, high levels of corticosterone and reduced levels of BDNF are both associated with increased levels of stress; however it has been shown that rats subjected to moderate concentrations of corticosterone, BDNF levels are increased whereas at higher concentration of corticosterone BDNF levels return comparable to control levels (Schaaf et al., 1997). This and similar findings are suggesting that the correlation between stress effects and "stress markers" is not always linear, but rather follows a U shaped curve distribution.

Furthermore, other neuronal systems have been correlated with stress vulnerability. Many candidates have been identified, but often the results were contradictory or not reproducible (for an example (Karg et al., 2011) further commented in (Hardy and Low, 2011; Blakely and Veenstra-VanderWeele, 2011). Recently SLC6A15, a neuron-specific neutral amino acid transporter (also known as v7-3 or B^0AT2), was proposed as a strong candidate gene for vulnerability to stress and major depression (Kohli et al., 2011; Schuhmacher et al., 2013).

To test the match/mismatch in individuals with different levels of responsiveness to stress effects, we here studied the interaction of early life experience and adult environment in the development of anxiety-like and depression-like behavior in female Balb/c mice. We hypothesized that animals which experienced either matched supportive environments or matched aversive environments (i.e. matched environments), would exhibit lower anxiety-like and depressive-like behavior compared to animals with mismatched early and adult life environments. Furthermore, we investigated the expression of stress vulnerability related genes in the brain, like BDNF and SLC6A15.

2. Experimental procedures

2.1. Animals and breeding procedures

Male and female Balb/c mice (n=30/sex) were purchased from Charles River, Germany. The experiments were carried out in accordance with European Communities Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during

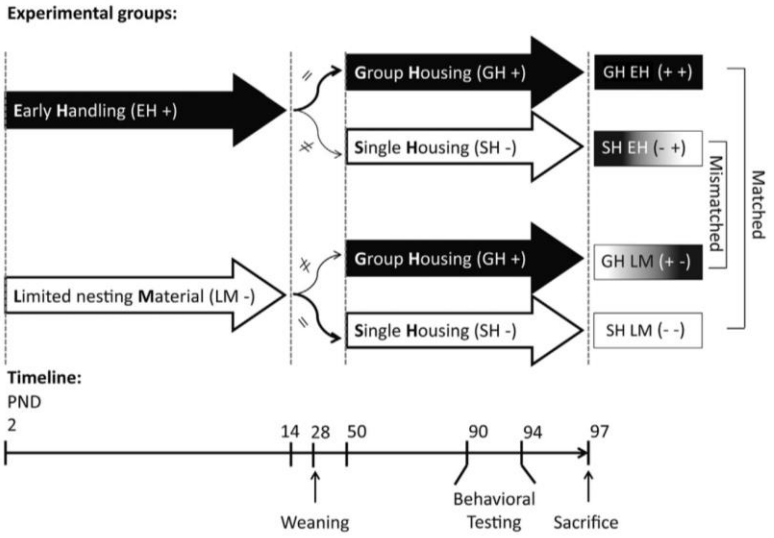

Figure 1 Experimental groups. The chosen experimental design results in four experimental groups: two matched groups (positive: early handled/group housed, GH EH + +; negative: limited nesting and bedding material/single housed, SH LM − −) and two mismatched groups (with early enriched environment: SH EH, − +, and with aversive early environment: GH LM, + −). Timeline. PND2 litters were assigned randomly to EH or LM. On PND14, all animals were returned to standard housing conditions. PND50 animals were assigned to either SH or GH. Behavioral tests were performed between PND90 and PND94. All animals were sacrificed on PND97.

the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany. Food and water were provided ad libitum. Upon arrival, animals were single housed in an air conditioned room with constant temperature (23±2 °C) under 12 h light and 12 h dark cycle conditions (lights on at 0800 h). After 1 week of acclimatization, breeding pairs (one male and one female mice) were formed. 16 days later, the male was removed from the cage.

2.2. Experimental design

In order to successfully test the match/mismatch hypothesis it is essential to generate opposing environmental conditions, which are either supportive or aversive (see Figure 1). For the early life manipulation we therefore chose the early handling (EH) paradigm, which was shown to increase maternal care (Caldji et al., 1998; Liu et al., 1997), and the limited nesting and bedding material paradigm (LM), which results in fragmented maternal care (Rice et al., 2008). Those paradigms were chosen for their long term effects on many domains, in particular on social behavior. During adulthood, animals were housed under social isolation (aversive situation) or in stable social groups (supportive situation). The inclusion of non-manipulated additional "control" groups (as e.g. animal facility reared animals) was specifically avoided, as the effects of a more neutral environment on later stress susceptibility are irrelevant to the match/mismatch hypothesis and not the aim of the current study.

2.3. Early life manipulations

On the day of the delivery (Postnatal Day 0, PND0), the litter was assigned randomly to either limited nesting and bedding material (LM) or early handled (EH) group. The LM paradigm was carried out as described previously (Rice et al., 2008). Briefly, on the morning of PND2, dams (n=13) were provided with a limited quantity of nesting material (half a square of Nestlets; Indulab, Gams, Switzerland), which was placed on a fine-gage aluminum mesh platform (McNichols, Tampa, FL, USA). All litters remained undisturbed during PND2-PND9. On PND9, all LM dams were provided with standard nesting and bedding material. For EH litters (n=18) all pups were separated from their dam for 15 min each day from PND2 until PND14. The handling procedure consisted of removing pups from their cage and housing them as a group in a clean cage. Offspring from both groups was weaned on PND28 and group housed at three to four per cage (Figure 1).

2.4. Adult life manipulations

On PND50 animals were assigned to either single housing (SH) or group housing (GH): SH females were transferred in a clean cage, while GH animals were placed in a new cage with one of the previous littermates, with a 2 × 2 design, respective to the early life manipulations. Behavioral testing was performed between PND91 and PND94 (Figure 1).

2.5. Estrous cycle determination

20 μl of Ringer's solution was used to elute cells from the female's vagina, spread on a glass slide and analyzed directly after sampling by means of a light microscope with a 10 × total magnification. The course through a standard 4-day cycle includes the regular recurrence of a few distinctive cell types, which correlate to the status of the vaginal mucosa and ovaries. After every behavioral test, we assessed the cycle stage of every female following published protocols (Goldman et al., 2007), in order to assign each animal to the respective group for the statistical analysis. In cases where the cycle stage could not be clearly allocated the animals were excluded from the statistical analysis (detailed report of the group size for each behavioral test is given in Supplementary material).

2.6. Behavioral analyses

Behavioral tests were carried out between 0830 h and 1230 h in the same room in which the mice were housed (Figure 1 illustrates the temporal order of the tests). All tests were performed using an automated video-tracking system (Anymaze 4.20, Stoelting, IL, USA).

2.6.1. Elevated plus maze

The elevated plus maze (EPM) was conducted on PND91, thus 6 weeks after the start of the adult stress procedure. The device consisted of a plus-shaped platform with two opposing open arms (length: 30 cm × width: 5 cm × height: 0.5 cm) and two opposing enclosed arms (length: 30 cm × width: 5 cm × height: 15 cm), made of gray polyvinyl chloride (PVC), which were connected by a central area (5 cm × 5 cm). The whole device was elevated 50 cm above the floor. The illumination was 25 lx in the open arms and less than 10 lx in the closed arms. Testing duration was 5 min and all mice were placed into the center zone facing one of the enclosed arms at the start of the test. The time spent in the open arms compared to the total arm time as well as the number of open arm entries was analyzed.

2.6.2. Open field test

On PND92, the open field test (OF) was performed. Testing was carried out in an empty open field arena (50 cm × 50 cm × 50 cm) made of gray PVC, which was evenly illuminated with 15 lx. Testing time was 15 min. Parameters of interest were the total distance traveled as well as the inner zone time (inner zone size 25 cm × 25 cm).

2.6.3. Sociability test

The sociability test was performed on PND93 as previously described (Moy et al., 2004; Sankoorikal et al., 2006), with slight modifications. The apparatus, a three chamber box (50 × 25), contained one center and two outer compartments (left and right chamber 19 cm × 25 cm × 40 cm; center chamber 12 cm × 25 cm × 40 cm). Two small openings with doors served as access points from the center chamber to the others. The apparatus was filled with bedding and evenly illuminated with 3–5 lx. The test was employed to assess the preference between a social target (unfamiliar juvenile C57Bl/6N mouse) and a non-social object (a toy mouse) which were placed under wire cages (Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH) in the two outer chambers. The test consisted of three separate trials. In the two initial habituation trials, the animals were allowed to freely explore the apparatus on two consecutive days for 10 min. Only the empty wire cages were present in the chambers at this time. On the third day of testing, the sociability trial took place. This time an unfamiliar male juvenile C57Bl/6N mouse was introduced into one of the chambers, enclosed in the wire cage, while a toy mouse was placed in the opposite chamber (alteration occurred every 3 consecutive trials, to avoid possible side bias). The test mouse was placed in the middle chamber for 5 min with both trap doors being shut. Afterwards, the doors were opened and the test animal was allowed to explore the rest of the apparatus for an additional 10 min. An experienced observer scored the time the mouse spent in the zone with the social target and the time spent in the zone with the non-social object. The discrimination index in % was calculated as follows: [time in zone with social target (s)/(time in zone with social target (s)+time in zone with non-social object (s))] × 100.

2.6.4. Forced swim stress

In the forced swim stress (FST), performed on PND94, each mouse was placed into a 2 l glass beaker (diameter: 13 cm and height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 6 min. The parameters time floating, time swimming, time struggling and latency to 1st floating were scored by an experienced observer, blind to the condition of the animals.

2.7. Acute stress response

The FST also served as an acute challenge in order to determine the stress response by measuring corticosterone plasma concentrations. Blood samples were taken by tail cut 30 min (stress response) and 90 min (stress recovery) after the onset of the FST according the procedure described by Fluttert et al. (2000). Samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled microcentrifuge tubes and stored at −20 °C until further processing.

2.8. Sampling procedure

All animals were sacrificed on PND97 in the circadian nadir by decapitation following quick anesthesia by isoflurane. Trunk blood was collected in labeled 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany) for basal corticosterone levels. All blood samples were kept on ice until centrifugation at 4 °C and 8000 rpm for 15 min. After centrifugation, the blood plasma was transferred to a new, labeled 1.5 ml microcentrifuge tube. All plasma samples were stored at −20 °C until the determination of corticosterone by radioimmunoassay (MP Biomedicals Inc.; sensitivity 6.25 ng/ml). Brains were removed, snap-frozen in isopentane at −40 °C, and stored at −80 °C for in situ hybridization. Adrenal and thymus glands were removed, dissected from fat and weighed.

2.9. In situ hybridization

Gene expression analysis was performed on 62 animals (detailed description of the respective group size is reported in Supplementary material). Frozen brains were sectioned at −20 °C in a cryostat microtome at 18 μm at the level of the dorsal hippocampus, thaw mounted on Superfrost Plus slides, dried and stored at −80 °C. In situ hybridization using ^{35}S UTP labeled ribonucleotide probes (BDNF, SLC6A15) was performed as described previously (Schmidt et al., 2007). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes were transcribed from a linearized plasmid. Tissue sections were saturated with 90 μl of hybridization buffer containing approximately 3–5 × 10^6 cpm ^{35}S labeled riboprobe. Brain sections were coverslipped and incubated overnight (14 h) at 55 °C. The following day, the sections were rinsed in 2 × standard saline citrate (SSC), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 × SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software (http://rsbweb.nih.gov/ij/). We performed two measurements (left and right for the hippocampus) for each brain slice and assessed two brain slices per animal. The data was analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest.

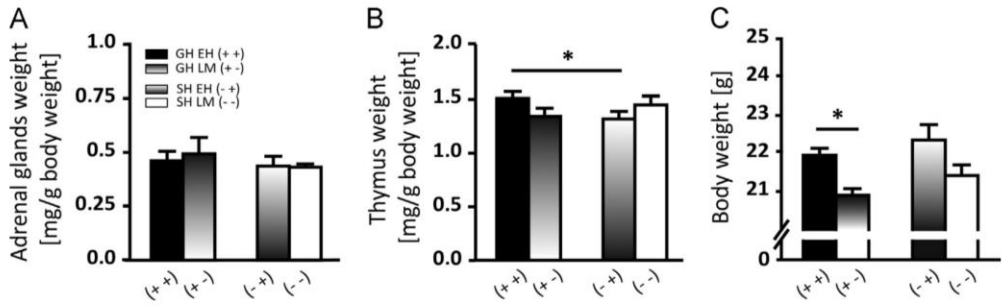

Figure 2 Effect of matched or mismatched environments on organ and body weights. (A) Matched or mismatched environments have no effect on adrenal gland weight, while (B) thymus weights were lighter under mismatched environments. This effect was significant between SH EH with GH EH. (C) Early life stress resulted in lower body weight only in animals under mismatched conditions compared to the positively matched group (GH LM vs. GH EH), while mismatched animals with positive early environment did not differ from negatively matched (SH EH vs. SH LM). Data are given as means and SEM (*=$p<0.05$).

2.10. Statistical analysis

All results are shown as mean+SEM and were analyzed by the software SPSS 16.0. All data were analyzed by 2-way ANOVA with early life and adult life conditions as independent factors. Whenever the ANOVA indicated a main or interaction effect (accepted with $p<0.05$ or $p<0.1$, respectively), Student *t*-tests were calculated to locate simple effects with a level of significance at $p<0.05$. Statistical outliers were identified with the Grubbs' test and excluded from the analysis.

3. Results

3.1. Physiological parameters

Organ weights were measured on the day of sacrifice, regardless of the estrous phase, which was unlikely to have an effect. Adrenal gland weight was not significantly affected by early life or adult life or interaction of the two factors ($F_{3,69}<1$, p=non-significant) (Figure 2A). ANOVA revealed a significant interaction effect of early life × adult life in thymus weight ($F_{3,68}=4.825$, $p<0.05$). The post hoc analysis showed that early handled single housed animals had significantly lower thymus weight compared to GH EH animals (Figure 2B). Body weight was affected by early life and adult life ($F_{1,70}=12.381$, $p<0.05$ and $F_{1,70}=4.026$, $p<0.05$, respectively). Tests with contrasts indicated that GH LM animals have significantly lower body weight compared to GH EH mice (Figure 2C).

3.2. Neuroendocrine analysis

Basal levels of corticosterone were measured in blood plasma at the circadian nadir. Moreover, response and recovery blood samples were collected 30 min and 90 min after the onset of the FST. In the non-estrous phase, an interaction early life × adult life was identified for basal corticosterone levels ($F_{3,48}=3.477$, $p=0.068$). SH EH mice have lower corticosterone levels, compared to GH EH mice (tests with contrasts, $p<0.05$). No group effects were detected for the response and recovery levels ($F_{3,41}<1$, p= non-significant and $F_{3,41}<1$, p=non-significant) (Figure 3A). In the estrous phase, no significant effects were observed for any of the three time points (respectively, $F_{3,21}<1$, p=non-significant; $F_{3,28}<1$, p=non-significant and $F_{3,28}<1$, p=non-significant) (Figure 3B).

3.3. Behavioral analysis

Four tests were conducted in order to investigate anxiety, exploration, social preference and stress-coping behavior (EPM, OF, sociability, and FST).

3.3.1. Elevated plus maze test

The EPM was used to investigate anxiety-related behavior. In the non-estrous phase ANOVA analysis did not reveal any significant differences in the percentage of the entries in the open arm ($F_{3,40}<1$, p=non-significant). Interestingly, during the estrous phase we found a significant effect of early life and a significant interaction of early life × adult life ($F_{1,27}=11.959$, $p=0.002$ and $F_{3,27}=6.367$, $p=0.018$). Post hoc contrasts tests revealed that GH LM animals show less entries in the open arm compared to GH EH mice ($p<0.05$) (Figure 4A).

3.3.2. Open field test

The open field test was used to assess arousal and locomotor activity in a novel environment. For the total distance traveled, during the non-estrous phase ANOVA revealed a significant early life × adult life interaction effect ($F_{3,37}=4.415$, $p=0.042$). SH EH mice traveled more in the arena compared to GH EH mice (tests with contrasts, $p<0.05$). During the estrous phase there were no statistical significant differences between the groups ($F_{3,28}<1$, p=non-significant) (Figure 4B).

3.3.3. Sociability test

The sociability test was performed to assess the social preference between an unfamiliar juvenile C57Bl/6N mouse and a non-social object. The time spent in the zone with the social target during non-estrous phase was not affected by adult life ($F_{3,32}<1$, p=non-significant). Regarding the estrous phase, ANOVA revealed a significant early life- × adult life interaction effect ($F_{3,28}=9.317$, $p=0.05$). GH LM mice showed significantly lower social interaction compared to SH LM and GH EH mice (tests with contrasts, $p<0.05$) (Figure 4C).

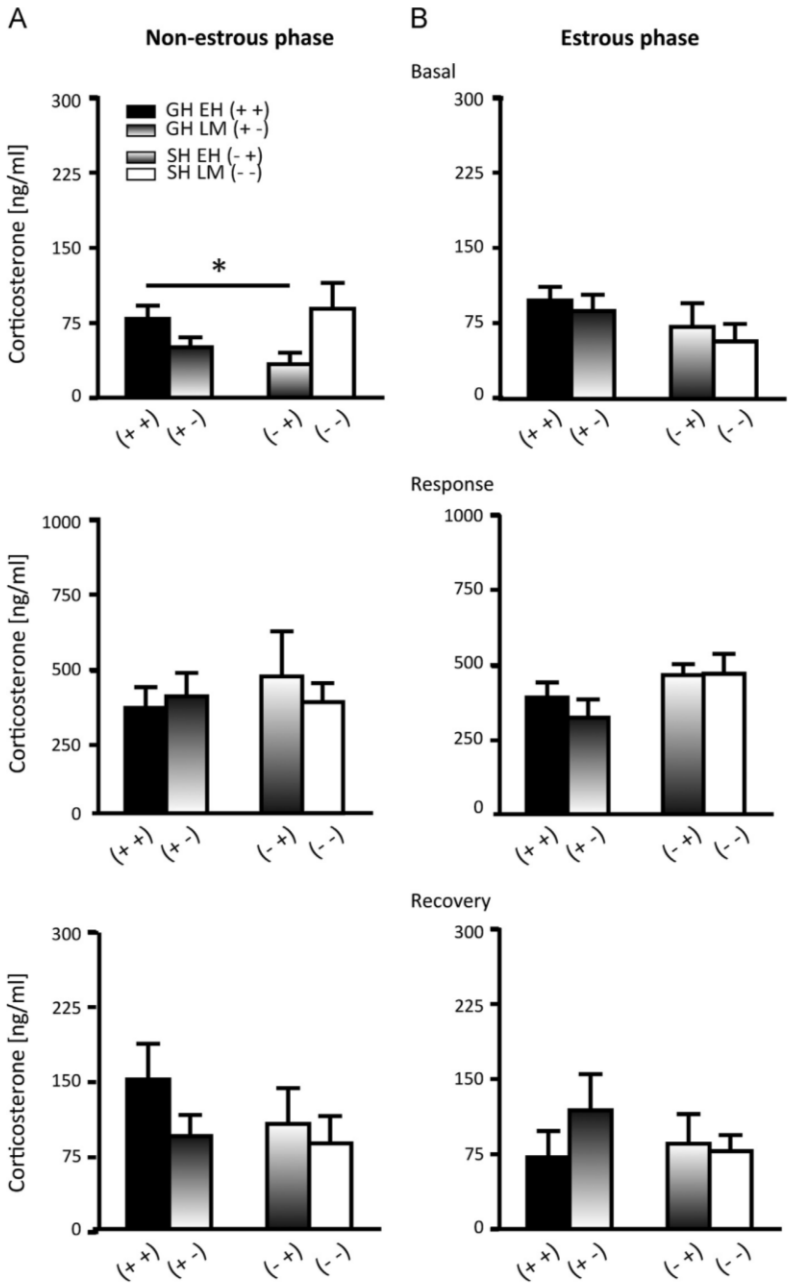

Figure 3 Plasma corticosterone levels. Corticosterone levels were measured in the plasma under basal conditions, 30 min (response) and 90 min (recovery) after an acute stressor (FST). (A) Levels during the non-estrous phase. Basal levels are reduced in mismatched animals that were exposed to early enriched environment, compared to their positively matched counterparts (SH EH vs. GH EH). Corticosterone levels in the response and recovery were not different between groups. (B) Levels during estrous phase. No significant effect of matched or mismatched environments was found in any of the time point measured. Data are given as means and SEM.

3.3.4. Forced swim stress

We investigated the animals' stress coping behavior using the FST. During the non-estrous phase, analysis of the latency to floating indicated an interaction of early life × adult life ($F_{3,36}=2.970$, $p=0.093$). Post hoc tests revealed that SH EH animals displayed a shorter latency to floating compared to GH EH mice (tests with contrasts, $p<0.05$). No significant effects were observed in the animals in the estrous phase ($F_{3,28}<1$, $p=$non-significant) (Figure 4D).

3.4. Gene expression analysis

We conducted in situ hybridization to investigate expression patterns of genes implicated in depression. For BDNF, a significant effect of adult life was found in CA1 and CA3 (adult life main effect, $F_{1,59}=12.140$, $p=0.001$, and $F_{1,59}=5.083$, $p=0.003$, respectively). Post hoc analyses revealed a decrease in expression in the CA1 in GH EH compared to SH EH and in GH LM compared to SH LM animals (post hoc $p<0.05$). Also in the CA3 a similar significant effect has been observed: a decrease in expression in GH EH animals compared to SH EH mice (adult life main effect, $F_{1,59}=15.213$, $p=0.001$). No significant effects were observed in the DG ($F_{3,59}<1$, p=non-significant) (Figure 5A). In addition, we measured the expression of SLC6A15, a new candidate gene for depression vulnerability, which in the CA3 showed a significant interaction effect early life × adult life conditions ($F_{3,54}=4.268$, $p=0.044$). A similar indication was also observed for the DG ($F_{3,54}=3.091$, $p=0.084$). Post hoc tests indicated that SH EH and GH LM animals have higher levels of SLC6A15 compared to GH EH and SH LM animals in the CA3, as well as in the DG (tests with contrasts, $p<0.05$). No significant effects were observed in the CA1 ($F_{3,54}<1$, p=non-significant) (Figure 5B).

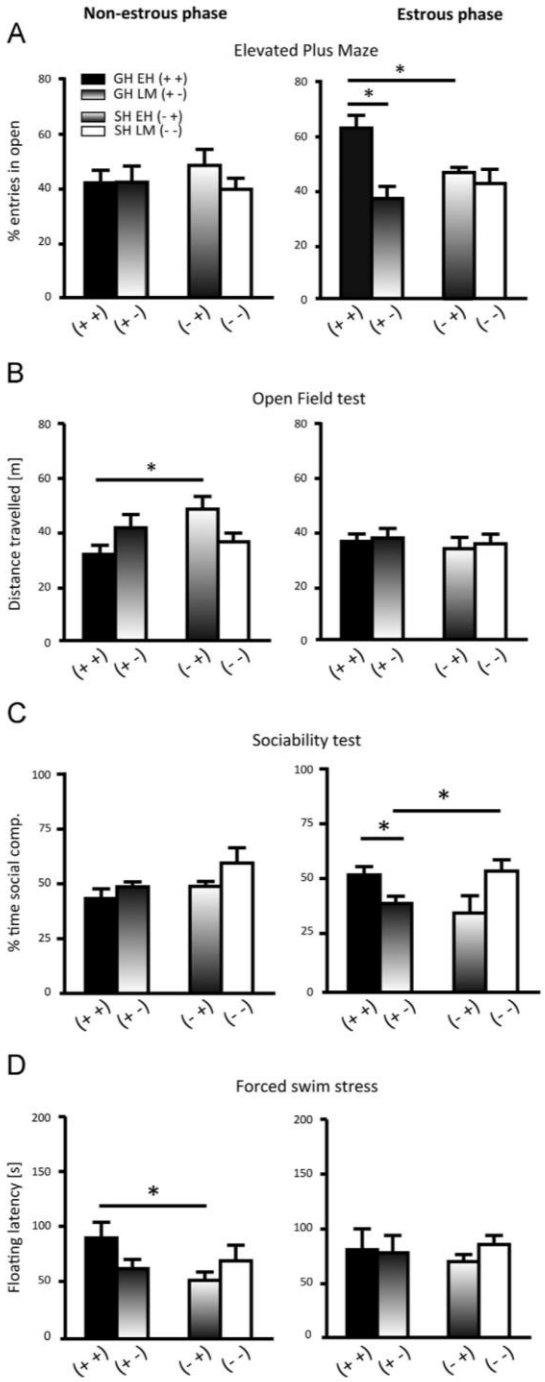

Figure 4 Behavior. Data are showing the effects of matched or mismatched environments on different behaviors during either non-estrous phase (on the left) or estrous phase (on the right). (A) Anxiety-like behavior in the elevated plus maze. In the non-estrous phase, all groups show similar levels of anxiety-like behavior. In the estrous phase, matched GH EH animals show lower anxiety-like behavior compared to both mismatched groups (GH LM and SH EH). (B) Locomotor activity in the open field test. In the non-estrous phase we observed a higher spontaneous activity in mismatched animals exposed to an early enriched environment compared to their positively matched counterparts. During the estrous phase no differences between groups were detected. (C) Sociability test. During the non-estrous phase, no significant effect of matched or mismatched environments was detected on the time spent in the social compartment. In the estrous phase, both positively and negatively matched groups showed higher sociability levels compared to mismatched animals with an aversive early environment, as indicated by the increase in time GH EH and SH LM spent in the social compartment compared to GH LM. (D) Stress coping in the forced swim test. During the non-estrous phase, mismatched animals that were raised in positive early life environment showed higher levels of stress coping behavior compared to positively matched animals, as indicated by the lower latency to floating of SH EH compared to GH EH. During the estrous phase, no significant effect of matched or mismatched environments was detected. Data are given as means and SEM.

4. Discussion

The aim of the current study was to investigate the interplay of supportive or aversive environments in early life and adulthood, thereby testing the cumulative stress and the match/mismatch hypotheses. Overall, our results indicate that the development of anxiety-like and depressive-like behavior in female Balb/c mice at adulthood is shaped by both early life and adult life experiences in a non-additive way, as would be expected according to the match/mismatch hypothesis. Furthermore, the differences between animals that experienced matched environments and those

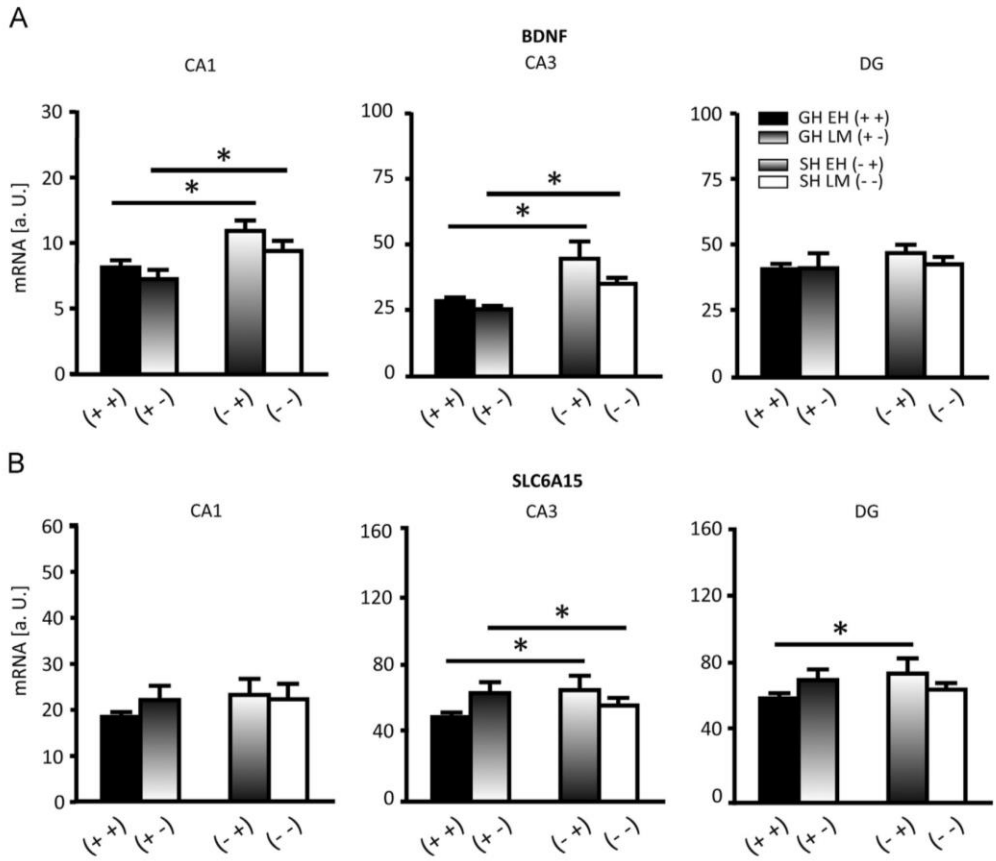

Figure 5 Gene expression profile in the dorsal hippocampus. (A) In situ hybridization revealed that a mismatched environment after an early enriched environment increases BDNF mRNA levels in the CA1 and CA3 compared to animals raised in positively matched environments as well as negatively matched environments raise BDNF mRNA levels compared to mismatched environment after an early aversive environment. This effect was not observed in the DG. (B) In situ hybridization for SLC6A15 in CA1 did not show an effect of matched or mismatched environments. In the CA3, the mRNA levels were increased in the mismatched groups, compared to the matched group with the same early environment. SLC6A15 levels in the DG were increased in animals raised in mismatched environments with early life enrichment compared to animals raised in positively matched environments. Data are given as means and SEM (*=$p<0.05$).

who experienced mismatched environments are modulated by the estrous cycle.

4.1. Effects of adverse life experiences are more pronounced under mismatched conditions

One of the most consistent findings of the current study is that adverse life experiences do not seem to have an additive effect on the measured physiological and behavioral parameters. Animals that were raised under matched aversive conditions are similar to animals that were raised in a matched supportive environment, while significant differences are most likely to be observed under mismatched conditions. Therefore, our data are in support of the match/mismatch hypothesis, stating that early life negativity shapes adult coping strategies, providing higher resilience to stress throughout life. There is a growing body of literature that supports the match/mismatch hypothesis: in rodents, the effects of early life stress are less pronounced under negative juvenile and adult environment (Buwalda et al., 2013; Daskalakis et al., 2012; Raftogianni et al., 2012; Ricon et al., 2012). Also in humans there are indications that life time adversity can buffer the response to an acute adult life stress (Elzinga et al., 2008; Resnick et al., 1995). Indeed, even though mostly unintentional, most of the early studies on early life or adult stress only used one stress procedure (either early in life or in adulthood), thereby always creating developmentally mismatched environmental conditions. However, there is also abundant and reliable evidence for the validity of the cumulative stress hypothesis (Walker et al., 2009). There are a number of possible factors that may contribute to these seemingly contradicting findings. First, the nature and the duration of the used stressors seem to be highly relevant (Branchi et al., 2013). While individuals are more likely to adapt to mild or modest stressors, the adaptive capacity for more severe stressors for a sustained period of time may be

limited. Second, the applicability of either the match/mismatch or the cumulative stress hypothesis is likely to be highly dependent on the genetic background of the individual (Van der Doelen et al., 2013). Third, it becomes evident that both the match/mismatch and the cumulative stress hypotheses can be applicable even in the same animal. For example, while a specific endophenotype (e.g. high levels of anxiety-like behavior) could be adaptive under matched aversive environments, another endophenotype (e.g. memory impairments) could be increasingly affected following both early and adult life stress exposures. Finally, the gender of animals and more specifically the hormonal status are clearly influencing the effects of matched vs. mismatched environments during development and at adulthood.

4.2. The estrous cycle modulates the effects of early and adult life experiences

Interestingly, our results clearly show that behavioral profiles of female mice differ significantly between estrous and non-estrous phases. While the importance of considering the estrous cycle with regard to anxiety- and depression-like behavior has previously been demonstrated (Galeeva and Tuohimaa, 2001; Ter Horst et al., 2012), many behavioral studies also neglect the influence of the cycle stage (Campbell et al., 2003; Eiland et al., 2012; Raftogianni et al., 2012). Intriguingly, we now find that while anxiety-related behavior and social behavior seem to be altered in mismatched environments mainly during the estrous phase, other behavioral domains as locomotion or stress coping behavior are rather altered during the non-estrous phase. This may have an adaptive value during critical phases for the survival of the animal itself - e.g. lower anxiety and higher social interactions are advantageous during mating periods.

4.3. Genes associated with depression are also regulated according to the match/mismatch hypothesis

An important additional finding of the current study is that genes related to depression in humans are also differentially altered under mismatched compared to matched environments. SLC6A15 has recently been linked to an increased depression risk (Kohli et al., 2011; Schuhmacher et al., 2013). While the role of this neutral amino acid transporter in relation to depression is still largely unclear, the here observed expression differences further support the potential role of SLC6A15 in mood and emotionality. We also investigated BDNF expression, as there is abundant evidence for an involvement of this neurotrophic factor in stress and depression (Adachi et al., 2008; Berton et al., 2006; De Kloet et al., 2005; Duman and Monteggia, 2006; Sairanen et al., 2005). Here, adult life experiences seem to have a more pronounced effect on BDNF expression compared to early life stress. This is in contrast with the situation following chronic stress in adulthood, which has generally been reported to result in a decrease of BDNF expression (Duman and Monteggia, 2006). However, also single housing has been associated with an increase in BDNF expression (Han et al., 2011; Pisu et al., 2011), which could mask some of the effects in our current study. In addition, gene expression analyses could not be subdivided by estrous cycle due to the low number of animals per group, which limits some possible conclusions. Further research is therefore needed to address the role of the nature of the stress exposure and of compensatory effects on BDNF levels.

4.4. Adaptive or maladaptive programming?

It is tempting to interpret the findings of the current study from a pathophysiological point of view, concluding that the observed behavioral and physiological alterations under mismatched conditions reflect a maladaptive, disease-like phenotype. However, this conclusion is in our view not warranted. Rather, it becomes evident that the behavioral alterations observed under the different environmental conditions are all within the normal range of behavioral adaptation, and may be beneficial and adaptive for the animals under specific environmental conditions. Some studies already addressed this issue, stressing the importance of taking into account the environmental features in which the individual is living, when discussing the adaptive/maladaptive value of certain behaviors (Belsky and Pluess, 2009; Kendig et al., 2011). Thus, whether or not mismatched environmental conditions increase the risk or vulnerability to disease will depend very much on the challenges within this environment.

4.5. Limitations of the current study

The investigation of complex gene × development × adulthood interactions is challenging and also the current experimental design comes with a number of limitations. For example, while there is substantial literature on the stressful effects of single housing in female rats (Brown and Grunberg, 1995; Kim and Kirkpatrick, 1996; Palanza et al., 2001), the fact that this environmental condition is indeed perceived as aversive by mice is still largely based on assumptions. There is a lively discussion in the scientific community on the differentiation of the term “stressor” and “enrichment” (Buwalda et al., 2013; Koolhaas et al., 2011; Würbel, 2001). Indeed, group housing could be perceived both as positive social enrichment or as social stress, probably based on the nature of the social interaction (housing siblings together for all their life span is not the same as group housing non-related adult male mice after single housing). Furthermore, results from different species, for example rat and mice, should be critically compared, due to the different adult social niches in those two species (Blanchard et al., 2001; Van der Veen et al., 2007). Thus, our results should be interpreted with caution and cannot be generalized e.g. to males, where the same paradigm is likely to have very different effects (Arndt et al., 2009; Palanza et al., 2001).

Due to the complexity of the design we also chose to test animals repeatedly in a number of behavioral tests, which could be a confounding factor for the later tests (Voikar et al., 2004). In addition, group sizes are varying due to the determined estrous phase for each animal, which is in some cases also affecting the statistical power of the tests.

Finally, we are using only female mice in the current study as "stress-vulnerable" individuals, but cannot causally link our results to a specific genetic or functional system.

4.6. Overall conclusion

Taken together, the current data indicate that continuous exposure to adverse environments is not necessarily resulting in a more pronounced phenotype compared to a continuous supportive environment. Rather, animals with mismatched environmental conditions generally differed from animals with matched environmental conditions, especially on behavioral parameters. These results further support the match/mismatch hypothesis and illustrate how mild or moderate aversive conditions during development can shape an individual to be optimally adapted to similar conditions later in life.

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The authors have nothing to disclose.

Contributors

SS designed the study, performed the experiments, analyzed the data and wrote the paper.
SL performed the experiments and analyzed the data.
XDW, SHS, KW, CL, and JH, helped performing experiments.
MVS and FH wrote the paper.
All authors have approved the final manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2014.02.002.

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The match/mismatch hypothesis of psychiatric disorders: evidence in male mice

Sara Santarelli[1], Georgia Kalideris[1], Sylvie L Lesuis[1], Andrés Uribe[1], Carine Dournes[1], Georgia Balsevich[1], Jacob Hartmann[1], Mercé M Nadal[1], Mathias V. Schmidt[1]

[1] Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, 80804 Munich, Germany

Manuscript in preparation

1. Abstract

Chronic stress is considered one of the main risk factors for depression. Interestingly, not all individuals develop psychopathology after chronic stress. In contrast to the prevailing view that stress effects are cumulative and increase stress vulnerability later in life, the recently formulated match/mismatch hypothesis of psychiatric disorders proposes that individuals experiencing high levels of psychosocial stress early in life are programmed for dealing with high psychosocial stress and are therefore resilient to high stress levels in later life. We here tested this hypothesis by comparing the developmental effects of two opposite early life conditions, when followed by two opposite adult environments. For this study we used male Balb/c mice that underwent either adverse early life conditions (limited nesting material) or a supportive environment (early handling). At adulthood, the animals of each group were either housed with an ovarectomized female (supportive environment, OVX +) or underwent chronic social defeat stress (socially adverse environment, CSDS -) for three weeks. At the end of the adult manipulations, all the animals returned to standard housing conditions and we then compared the neurobehavioral effects of the interaction between early and adult environment, in particular on the HPA axis responsiveness and on social behavior. Our study showed that also in male mice early life negativity does not necessarily result in increased vulnerability to stress. Specific endophenotypes, like corticosterone elevation after an acute challenge, resulted buffered by high levels of adversity throughout life. On the other hand, aggressive and anxiety-like behaviors are markedly increased in negatively matched individuals, supporting the cumulative stress theory. Interestingly, it has been speculated that being raised in a stressful environment prepares the offspring to better cope with a challenging adult environment, as the increase in anxiety-like behavior and aggressiveness may suggest. Overall, these results clarify the long term effects of aversive rearing and emphasize the role of early life experiences in shaping adult responsiveness to stress.

2. Introduction

Many mental illnesses, depression in particular, have their roots in the exposure to stressful experiences. Especially early stages of life are more sensitive to stress and negative early experiences have long lasting effects on the development of the offspring throughout adulthood (de Kloet et al., 2005; Levine, 2006; Suchecki, Rosenfeld, & Levine, 1993). Furthermore, it has been reported that chronic stress in adulthood precipitates neuroendocrine alterations caused by disrupted early life experiences (Christine Heim, Newport, et al., 2008; McEwen & Wingfield, 2003). Consequently, it has been proposed that the negative effects of stress add up over time ("cumulative hypothesis") and that there is a positive direct correlation between the number of stressful events during the lifetime course and the probability to develop psychopathology. On the other side, only a limited percentage of the individuals who experiences a negative event develop a stress-related illness. Experimental evidence from early life stress studies also reported a "paradoxical" protective effect of stress on later adverse life experiences (D. L. Champagne et al., 2008; Cicchetti, 2010). Recently, a new hypothesis has been formulated to integrate such seemingly contrasting findings. Taking its roots from a developmental-evolutionary perspective this theory suggests that during early life there is a heighted sensitivity to the context, in order to favour developmental trajectory adjustments to environments similar to the one experienced during early life stages. From this point of view, early stressful experiences could improve coping skills necessary at adulthood to face an aversive adult life environment. The match/mismatch hypothesis of psychiatric disease integrates this ethological approach in a broader context: when a mismatch between the early life experiences and the adult life environment occurs, the individual lacks adaptive strategies to the new environment. Accordingly, a moderately stressful rearing environment will be helpful to develop effective coping skills necessary to face adversities later in life. Therefore, psychiatric disorders could emerge from a mismatch between the early and the adult environment. However, high levels of stress are still considered detrimental, whereas short, controllable stressful experiences could fall into a match/mismatched case. We have recently reported that in female Balb/c mice moderate early life adversity indeed increased resilience to adversity in adulthood (Santarelli et al., 2014). However, the available data are still sparse and it remains unclear, whether early life adversity could also be beneficial for resilience to more severe social stressors. We addressed this question by comparing four groups of animals: two matched (either always exposed to positive environments or always exposed to adversities) and two mismatched (experiencing only once adverse period, either in early or adult life). We used limited nesting and bedding material (LM) as aversive early life environment and daily early handling (EH) as positive stimulus for the pups. At adulthood, chronic social defeat stress (CSDS)

was used to stress the experimental animals, whereas housing with an ovarectomized female (FH) served as positive stimulus. At the end of the adult manipulations, the neuroendocrine and behavioral profiles of the animals from the different groups were compared.

3. Material and Methods

3.1 Experimental animals and breeding

Balb/c mice of both genders (n=40/sex) were purchased from Charles River, Germany. The animal facility provided a 12 h light and 12 h dark cycle (lights on at 0700 h), constant temperature (21 °C ± 2 °C) and 40 % relative humidity. Upon arrival, animals were housed in standard cages (21 cm x 15 cm x 14 cm, Plexiglas) filled with sawdust, bedding and nesting material, and closed with wire lids, containing food (Altromin 1412, Altromin GmbH, Germany) and water *ad libitum*. Upon arrival, males were single housed and females group housed (four per cage). After 15 days of acclimatization, each female mouse was housed in the cage of one male mouse, forming 40 breeding pairs. Males were removed 18 days after the pairing, and the females were left undisturbed in their home cages. After the separation, pregnancy status was checked daily. The day of the delivery was defined as postnatal day zero (PND 0). Only male pups were later used as experimental animals, whereas females were used as social stimulus for the ovarectomized group. All experiments were conducted in accordance with the European Communities Council Directive 2010/63/EU. The applied protocols have been approved by the committee for Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

3.2 Early life conditions

On PND 2 litters were randomly assigned to two groups: one was provided with increased maternal care (defined as early handled (EH +)), whereas the other with an impoverished early life environment (limited bedding and nesting material (LM -)). The EH consisted in 15 minutes separation of the litter and the nest from the dam. The separation was performed at random times between 08:00 and 18:00 (adapted from (Millstein & Holmes, 2007). In addition to the standard bedding material, all the EH dams were provided with two nestlets (5 cm x 5 cm from Indulab, Gams, Switzerland). For the LM litters, on PND 2 the standard bedding material was substituted by reduced amount of sawdust (two falcon tubes), overlaid by a fine-gauge aluminium mesh platform (McNichols, Tampa, FL, USA) and only a half nestlet was provided (Rice et al., 2008). The dams and litters were left undisturbed until PND 9. On PND 9, all the animals returned to standard rearing conditions. On PND 23 the pups were weaned, and housed in groups (4 animals/cage of the same sex). On PND 67 the adult life manipulation started.

3.3 Adult life conditions

Males (EH+, LM-) were further separated in two groups: the aversive chronic social defeated stress group (CSDS -) and the supportive environmental group, which was housed together with ovarectomized females (OVX +) for three weeks. Therefore, two matched experimental groups (EH OVX ++ and LM CSDS --) and 2 mismatched groups (EH CSDS +- and LM OVX -+) were formed. The experimental design and experimental groups are summarized in the figure 1. Twenty two males (11 from EH and 11 from LM) were assigned to the adult life enrichment group (OVX +): each animal was housed with an ovariectomized female in double sized cages (42 cm x 30 cm x 28 cm), separated by a wire mesh allowing continuous visual and olfactory contact. Once per day the mesh was removed, allowing interaction between the two animals. In order to create a stressful adult environment, the chronic social defeat stress paradigm (CSDS -) was used (Wagner et al., 2012). Briefly, experimental animals (11 from EH and 11 from LM) were housed with one CD-1 dominant mouse in double sized cage (42 cm x 30 cm x 28 cm), kept physically separated by a wire mesh, to guarantee sensory interaction but no physical contact. The animals were allowed to interact once per day, until the experimental animal was defeated. Afterwards, the animals were separated and then left undisturbed until the next defeat.

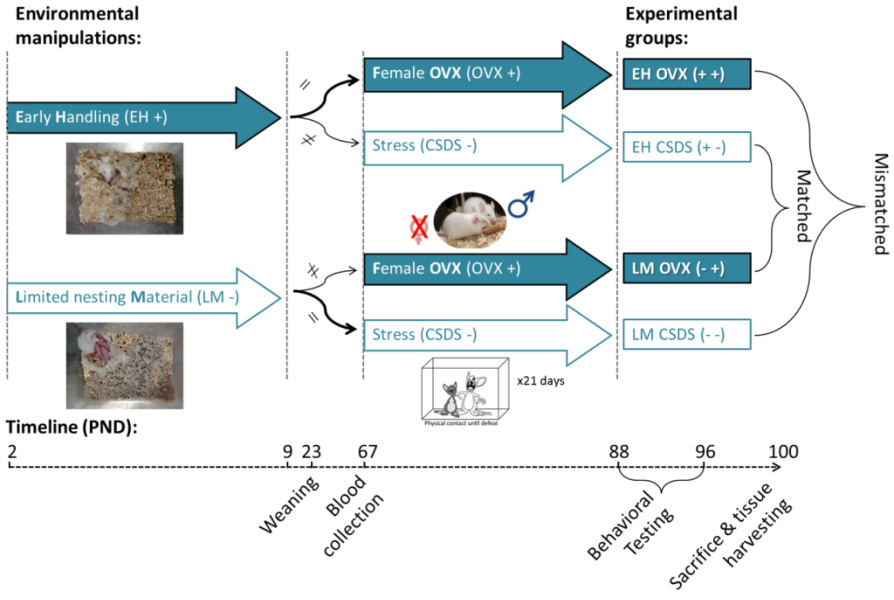

Figure 1: Timeline and experimental groups. At the end of the environmental manipulations the experimental groups were: two matched groups (positive: early handled/group housed, EH OVX + +; negative: limited nesting and bedding material/chronic social defeat, LM CSDS - -) and two mismatched groups (with early enriched environment: EH CSDS, + -, and with aversive early environment: LM OVX, - +). On PND 2, the litters were assigned either to EH or LM groups, until PND 9, when they returned to standard house condition. All the litters were weaned on PND 23. At adulthood (PND 67) the animals were further assigned to CSDS or OVX for three weeks (until PND 88). Behavioral testing took place in the following week (between PND 89 and 92). Animals were sacrificed on PND 100.

3.4 Body weight and fur status

Body weight was taken with an electronic precision scale (Scout Pro; readability 0,1 g) during the adult life treatment and before behavioral testing. Simultaneously with the body weight, fur status was recorded. A 4-point scale was used to determine the fur status, where 1 was given for a clean and well-groomed fur and 4 for a stained and dirty fur that may have some bold patches. Intermediate scores were assigned for deteriorated furs between 1 and 4 status (Mineur, Prasol, Belzung, & Crusio, 2003).

3.5 Acute stress response

Corticosterone (CORT) plasma levels were assessed under basal condition at 2 times: 1 week before the start of the adult manipulation, by tail cut, and at the end of the experiment, collecting trunk blood. Furthermore, CORT was measured after two acute challenges, namely after the first adult manipulation and after the forced swim test (FST). In both situations, blood samples were taken by tail cut 30 min (stress response) and 90 min (stress recovery) after the onset of the acute stressor (Fluttert et al., 2000). Samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled microcentrifuge tubes and stored at −20 °C until the determination of corticosterone by radioimmunoassay (MP Biomedicals Inc.; sensitivity 6.25 ng/ml).

3.6 Behavioral testing

Behavioral testing was performed at the end of the adult manipulation, between 08:00 and 14:00. The behavioral testing consisted of the Dark-Light test (DaLi), the Open Field test (OF), Sociability test (SOC), Social Avoidance (SA) and Forced Swim test (FST). All tests were recorded and analyzed using an automated video-tracking system (Anymaze 4.20, Stoelting IL, USA). An experienced observer scored the videos, blind to the condition of the animals.

3.6.1 Dark-Light Test

To assess anxiety-like behavior, on PND 90 the DaLi was performed. The apparatus consisted of a four square arena (46 cm × 27 cm × 30 cm), separated in two chambers: one bright illuminated compartment (2/3 of the entire apparatus) and a dark compartment (1/3 of the apparatus) (Bourin & Hascoët, 2003), connected with an opening. The bright compartment was approximately illuminated with 600 lux and the dark compartment was less than 10 lux. Animals were placed in the corner of the dark chamber, facing the wall. The mice were tested for 5

minutes and the latency to the first entry to the lit zone and the time spent in the lit zone were recorded.

3.6.2 Open Field

To measure locomotion and general activity, the OF was performed on PND 91. The animals were placed in an open area, consisting of grey PVC (50 cm x 50 cm x 50 cm) and evenly illuminated conditions (6-10 lux), facing the corner of the device. The area was separated in two virtual zones, an inner (25 cm x 25 cm) and an outer zone (Cryan & Holmes, 2005). The test lasted for 15 minutes and the percentage of time spent in the inner zone, the distance travelled and the immobile time were measured.

3.6.3 Sociability test

The test was used to assess the tendency to interact with a non-aggressive conspecific. SOC test was performed on PND 94. The apparatus, a three chamber box (50 cm × 25 cm), contained one center and two outer compartments (left and right chamber 19 cm × 25 cm × 40 cm; center chamber 12 cm × 25 cm × 40 cm). Two small openings with doors served as access points from the center chamber to the others. The apparatus was filled with bedding and evenly illuminated. The social target (unfamiliar juvenile C57Bl/6N mouse) and a non-social object (a toy mouse) which were placed under wire cages (Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH) in the two outer chambers. Two habituation trials were performed to allow the animals to familiarize with the arena on two consecutive days for 10 min. Only the empty wire cages were present in the chambers at this time. On the third day, the test took place with an unfamiliar male juvenile C57Bl/6N mouse enclosed in the wire cage, while a toy mouse was placed in the opposite chamber (alteration occurred every 3 consecutive trials, to avoid a possible side bias). The test mouse was placed in the middle chamber for 5 min with both trap doors being shut. Afterwards, the doors were opened and the test animal was allowed to explore the rest of the apparatus for an additional 10 min. Time interacting with the social stimulus and the latency to the social compartment were measured.

3.6.4 Social Avoidance Test

The SA was performed on PND 95 to examine the agonistic behavior. A wire cage with a non-aggressive CD1 mouse was placed close to one wall of the OF apparatus described before (Golden, Covington, Berton, & Russo, 2011). To analyze the behavior, the arena was virtually divided in an interaction zone (26 cm x 13 cm, where the CD1 was placed) and non-interaction

zone (the rest of the arena). At the beginning of the test, the experimental animal was placed in one corner far away from the CD1 facing the walls. The testing lasted for 5 min and the latency to the first interaction with the CD1 and the time spent in the interaction zone were measured.

3.6.5 Forced Swim test

In the FST, performed on PND 96 to assess despair-like behavior, each mouse was placed into a 2 l glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 6 min. The parameters latency to 1st floating and time struggling were scored.

3.7 Sampling procedure

All animals were sacrificed on PND 100 during the circadian nadir by decapitation following quick anesthesia by isoflurane. Adrenal and thymus glands were removed, dissected from fat and weighed for further processing.

3.8 Statistics

All behavioral and physiological data were analyzed and evaluated using the program SPSS 18.0 (SPSS inc., California, USA). The univariate 2-way ANOVA test was used, with significance level for main effects at $p < 0,05$ and for interaction effects at $p < 0.1$. Early life and adult life conditions were set as independent factors. If a main effect was present, post hoc tests revealed the specification of the effect ($p<0.05$). For body weight and fur status, repeated measures ANOVA was used. All results are presented as mean ± SEM.

4. Results

4.1 Body weight, fur status and organ weight

To investigate the general health status of the animal, body weight and fur status were observed during the adult manipulation period. No statistical effect of the environmental conditions was observed (Figure 2A-B). To have an indication of the effectiveness of the adult life manipulations adrenal glands weight were measured. Adrenal glands weight was affected by the adult life * early life interaction ($F_{(1,38)}$=3,97, p=0,054). Post hoc tests revealed that in early handled offspring, chronic social defeat stress increased adrenal weight compared to housing with an ovarectomized female, while no effect was observed in limited nesting offspring (Figure 2C).

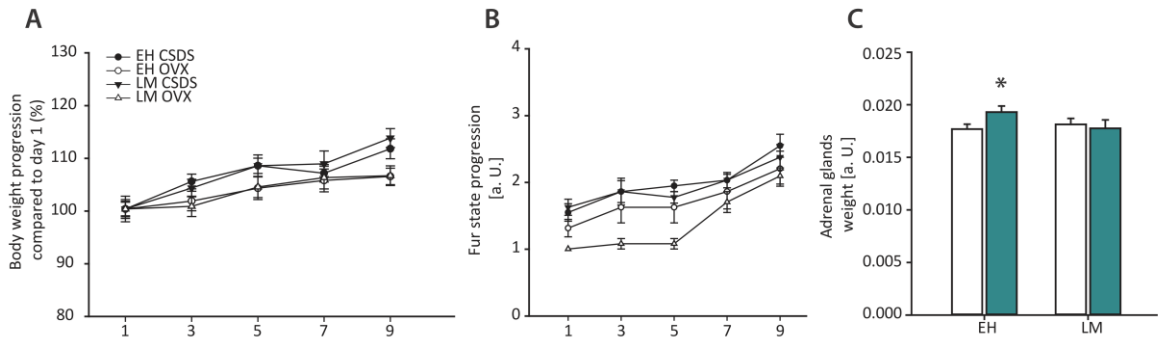

Figure 2: Effect of different environments on organ and body weights. (A-B) Matched or mismatched environments have no effect on body weight progression and fur status. **(C)** Mismatched environment with early enriched environment resulted in a significant increase of the adrenal glands when compared to both positively and negatively matched environments (EH CSDS vs EH OVX, EH CSDS vs LM CSDS). Interestingly, mismatched environment with early adversities and negatively matched ones, resulted in comparable adrenal glands weight (LM OVX vs LM CSDS). Data are given as means and SEM (* = p<0.05).

4.2 Corticosterone levels

Before the beginning of the adult manipulation, no significant differences in CORT were observed between early handled and limited nesting offspring (Figure 3A). After the first acute challenge, adult life experience significantly affected both response ($F_{(1,43)}$=29,7, p<0,05) and recovery CORT levels ($F_{(1,43)}$=21,25, p<0,05). Post hoc test indicated that chronic social defeated animals showed significantly higher levels of CORT compared to housing with an ovarectomized female, independently from the early life history (Figure 3B-C). Plasma CORT was measured again at the end of the adult manipulations. Under basal condition, no statistical difference between the experimental groups were observed (Figure 3D). After the FST, response levels of CORT were significantly affected from the adult life ($F_{(1,38)}$=7,86, p<0,05) and from early life * adult life interaction ($F_{(1,38)}$=3,39, p<0,1). Post hoc tests revealed that chronic social stress exposure

significantly increased the CORT response in early handled offspring, while no effect was observed in limited nesting offspring (Figure 3E). Ninety minutes after the acute stressor, no statistical difference was observed between the groups (Figure 3F).

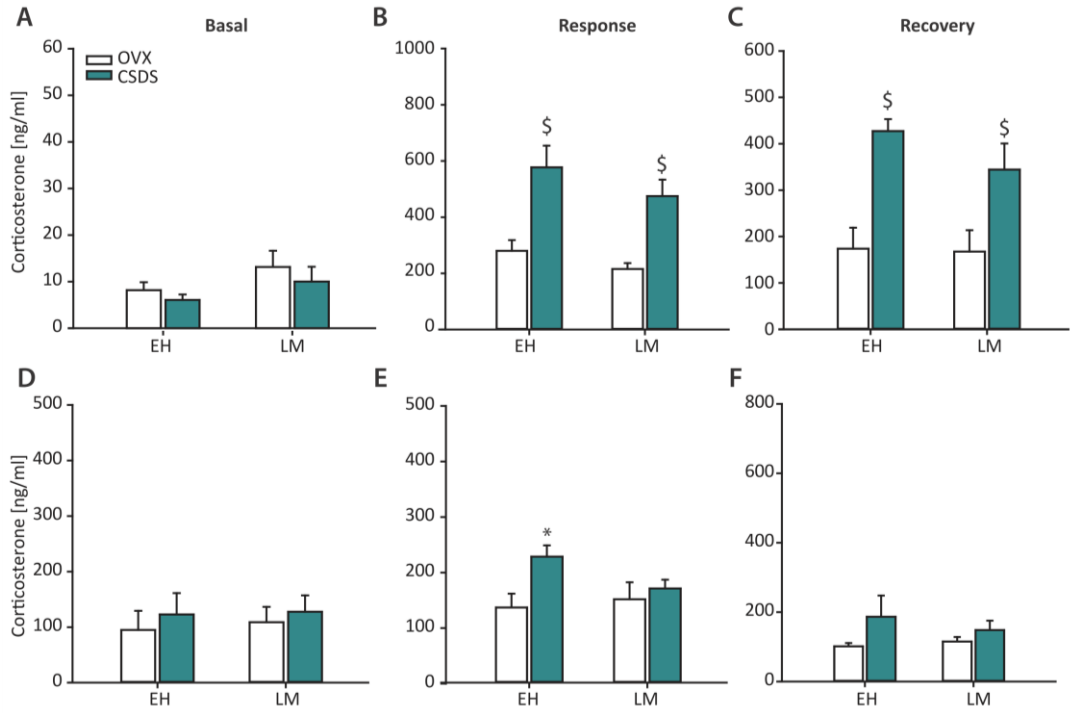

Figure 3: Plasma corticosterone levels. Corticosterone levels were measured in the plasma under basal conditions, 30 minutes (response) and 90 minutes (recovery) after an acute stimulation. **(A)** Before the onset of the adult life manipulations, basal levels of corticosterone were comparable between groups. **(B-C)** After the first adult manipulation, defeated animals (CSDS) had higher levels of plasma corticosterone, independently from the early life experience **(D).** On the sacrifice day, another basal measurement of corticosterone was performed and there was still no difference between the groups. **(E)** 30 minutes after the acute physical stressor (FST), mismatched environment with early enriched environment resulted in a significant increase of corticosterone when compared to both positively and negatively matched environments (EH CSDS vs EH OVX, EH CSDS vs LM CSDS). **(F)** This difference was completely abolished 90 minutes after the FST. Data are given as means and SEM (* = p<0.05).

4.3 Anxiety-like behavior

Time spent in the bright compartment of the Dark-Light box was measured, but no significant effects were observed. Latency to enter in the bright compartment was measured, and statistical analysis has revealed a significant early life*adult life interaction effect ($F_{(1,40)}$=5,01, p<0,05). Chronic social stress exposure resulted in a decreased latency to enter the lit compartment in early handled offspring, but not in limited nesting offspring (Figure 4A). Regarding the OF test, a main effect of adult life ($F_{(1,39)}$=11,16, p<0,05) and an interaction effect between adult life*early life ($F_{(1,39)}$= 5,30, p <0.05) on the latency to entry the inner zone were found. Post hoc tests revealed no difference is observed in EH offspring due to adult life condition, whereas LM CSDS animals showed a significant increase in the latency to the first entry

in the bright compartment, compared to LM OVX animals (p<0,05) (Figure 4B). In addition, a main effect of adult life was observed on the distance travelled in the arena ($F_{(1,39)}$= 3.67, p<0.05) and on the time spent immobile ($F_{(1,39)}$=55,88, p<0.05). Post hoc tests revealed that CSDS animals travelled less and spent more time immobile compared to the respective EH group (Figure 4C-D).

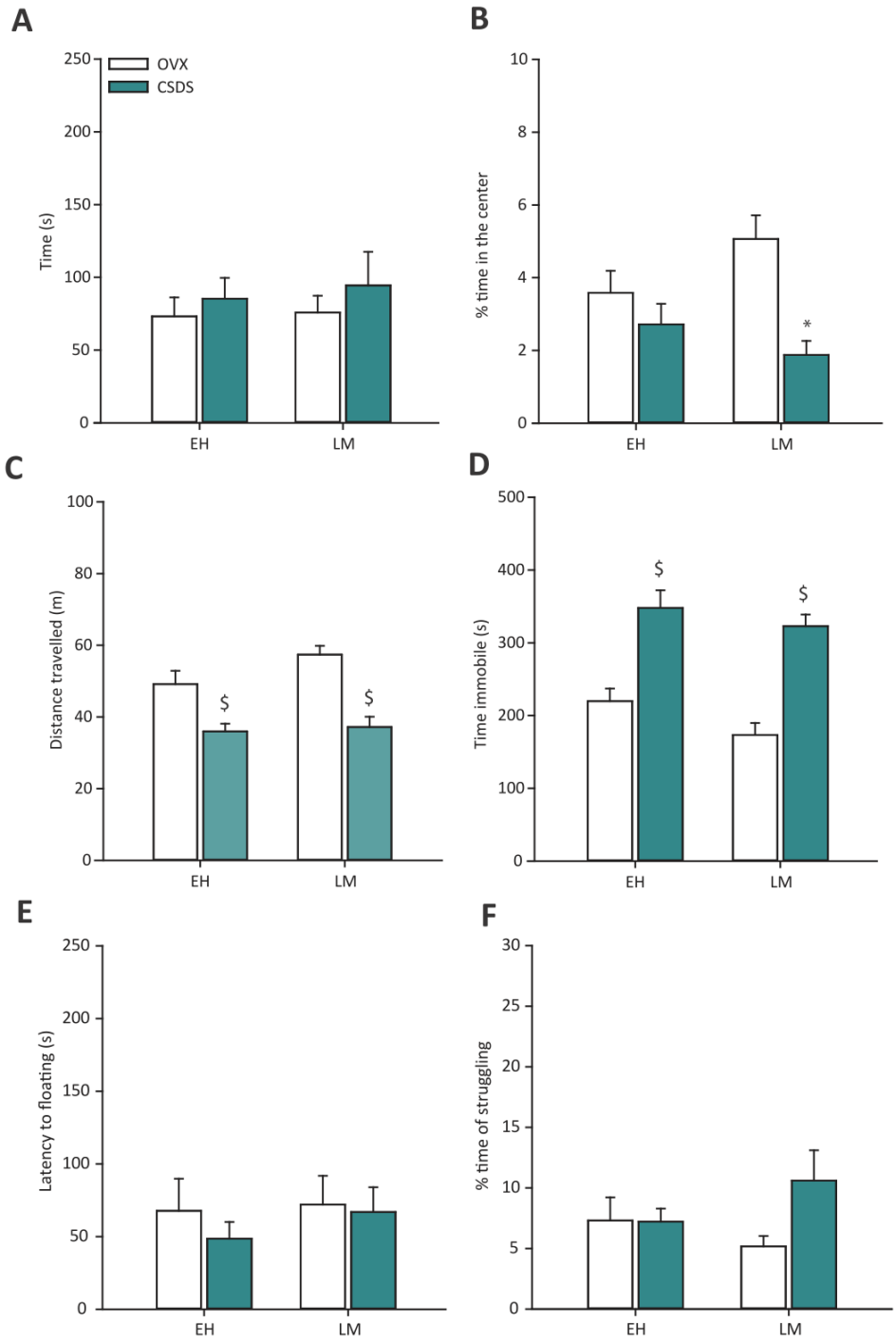

Figure 4: Emotional behavior. (A) No significant effects have been observed in the DaLi test. **(B)** The OF revealed cumulative effects of stress, with negatively matched animals spending reduced time in the inner zone compared to mismatched animals with early negativity (LM OVX). **(C-D)** A predominant effect of the adult life was observed in locomotion and time immobile. CSDS animals travelled significantly less time and spent more time immobile, compared to OVX. **(E-F)** FST was performed to assess the coping behavior toward a new acute stressor. No statistical difference was observed between the groups. Data are given as means and SEM (* = p<0.05).

4.4 Stress-coping behavior

Statistical analysis of the FST test has revealed no difference in the behaviors scored (time struggling and latency to 1st floating) between the experimental groups (Figure 4 E-F).

4.4.1 Social behavior

In the SOC test, ANOVA test did not reveal any significant effects of early and adult life or interaction on the time spent in the social chamber and in the interaction with the juvenile (Figure 5 A-B). We also investigated agonistic behavior using the SA test. A significant interaction effect early life*adult life ($F_{(1,36)}$=3,94, p=0.055) was detected on the time spent in the interaction zone. Post hoc analysis revealed that chronic social stress reduced the time in the interaction zone, compared to housing with ovarectomized female in limited nesting offspring. Early handled offspring, independently from the adult manipulation, spent comparable time to negative matched animals in the interaction zone (Figure 5A). A tendency for adult life effect was observed in the latency to enter in the interaction zone ($F_{(1,34)}$=4,09, p=0.052), with CSDS animals showing a longer latency compared to ovarectomized female animals (Figure 5B).

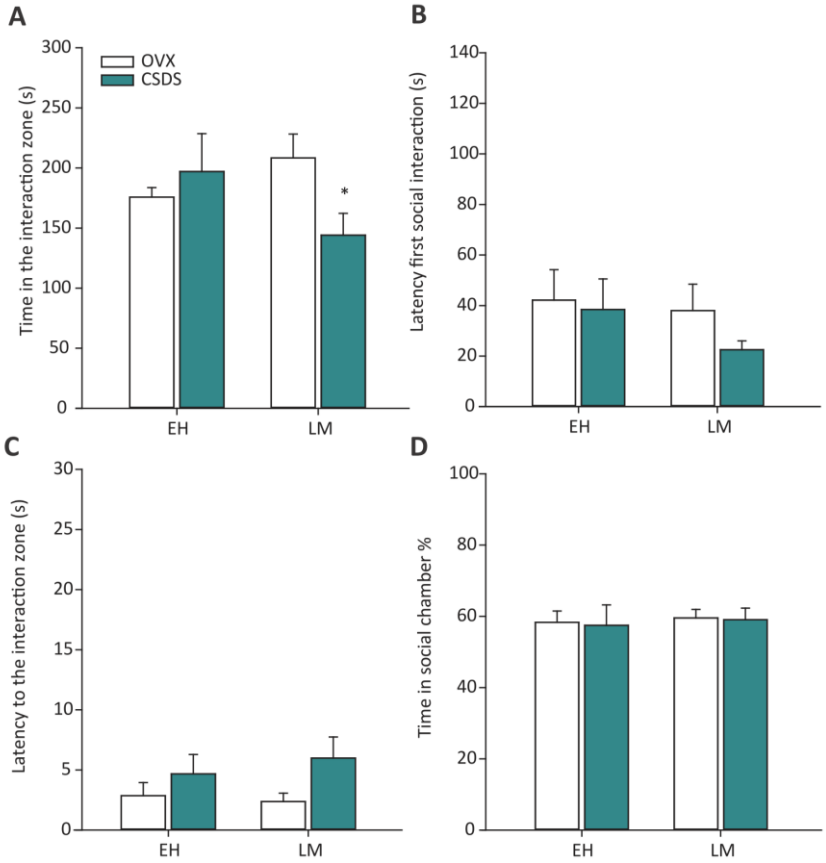

Figure 5: Social behavior. The SA and SOC were performed to investigate the role of matched or mismatched environment on social behavior. **(A)** In the SA, negatively matched environment significantly reduced the time spent interacting with the aversive stimulus compared to mismatched with negative early adversities **(B)** but no difference was observed in the latency to the interaction zone was observed. **(C-D)** In approaching a young, non-aversive male, no differences were observed between the groups. Data are given as means and SEM (* = p<0.05).

5. Discussion

Chronic stress experiences are recognized as major risk factors for depression. However, there are contrasting theories about the effects of repeated stressful experiences during different stages of life. In this study, we addressed the effects of different rearing conditions, on the response to opposite adult life environments in male mice. Overall, our study shows that early life negativity provides resilience against the neuroendocrine effects of adult chronic stress, following the match/mismatch theory. On the other hand, the behavioral phenotype observed after early chronic social stress or positive environment, are in support of the cumulative stress theory.

5.1 General physiology and endocrine differences

As general indicators of the status of the animals, we investigated the body weight and the fur status progression. No significant effects of early life and adult life experiences were observed. Adrenal glands weight was increased by adult chronic stress, but previous early life stress buffer this effect, supporting the mismatch hypothesis. This is an important indication that early life stress might prepare the organism to confront aversive experiences in adulthood. The adrenal glands phenotype suggested alterations in the HPA axis therefore corticosterone levels were measured. In our study, early life experiences had no significant effect on corticosterone basal levels and on corticosterone elevation due to the first acute social defeat. In addition, CORT levels after the first encounter with an ovarectomized female are lower than after the first social defeat, confirming that exposure to an ovarectomized female is not an aversive event, but rather represents a positive stimulus. Hyper production of corticosterone in response to an acute challenge, like after the FST, is observed after chronic social stress, as it was already reported in literature (Keeney et al., 2006). Interestingly, previous early life adversities buffer this increase in corticosterone, with a similar reactivity to animals which did not undergo chronic social defeat. Those data corroborate the adrenal glands findings and are in support of the mismatch hypothesis, demonstrating that early life stress could promote resilience to adult life stress.

5.2 Anxiety and depressive like behavior

As next step, emotional behavior was investigated. Different behavioral tests indicated a significant early * adult environment interaction effect on anxiety-like behavior. In the Open Field test negative matched animals showed the highest levels of anxiety-like phenotype, whereas Dark-Light test exerted no difference between the groups. Careful conclusions should be made interpreting those data: in the Dark Light box test, the light conditions are more aversive then in the Open Field, where the test is performed under dim light. Mice are very sensitive to light

condition, and tend to avoid strong illuminated areas. Therefore, a test performed in more aversive conditions could exert different behavioral responses when compared to test performed in milder conditions. More experiments are needed to clarify this issue, for example using more salient context, like novelty-related anxiety, to investigate the direction of the effects. Overall, the behavioral data pointed to cumulative stress effects, and this is in line with other results from female mice (*Chapter 2* of this thesis).

5.3 Social and agonistic behavior

Social interactions are also known to be affected by rearing experiences. Therefore, we investigated the behavior of experimental animals in different social contexts: during the interaction with a non-aversive conspecific and with a potential aggressive competitor. No differences were found in the interaction with a juvenile, non-aggressive conspecific between the experimental groups. Interestingly, when confronted with a potential male competitor, the strongest chronic social defeat effects in terms of reduced social interaction time was observed in animals with an aversive early life history (limited nesting offspring that underwent chronic social defeat at adulthood), suggesting the more pronounced depressive-like phenotype On the other hand, early positive up bringing buffered the effects of the chronic social defeat stress, as showed by similar interaction time between the two EH groups. Hence, we concluded that chronic social defeat stress effects on social skills are more pronounced in animals with a stressful life trajectory, supporting the cumulative stress hypothesis, whereas positive rearing buffers the effect of adult chronic stress. However, the interpretation of the social avoidance in the social avoidance test should be careful. It can be speculated that animals raised under constant aversive environment, that in a more naturalistic settings can be a higher number of intra specific competitors or limited amount of resources, characterized by higher social avoidance are more likely to avoid aggressive interaction, potentially leading to wounds, and therefore increase the chances to survival. From this point of view, an increased social avoidance may represent a more successful strategy when compared to less anxious, more riskful behaviors. From this point of view, the cumulative stress hypothesis applies and it is further supported from an ethological point of view.

5.4 Individual resilience versus context specificity

Opposite environments like early enrichment and mild early life stress have been shown to increase resilience against stress at adulthood (Lyons et al., 2010). Nevertheless, the effects of the rearing experiences can vary depending on individual sensitivity to context (i.e. genetic background) and on the adult living environment. Our study revealed that different adult

environments require a different set of coping skills for adaptation. Overall, we could here show that early life adversity provides resilience to cope with stressful adult environments, in an integrated framework of the cumulative and match/mismatch hypotheses. Matched life experiences provided protection against neuroendocrine effects of stress, whereas social avoidance was increased following exposure to repetitive chronic stress. In conclusion, these data support the integration of the cumulative hypotheses and the match/mismatch in a broader theoretical framework, reinforcing the relevance of studying resilience mechanisms both in humans and preclinical studies. In addition, preclinical models lack of approaches to evaluate positive emotions of experimental animals, creating a bias towards the negative effects of stress, leaving unassessed potential improvements in mood status, one of the core parameters of resilience in humans.

6. Acknowledgments

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The amino acid transporter SLC6A15 is a regulator of hippocampal neurochemistry and behavior

Sara Santarelli[1], Christian Namendorf[1], Tamara Gerlach[1], Elmira Anderzhanova[1], Benedikt Bedenk[1], Sebastian Kaltwasser[1], Klaus Wagner[1], Christiana Labermaier[1], Jana Drgonova[2], Michael Czisch[1], Manfred Uhr[1], Mathias V Schmidt[1]

[1] Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, 80804 Munich, Germany

[2] NIDA-IRP/NIH, 333 Cassell Drive, Baltimore, USA

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1. Abstract

Although mental disorders are highly prevalent worldwide, their molecular, neurochemical and functional underpinnings often remain elusive. Recently, the neutral amino acid transporter SLC6A15 was identified as novel candidate gene, with a reported 1.42 fold increased risk of suffering from depression. The risk polymorphism was also found to affect hippocampal morphology, integrity, and hippocampus-dependent memory. However, the function of SLC6A15 in the brain is so far largely unknown. To address this question, we investigated if alterations in SLC6A15 expression, either using a full knockout or a targeted hippocampal overexpression, affect hippocampal neurochemistry and consequently behavior. We could show that a lack of SLC6A15 reduced hippocampal levels of proline and other neutral amino acids and consequently led to a decreased availability of glutamate and glutamine. In contrast, SLC6A15 overexpression increased glutamate/glutamine concentrations. These neurochemical alterations could be linked to behavioral abnormalities in sensorimotor gating, a key behavioral phenotype indicative of glutamate system dysregulation. Overall, our data supports SLC6A15 as a crucial factor controlling the neuroactive amino acid content in the hippocampus, thereby likely interfering with glutamatergic transmission and behavior. These findings emphasize SLC6A15 as pivotal risk factor for vulnerability to psychiatric diseases.

2. Introduction

Psychiatric diseases affect millions of people worldwide and are regarded as the number one burden in western societies (Gustavsson et al., 2011; Lopez AD, Mathers CD, Ezzati M, Jamison DT, 2006), but their molecular, neurochemical and functional underpinnings are often still unclear. Recently, the novel candidate gene SLC6A15 was associated with psychiatric disorders as depression (Kohli et al., 2011), stress system activity as well as memory and attention (Schuhmacher et al., 2013). SLC6A15 belongs to the solute carrier family 6 (SLC6A) and encodes for a sodium-dependent transporter for neutral amino acids. Its expression has been mainly localized in neurons. The chemical and structural features of SLC6A15 have been well characterized (Bröer et al., 2006), but its physiological function is still poorly understood. The study from Kohli and colleagues also reported an effect of the risk polymorphism on glutamate levels and hippocampal volume, suggesting a potential link of SLC6A15 and glutamate signaling (Kohli et al., 2011). In mice, deletion of SLC6A15 did not result in an obviously different behavioral endophenotype compared to wild-type littermates under basal condition, except for the partial alteration in anxiety-like behavior after acute stress (Drgonova et al., 2007). So far, no experimental proof of a connection between the SLC6A15 transporter and glutamate signaling was published.

Among different hypotheses explaining mood disorder vulnerability, the glutamate hypothesis has recently gained more attention. For example, the importance of the glutamatergic system in the hippocampus in depression vulnerability has been extensively described (Sanacora et al., 2012). However, an altered glutamate signaling or an imbalance of excitatory and inhibitory neurotransmitters has been proposed as central mechanism for a number of psychiatric disorders, including also schizophrenia (Moghaddam & Javitt, 2012), autism (Tebartz van Elst et al., 2014) or bipolar disorders (Chen, Henter, & Manji, 2010). Interestingly, proline, one of the main amino acids transported by SLC6A15, is involved in glutamate synthesis (Pérez-Arellano et al., 2010). This is of specific interest as a number of studies have already correlated proline levels in the brain with differences in sensorimotor gating and memory (Cherkin et al., 1976; Gogos et al., 1999; Roussos et al., 2009), phenomena determined by glutamatergic neurotransmission (Geyer, Krebs-Thomson, Braff, & Swerdlow, 2001; Riedel, Platt, & Micheau, 2003) and highly relevant for psychiatric disorders such as e.g. schizophrenia (Braff, 1990). Sensorimotor gating refers to the state-dependent regulation of transmission of forwarding the sensory information to the motor system (Nusbaum & Contreras, 2004). Sensorimotor gating tests are a powerful tool used both in clinical and preclinical studies to assess integrity of the neural circuits.

To test whether SLC6A15 is modulating brain neurochemistry, in particular of the glutamatergic system, and consequently behavior, we here studied the effects of either SLC6A15 complete deletion or hippocampus-specific SLC6A15 overexpression in mice. We report the differences in hippocampal amino acid content, including proline, alteration of glutamate and glutamine levels, and impairments of the sensorimotor gating behavior after manipulation of SLC6A15, thereby providing the first direct experimental support for the involvement of SLC6A15 in glutamate-related pathophysiology.

3. Materials and Methods

3.1 Animals and husbandry

SLC6A15Knockout mice were kindly provided (Drgonova et al., 2007) and kept as *in house* colony. SLC6A15KO mice (from now on abbreviated as SLC-KO) did not show overt reproductive failures and no abnormalities in the survival rate. Mice used for the experiments were obtained from heterozygous breeding pairs and only male animals were used in this study. SLC6A15 wild-type littermates (abbreviated as SLC-WT) were used as a control group. Genotypes were verified by PCR analysis of tail DNA following published protocols (Drgonova et al., 2007). For hippocampal SLC6A15 overexpression studies, male C57Bl/6N mice (Charles River Laboratories, Maastricht, the Netherlands) were used. All animals were between 10-17 weeks old and single housed for at least 1 week before testing. Mice were held under reversed light conditions (12 light: 12 dark light cycle, lights on at 8 pm, temperature at 23 ± 2°C). Food (Altromin 1324, Altromin) and tap water were available *ad libitum*. At the end of each experiment, animals were euthanized with isoflurane and brains were collected. Subsequently, hippocampi and prefrontal cortices were dissected on ice using an optical microscope, weighted and stored at -80 °C before further processing. All the procedures involving animals were carried out according to the European Communities Council Directive 2010/63/EU and approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

3.2 Experimental design

In *Experiment 1*, we used either SLC-KOs or animals with hippocampal SLC6A15 overexpression (SLC-OE) and their controls (SLC-WT and SLC-empty, respectively) to assess the content of tissue amino acids, in particular the ones transported by SLC6A15. Therefore, we measured the levels of proline, leucine, isoleucine and methionine using mass spectrometry (n=9-11 per group). Next, we tested in *Experiment 2* whether alterations in amino acid levels are reflected by changed tissue contents of neuroactive amino acids, specifically glutamate, using HPLC, in SLC-KO and SLC-OE animals (n=6-11). To further confirm those data in an independent sample and to compare them with the human findings, in *Experiment 3* we subsequently performed spectroscopy and volumetry of the hippocampi via manganese-enhanced magnetic resonance imaging (MEMRI), both in SLC-KOs and SLC-OE mice (n=8-10). Finally, we enquired in *Experiment 4* how the observed neurochemical alterations affect behavior, testing the sensorimotor gating function in the prepulse inhibition/facilitation paradigm (PPI/PPF) (n=6-9). Each experiment was performed independently, using a different batch of animals.

3.3 Surgery

To induce an increase of SLC6A15 expression in the hippocampus, virus-mediated overexpression was employed as described previously (Schmidt, Schülke, et al., 2011). We used an adeno-associated AAV1/2 vector containing a CAG-HA-tagged-SLC6A15-WPRE-BGH-polyA expression cassette to induce SLC6A15 overexpression (abbreviated as SLC-OE). Control animals (SLC-empty) were injected with an empty construct (AAV1/2-CAG-Null/Empty-WPRE-BGH-polyA). All viral constructs used were designed and produced by GeneDetect, New Zealand (http://www.genedetect.com). For surgery, mice were anesthetized with isoflurane, installed in a stereotactic frame and injected with 0.5 µl of either AAV-SLC6A15 or AAV-Null/Empty (titers: 1.3×10^{12} genomic particles/ml) bilaterally in the dorsal hippocampus at 0.06 µl/min by glass capillaries with tip resistance of 2–4 MΩ. The following coordinates were used: 1.9 mm posterior to bregma, 1.3 mm lateral from midline, and 1.3/1.8 mm below the surface of the skull, targeting the CA1 and dentate gyrus (DG) regions of the dorsal hippocampus, respectively (George Paxinos, 2012). Experiments started 4 weeks after virus injection. Successful overexpression of SLC6A15 was verified by RT-qPCR.

3.4 *Ex vivo* mass spectroscopy

Hippocampal tissue samples were weighed and then homogenized in the sevenfold volume phosphate buffered saline (PBS), containing “Complete Protease Inhibitor Cocktail Tablets” (Roche, Penzberg, Germany) using a Minilys homogeniser (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The homogenates were analyzed using the combined high-performance liquid chromatography/mass spectrometry (HPLC/MS-MS) technique. Analysis was performed using an Agilent 1100 Series (Agilent, Waldbronn, Germany) liquid chromatograph which was interfaced to the ESI source of an Applied Biosystems API 4000 (ABSciex, Darmstadt, Germany) triple quadrupole mass spectrometer. All samples were prepared using Ostro protein precipitation and phospholipid removal plates (Waters, Eschborn, Germany). L-Proline-13C5,15N was used as internal standard for proline and L-Leucine-5,5,5-d3 was used for leucine, isoleucine and methionine. Chromatography was accomplished using a gradient elution in a Atlantis T3 column (2.1 x 100 mm, 3 µm, Waters, Eschborn, Germany) at a flow rate of 0.3 ml/min and 30 °C.

3.5 High-performance liquid chromatography

Determination of free amino acid contents in supernatants of hippocampus and prefrontal cortex homogenates (1:10 w/v) (which were stored at −80 °C prior the analysis) was done by detecting the o-phthaldialdehyde (OPA) amino acid derivatives (Smith & Sharp, 1994). Biocompatible UltiMate3000 HPLC system was coupled with coulometric detector CoulochemIII (ThermoFischer, USA). The mobile phase consisted of 50 mM Na_2HPO_4, 0.25 mM 1-octanesulfonic acid sodium salt, 0.01 mM Na_2-EDTA and 12 % methanol (v/v) and pH was adjusted to 6.5 with orthophosphoric acid. All reagents used for the mobile phase were of analytical grade and obtained from Sigma-Aldrich or Fluka (USA). The mobile phase was filtered through a 0.22 µm nylon filter (Merck Millipore, Merck KGaA, Germany) and degassed. Automatic derivatization was done with OPA-2-mercaptoethanol reagent in the presence of 0.025 M borax buffer (pH=9.6) (Agilent Technologies, Inc., USA/Switzerland) at 6°C for 5 minutes. Amino acids were separated on HR-80-3 analytical column, C18, 80 mm x 3.2 mm, 3 µm (ESA, Inc., USA) at the mobile phase flow rate of 1.0 ml/min. Glutamate was detected at the coulometric cell with E1 and E2 potentials set at +400 and +600 mV, respectively with a signal gain of 200 nA. Guard cell potential was set at + 700 mV. Detection limits for glutamate was <1 nM. Amino acid concentrations in the supernatant were calculated using an external standard curve calibration using peak area for quantification.

3.6 Manganese enhanced magnetic resonance imaging

Manganese enhanced magnetic resonance imaging (MEMRI) was performed accordingly to (Grünecker et al., 2010). Briefly, animals received intraperitoneal injections of 30 mg/kg $MnCl_2$ (Sigma, Germany) every 24 h for 8 consecutive days. MEMRI experiments were performed on a 7T Avance Biospec 70/30 scanner (Bruker BioSpin, Ettlingen, Germany). Imaging took place 12–24 h after the last injection. T_1-weighted (T_1w) brain images were acquired using a 3D gradient echo pulse sequence [TR = 50 ms, TE = 3.2 ms, matrix size = 128 × 106 × 106, zero filled to 128 × 128 × 128, field of view (FOV) = 16 × 16 × 18 mm^3, number of averages = 10, resulting in a spatial resolution of 125 × 125 × 140.6 μm^3 with a total measurement duration of 90 min]. Additionally, 3D T_2-weighted (T_2w) images were obtained using a RARE (rapid acquisition relaxation enhanced) pulse sequence [TR = 1000 ms, TE = 10 ms, matrix size = 128 × 112 × 112, zero filled to 128 × 128 × 128, FOV = 16 × 16 × 18 mm^3, number of averages = 2, RARE factor = 16, resulting in a resolution of 125 × 125 × 140.6 μm^3, with a measuring time of around 30 min].

Images were reconstructed using Paravision software (Bruker BioSpin, Ettlingen, Germany) and transferred to standard ANALYZE format. Further post-processing was performed using SPM8 (www.fil.ion.ucl.ac.uk/spm). All images were bias corrected to remove intensity gradients introduced by the geometry of the surface coil. T_2w images were then spatially normalized to a

custom-made master template deriving from 216 individual images. A binary mask containing the intracranial vault without large vessels (whole brain) was defined in MRIcro (www.sph.sc.edu/comd/rorden/mricro.html) on the T_2w master template and transformed to the native space (co-registered) of each individual animal. For further analysis, whole brains were extracted from the co-registered and bias corrected T_1w-images.

3.7 Sensorimotor gating

The acute startle response protocol was performed according to Yen and colleagues (Yen et al., 2013). Briefly, test naïve mice were placed into one out of seven identical startle set-ups, consisting of a non-restrictive Plexiglas cylinder. The startle amplitude was defined as the peak voltage output within the first 50 milliseconds after stimulus onset and quantified by means of SR-LAB software. Before startle measurements, we calibrated response sensitivities for each chamber to assure identical output levels. Startle stimuli and background (BG) noise were delivered through a high-frequency speaker placed 20 cm above each cage. The startle stimuli consisted of white noise bursts of 20-millisecond duration and 75, 90, 105 or 115 dB intensity presented in a constant background noise of 50 dB. Intensity was measured using an audiometer (Radio Shack, 33-2055; RadioShack, Fort Worth, TX, USA). On control trials, only background noise was present. After an acclimation period of 5 min, 10 control trials and 20 startle stimuli for each intensity were presented in pseudorandom order in each test session. The interstimulus interval was 15 seconds averaged (13–17 seconds, pseudorandomized). Plexiglass cylinders were cleaned thoroughly with soap water after each trial. Experiments were performed in the dark (0 lux) with red light in the experimental room. Acoustic startle responses (ASRs) to a given stimulus intensity were averaged separately for each mouse. On the next day relative changes of startle responses at different prepulse (PP) intensities were measured. Mice were placed into one of the cylinders used for the acoustic startle response. Startle amplitude was taken as the highest voltage during a time window of 20 ms. Mice were acclimated to the startle apparatus for 5 min before the first trial began. The first 20 trials consist of 20 startle pulses (white noise 115 dB) which served to habituate and stabilize the animals' startle response and were not included in the analysis. Each session consisted in 22 pulse-alone trials (115 dB), 210 prepulse (PP)-condition trials, and 18 prepulse-alone trials. The 250 discrete trials were presented in a pseudorandom order, with a variable inter-trial interval of a 15 s on average (ranging from 13 to 17 s). Fifteen different prepulse-condition trials were presented, each for 14 times. 55dB white noise was used as prepulse intensity with an inter-pulse interval (IPI, between onsets of the prepulse and pulse) of 5,

10, 25, 50, or 100 ms. Prepulse inhibition/facilitation was calculated as follows: % ASR = [(PP-condition – pulse-alone)/pulse-alone × 100%].

3.8 Statistical analysis

All results are shown as mean ± SEM and were analyzed by the SPSS 16.0 software. Independent *t* tests were used to investigate group differences (SLC-WT vs SLC-KO and SLC-empty vs SLC-OE). When the assumptions required for the parametric tests were violated, Mann-Whitney U tests was performed. Imaging data were analyzed using two-way ANOVA (hemisphere and genotype as independent factors). Behavioral data, input/output curve and prepulse inhibition and facilitation, were analyzed with repeated measure ANOVA, using intensities of the stimulus (BG, 75, 90, 105, 115 dB) and time (5, 10, 25, 50, 100 msec) as within-subjects factors and genotype as between-subject factor (SLC-WT vs SLC-KO). When the ANOVA was significant, effects were located with Bonferroni´s post hoc tests ($p<0,05$). Statistical outliers were identified with the Grubbs´ test and excluded from the analysis. If Shapiro-Wilk test of normality or Levene´s test of homogeneity were violated, data were Ln transformed. For each experiment, SLC-OE animals not showing increase in SLC6A15 levels were excluded from the analysis. P-values of $p<0,05$ were considered significant.

4. Results

4.1 Experiment 1: Amino acid concentrations in the hippocampus

The total levels of the main amino acids which are primarily substrates for SLC6A15 were measured in hippocampal extracts using mass spectroscopy. SLC-KO mice showed significantly reduced levels of proline, leucine, isoleucine and methionine compared to SLC-WT (proline: U_{pro}=20, p<0,05; leucine: U_{leu}=23, p<0,05; isoleucine: U_{ile}=20, p<0,05; methionine: U_{met}=20, p<0,05) (Figure 1A-D). On the other hand, SLC6A15 overexpression did not alter hippocampal levels of those amino acids compared to SLC-empty animals (Figure 1E-H).

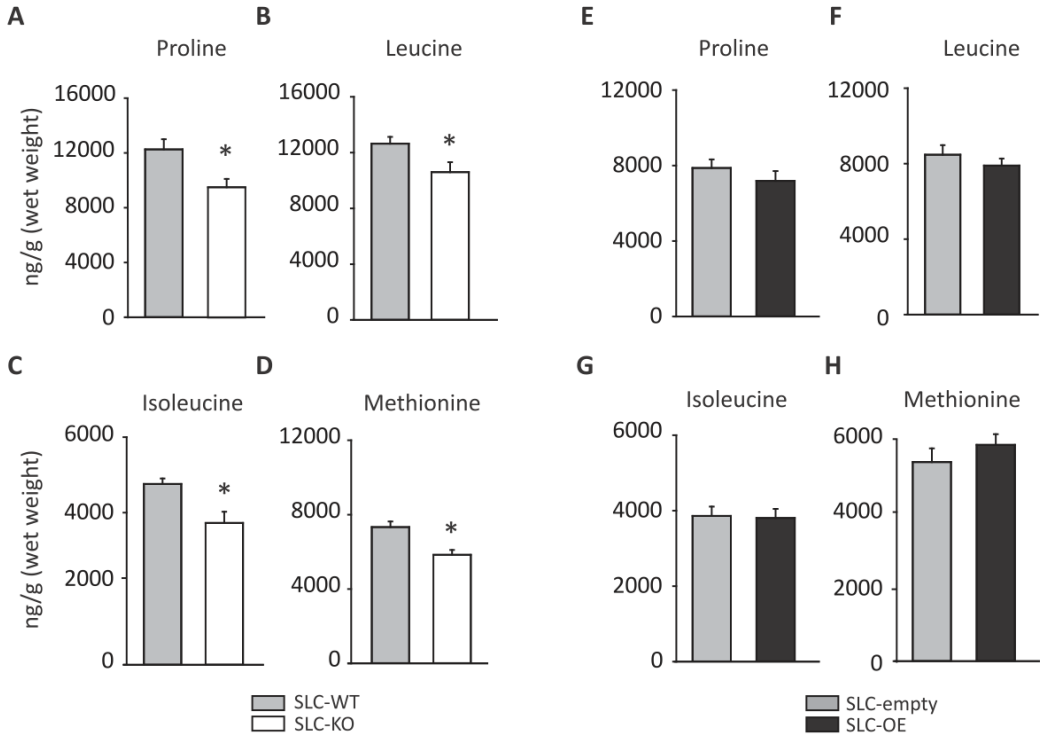

Figure 1: Mass spectroscopy. Abundance of SLC6A15 substrates in the hippocampus was significantly affected by the absence of the transporter. Tissue content of proline, leucine, isoleucine and methionine, was reduced in the SLC-KO compared to the SLC-WT **(A-D)**. Overexpression of SLC6A15 did not affect the total amount of those amino acids in the hippocampus **(E-H)**. Data are given as means and SEM (* = p<0.05).

4.2 Experiment 2: Glutamate content in the brain

The total glutamate levels in the forebrain were assessed in experiment 2. Using HPLC we determined a reduction of glutamate levels in the hippocampus of SLC-KO animals ($t_{(16)}$=3,028, p<0,05). To assess another brain region strongly implicated in many psychiatric disorders, we also investigated glutamate levels in the prefrontal cortex (PFC). Here, SLC-KOs also displayed lower glutamate levels ($t_{(11)}$=2,152, p=0,05) (Figure 2A-B). We next assessed the same parameters

following SLC6A15 overexpression in the hippocampus. SLC-OE mice showed a significant increase in glutamate tissue levels ($t_{(17)}$=2,921, p<0,05) compared to SLC-empty mice in the hippocampus, but no significant effect was found in the PFC (t test, p>0,05) (Figure 2C-D).

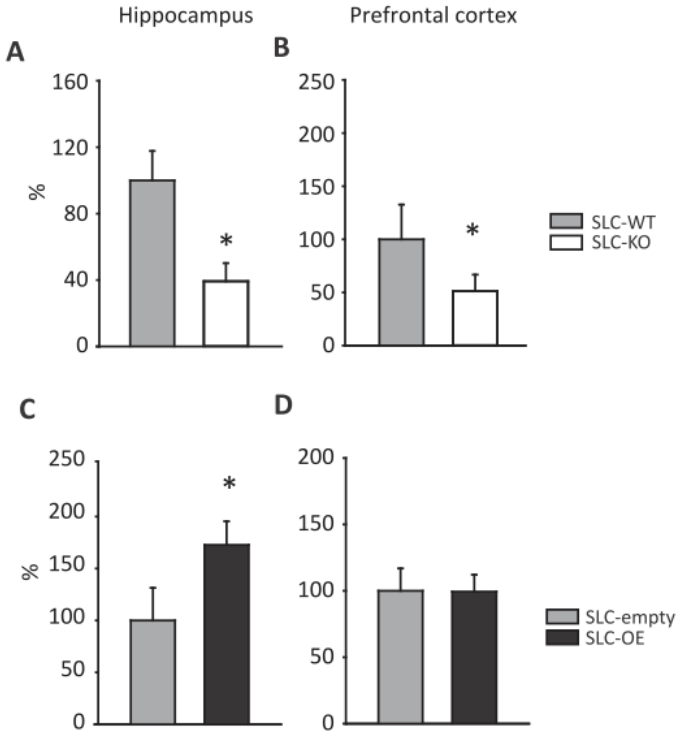

Figure 2: HPLC. Brain neurochemistry was evaluated via HPLC. Comparisons between SLC-WT and SLC-KO revealed a decrease in glutamate in SLC-KO, in both of the areas analyzed **(A, B).** On the other hand, overexpression of SLC6A15 in the hippocampus leads to an increase of glutamate selectively in the hippocampus **(C)** but not in the prefrontal cortex **(D).** Data are given as means and SEM (* = p<0.05).

4.3 Experiment 3: MR Imaging and MR-spectroscopy of the hippocampus

Manganese-enhanced magnetic resonance imaging and MR- spectroscopy were performed to investigate whether alterations of SLC6A15 expression would parallel the results observed in humans (Kohli et al., 2011). The spectroscopic profile of the hippocampus showed reduced glutamate and glutamine levels in SLC-KO mice ($F_{(1,31)}$=14,58, p<0,05) compared to SLC-WTs in both the left and right hemisphere (post hoc tests, p<0,05) (Figure 3A). Regarding the overexpression study, different changes in glutamate and glutamine were found in the hippocampus of SLC-OE compared to SLC-empty ($F_{(1,33)}$=82,10, p<0,05) (Figure 3B). Post hoc analysis revealed an increase in glutamate and glutamine levels in SLC OE mice (p<0,05). In addition, glutamate and glutamine levels were increased in the right compared to the left hemisphere, independently on the SLC6A15 expression. Volumetric analyzes did not reveal any statistical differences between the groups (ANOVA, p>0,05) (Figure 3C-D), illustrating that at least

at this level of analyzes hippocampal volume is not directly linked to SLC6A15 expression in mouse brain.

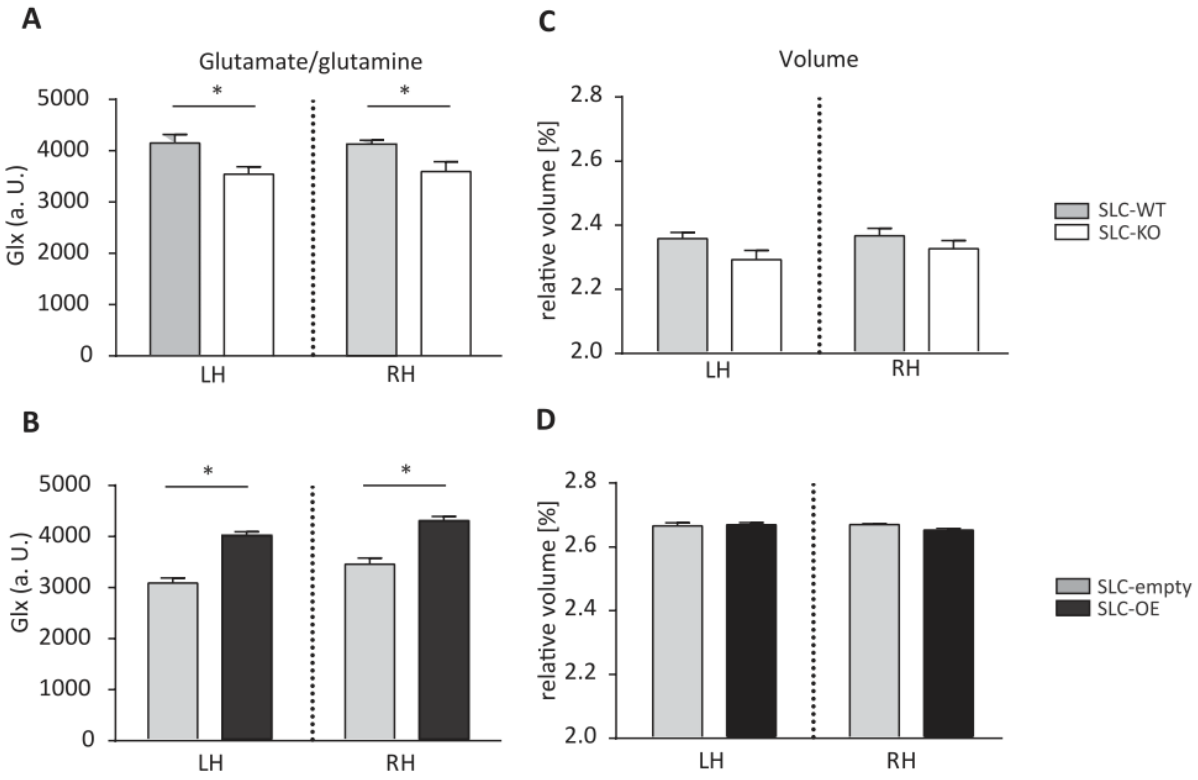

Figure 3: Imaging results. We performed MEMRI studies to investigate the spectroscopic properties and volume of the hippocampus. As it was indicated by the human findings in Kohli et al 2011, SLC-KO have significant reduction of Glx concentration compared to SLC-WT **(A).** Overexpression of SLC6A15 leads to increase of Glx concentration **(B)**, while hippocampus volume is not affected **(C, D)**. LH, left hemisphere; RH, right hemisphere; Glx, glutamate+glutamine. Data are given as means and SEM (* = p<0.05).

4.4 Experiment 4: Behavioral analysis

Finally, we aimed to obtain a behavioral correlate that was previously shown to be sensitive to alterations in glutamate signaling. Although the acoustic startle response showed no main effect of genotype (ANOVA, p>0,05), a significant interaction between tone intensity and genotype was observed ($F_{(1,13)}$=5,243, p<0,05), indicating that SLC-KOs show a different input/output curve response. At 115 dB a higher baseline startle amplitude was observed in SLC-KO compared to SLC-WT littermates (post hoc tests, p<0,05) (Figure 4A). Furthermore, sensorimotor gating evaluation revealed a main effect of genotype on prepulse inhibition/facilitation at 55 dB ($F_{(1,13)}$=13,82, p<0,05), as SLC-KOs showed reduced prepulse facilitation at 5 and 10 msec inter-pulse interval (post hoc tests, both p<0,05) (Figure 4B). No statistical differences in the basal startling reflex and sensorimotor gating test were found between SLC-OE and SLC-empty animals (Figure 4C-D).

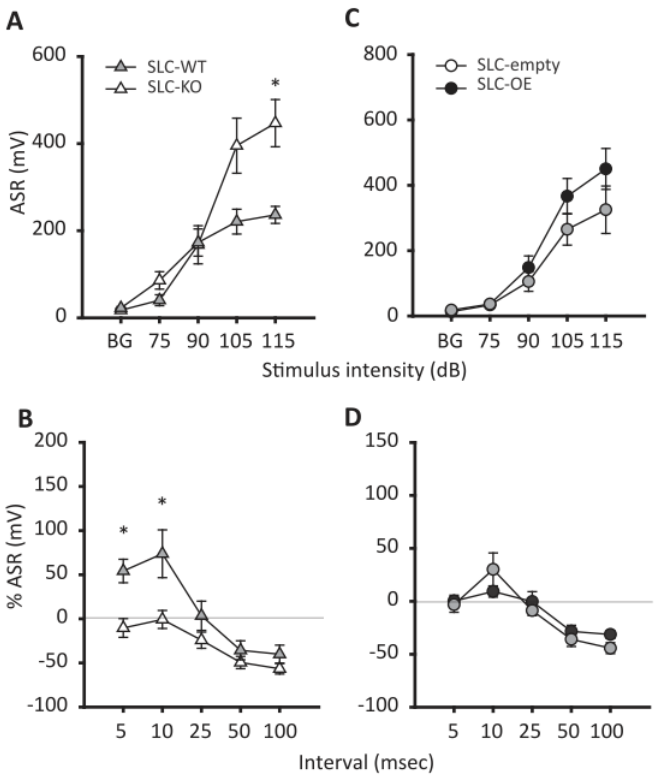

Figure 4: Behavior. According to the neurochemical profile we observed, we hypothesized that SLC-KO and SLC-OE have impairments in sensorimotor gating. Therefore, acoustic startle response was measured at 5 different acoustic intensities. SLC-KO showed significant increased startle response at 115 dB **(A)**. Also relative changes of startle responses at different prepulse intensities were assessed. At 55 dB, SLC-KO showed a significantly different behavioral response from SLC-WT after 5 and 10 msec interpulse interval **(B).** No significant difference was found between SLC-empty and SLC-OE animals in the startle curve or prepulse alterations **(C-D).** BG=background, ASR=acoustic startle response. Data are given as means and SEM (* = $p<0.05$).

5. Discussion

Recent studies have reported a role for SLC6A15 in psychiatric disorders (Kohli et al., 2011) and suggested a potential link to the glutamatergic system, but direct experimental evidence for this association was so far missing. We therefore investigated the role of this novel, highly promising candidate gene in regulation of brain neurochemistry and behavior. We show that modulation of SLC6A15 expression directly affects the glutamatergic system and a related behavioral phenotype, providing substantial evidence supporting SLC6A15 as a crucial factor contributing to psychopathologies that involve a dysregulation of glutamatergic signaling.

We started by investigating the primary substrates of SLC6A15, proline and other amino acids, in the hippocampus, the brain area where SLC6A15 is mostly expressed in the mouse brain. Previously, SLC6A15 availability was shown to correlate with amino acid uptake in synaptosomes (Bröer et al., 2006; Drgonova et al., 2007). We now determined that the presence (or the absence) of SLC6A15 itself is already sufficient to regulate the abundance of hippocampal amino acids, especially proline, which was significantly decreased in SLC-KO mice. The lack of changes in proline levels in SLC-OE animals may be due to a ceiling effect, as the higher intracerebral proline availability could be immediately channeled to downstream pathways, which is in line with the observed higher levels of glutamate. It is worth to mention that proline, a neuroactive amino acid, is linked to many behavioral and neurochemical alterations relevant to mood disorders (Baxter, Baldwin, Davis, & Flood, 1985; Cherkin et al., 1976; S. M. Cohen & Nadler, 1997; Moreira, Wannmacher, Costa, & Wajner, 1989; Roussos et al., 2009). In addition, this amino acid is of predominant interest as it can be converted to glutamate via 1-pyrroline-5-carboxylate dehydrogenase (Pérez-Arellano et al., 2010), representing one of the starting pools for glutamate *de novo* synthesis. In support to these findings, we demonstrated that SLC6A15 is crucial for glutamate availability in the brain. In addition, the effect of SLC6A15 modulation is site specific, as in SLC-OE animals glutamate levels were not affected outside the hippocampus. The robustness of the observed effects is further confirmed by employing different detection techniques in several independent cohorts of animals.

In order to align our findings with the previously published results in human risk allele carriers studies, we also performed a manganese-enhanced imaging study, to assess potential hippocampal volume changes. Kohli and colleagues had reported an interaction effect between genotype (risk allele carriers vs non carriers for the rs1545843 SNP) and condition (controls vs patients) on hippocampal volume. In our results, at least under basal conditions, no differences in volumes were observed. This may be due to a lack of gene * environment interaction, which may

be necessary to observe changes in hippocampal volume. In addition, the used model systems are clearly artificial and do not fully represent the human situation with the risk polymorphism. Interestingly, the group of Fredriksson reported effects of SLC6A15 polymorphisms only under specific environmental conditions (Hägglund et al., 2013), indicating that SLC6A15 may have a crucial role in adaptation to challenging situations. Further experiments are therefore needed to explore the conditions under which SLC6A15 may be involved in regulating structural changes in the brain and the possible role of SLC6A15 in stress vulnerability or resilience.

As we observed strong alterations in amino acid neurochemistry in the hippocampus, we investigated potential behavioral alterations following SLC6A15 regulation. According to the profile observed in glutamate levels and the absence of structural changes in the hippocampus, we tested sensorimotor gating, a well-characterized endophenotype regulated by neurochemistry of the hippocampus used both in humans and animals (Reynolds, Cochran, Morris, Pratt, & Reynolds, 2005). Intriguingly, SLC-KOs displayed a significant increase in the acoustic startle response and a reduction in the prepulse facilitation, indicating that the sensorimotor gating processes of SLC-KOs are altered compared to SLC-WT mice. Interestingly, also a direct modulation of proline levels has previously been reported to regulate sensorimotor gating (Gogos et al., 1999). In contrast, SLC-OE mice did not show a behavioral phenotype in this task. This could be due to the compensating involvement of other brain areas, for example the prefrontal cortex or bed nucleus of the *stria terminalis* (BNST), which are mediating ASR together with the hippocampus, but that are not affected by an overexpression of SLC6A15 in the hippocampus. Alternatively, an increased transport of proline may be quickly compensated via metabolic pathways in order to avoid proline´s neurotoxic effects (Cherkin et al., 1976; Delwing, Bavaresco, Wannmacher, et al., 2003; Henzi, Reichling, Helm, & MacDermott, 1992) and ultimately not result in significant behavioral alterations, which would be in line with the finding that SLC6A15 overexpression does not increase levels of proline. Further studies are needed to unravel the behavioral endophenotypes linked to SLC6A15 functionality and expression, in particular connected with the molecular profile observed.

In summary, we conclude that the recently identified risk factor for psychiatric disorders SLC6A15 is apparently crucial to maintain glutamate (and the excitatory neurotransmission) homeostasis in the brain. Abundance of SLC6A15 in the hippocampus is linked to neuroactive amino acids availability, which in turn has drastic consequences on the glutamate levels in the hippocampus. There is a growing body of evidence in support of the important role of the glutamatergic system in vulnerability to psychiatry disorders (Sanacora et al., 2012) and our data are fitting well into this framework. Our findings demonstrate that SLC6A15 expression changes

may exert a strong impact on specific behaviors and brain neurochemistry markers, making SLC6A15 manipulation a promising candidate to unravel the role of glutamate in hippocampus-mediated behavior and a good target for developing potential neuroactive drugs with an innovative mechanism of action.

6. Acknowledgments

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SLC6A15, a novel stress vulnerability candidate, regulates anxiety and depressive like behaviors through glutamatergic system

Sara Santarelli[1], Klaus V Wagner[1], Christiana Labermaier[1], Andrés Uribe[1], Carine Dournes[1], Georgia Balsevich[1], Jacob Hartmann[1], Mercé M Nadal[1], Mathias V. Schmidt[1]

[1] Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, 80804 Munich, Germany

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1. Abstract

Major depression is a multifactorial disease, involving both environmental and genetic risk factors. Recently, SLC6A15 was proposed as new candidate gene for major depression and stress vulnerability. Risk allele carriers for a single nucleotide polymorphism (SNP) in a SLC6A15 regulatory region display altered hippocampal volume and glutamate levels and hypothalamus-pituitary-adrenal (HPA) axis activity, all markers of major depression vulnerability. Despite the strong correlation between SLC6A15 and depression, its functional role with regard to the disease is still unknown. SLC6A15 is coding for a neutral amino acid transporter, which is mainly expressed in neurons. The aim of the current study was therefore to characterize the role of Slc6a15 in brain and behavior, especially in relation to stress and mood disorders. We investigated the effects of slc6a15 manipulation using two mouse models, SLC6A15 total knockout (SLC-KO) and virus-mediated hippocampal slc6a15 overexpression (SLC-OE). Mice were tested under basal conditions and following chronic social stress. We found that SLC-KO animals displayed a similar behavioral profile to wild type littermates (SLC-WT) under basal condition. Interestingly, following chronic social stress SLC-KO animals had lower levels of anxiety- and depressive-like behavior compared to stressed WT littermates. In support of those findings, SLC-OE animals displayed, already under basal condition, increased anxiety- and depressive-like behavior. We also provided evidence supporting that GluR1 expression levels in the dentate gyrus are regulated by slc6a15 levels, possibly contributing to different stress responsiveness in SLC-KO animals. Taken together, our data demonstrate that variations in slc6a15 expression levels affect emotional behavior, likely by influencing the glutamatergic neurotransmission.

2. Introduction

Acute and chronic stress represent the most prevalent environmental risk factors for a number of mental disorders, particularly for major depression (de Kloet et al., 2005). However, although the majority of the population is exposed to stressful experiences, only a subset develops depression later in life. This may be attributable to differences in sensitivity to stress, e.g. due to genetic variances associated with stress vulnerability. Indeed, some genetic risk factors for depression have been identified, revealing that polymorphisms in genes involved in neurotransmitter systems and neurotrophic factors are associated with a significantly increased risk of developing mental disorders (Gatt et al., 2009; Gratacòs et al., 2007; K.-P. Lesch et al., 1996; Schinka, Busch, & Robichaux-Keene, 2004). It is nowadays accepted that gene * environment interactions are the basis for developing major depression (Caspi & Moffitt, 2006).

Recently, a genome-wide association study proposed SLC6A15, a neuron-specific neutral amino acid transporter, as new candidate gene for vulnerability to stress and major depression (Kohli et al., 2011). A single nucleotide polymorphism (SNP) in a regulatory region of SLC6A15, rs1545843, was significantly correlated with higher incidence of depression. In addition, risk allele carriers for this SNP have reduced hippocampal volume and glutamate levels (Kohli et al., 2011). Furthermore, hypothalamus-pituitary-adrenal (HPA) axis activity, memory and cognition are significantly impaired in depressed patients carrying the aforementioned SLC6A15 SNP (Schuhmacher et al., 2013). Support to the human findings comes also from animal studies, where deletion of *Slc6a15* leads to downregulation of glutamate levels in the hippocampus and alteration in emotional behavior (Santarelli et al, under revision). Therefore, accumulating evidence from both clinical and preclinical studies is pointing at a crucial involvement of SLC6A15 in regulating the glutamatergic system in the hippocampus and emotional behavior. However, while human findings suggest that SLC6A15 plays a major role under stressful conditions, animal studies have so far not addressed gene * environment interactions for *SLC6A15*. Therefore, the aim of this study was to characterize the involvement of SLC6A15, especially in relation to chronic stress exposure. We addressed this question by comparing the behavioral and neuroendocrine response of *Slc6a15* knockout (SLC-KO) mice to chronic social defeat stress (CSDS), compared to wild type littermates (SLC-WT). To challenge the results obtained from SLC-KO under stress, we also studied mice with a targeted SLC6A15 overexpression in the hippocampus (SLC-OE) for alterations in the same phenotypic markers. Our results showed that SLC6A15 deletion attenuated stress-induced anxiety- and depressive-like phenotypes, whereas hippocampal SLC-OE results in increased anxiety- and depressive-like phenotypes already under basal conditions.

3. Material and Methods

3.1 Animal housing and husbandry

For experiment 1, Slc6a15 knock-out mice were kindly provided (Drgonova et al., 2007) and kept as in house colony. Slc6a15 knock-out mice (from now on abbreviated as SLC-KO) did not show overt reproductive failures and no abnormalities in the survival rate. The genotypes of the offspring corresponded approximately to the Mendelian ratios, as well as sexes were equally distributed. Mice used for the experiments were obtained from heterozygous breeding pairs. wild-type littermates (abbreviated as SLC-WT) were used as a control group. Genotypes were verified by PCR analysis of tail DNA following published protocols (Drgonova et al., 2007). For experiment 2, the hippocampal SLC6A15 overexpression studies, male C57Bl/6N mice (Charles River Laboratories, Maastricht, the Netherlands) were used. All animals were between 10-17 weeks old and single housed for at least 1 week before testing. Mice were held under normal light and temperature conditions (12 light: 12 dark light cycle, lights on at 8 pm, temperature at 23 ± 2°C). Food (Altromin 1324, Altromin) and tap water were available *ad libitum*. All the procedures involving animals were carried out according to the European Communities Council Directive 2010/63/EU and approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

3.2 Experimental design

Experiment 1: SLC-KO and SLC-WT littermates at the age of 12 to 14 weeks underwent 3 weeks of chronic social defeat stress. Behavioral testing took place during the last week of the stress procedure, in the following order: elevated plus maze, open field and forced swim test with at least one day between tests.

Experiment 2: Twelve-week old male C57Bl/6N mice underwent stereotactic surgery in order to induce viral overexpression of SLC6A15 bilaterally in the hippocampus. After surgeries, the animals were left undisturbed for four weeks. Afterwards, they were tested in the following order: elevated plus maze, open field and forced swim test with one day interval between tests.

3.3 Stress procedure

The chronic social defeat stress (CSDS) paradigm was conducted as described previously (Wagner et al., 2012). Briefly, the experimental mice were introduced into the home cage (45 cm × 25 cm) of a dominant resident mouse and defeated shortly after. After the defeat was achieved, the animals were separated by a wire mesh, preventing physical but allowing sensory contact for

24 h. Each day, stressed animals were defeated by another unfamiliar, dominant resident CD-1 mouse, in order to exclude a repeated encounter throughout the experiment. Experimental mice were always defeated by resident males during the entire experimental period. Control mice were housed in their home cages during the course of the experiment. All mice were handled daily; body weight and fur quality were assessed every 3–4 days prior and during the social defeat. The fur state was rated by an experienced investigator as previously described (Mineur et al., 2003). Briefly, ratings were classified according to a 4-point scale, where 1 represents a perfect, clean fur, while 4 stands for disheveled, scruffy fur, often including traces of wounds and scurf. Scores of 2 and 3 demonstrate intermediate fur states, respectively.

3.4 Surgery

To induce an increase of SLC6A15 expression in the hippocampus, virus-mediated overexpression was employed as described previously (Schmidt, Schülke, et al., 2011). We used an adeno-associated AAV1/2 vector containing a CAG-HA-tagged-SLC6A15-WPRE-BGH-polyA expression cassette to induce SLC6A15 overexpression (abbreviated as SLC-OE). Control animals (SLC-empty) were injected with an empty construct (AAV1/2-CAG-Null/Empty-WPRE-BGH-polyA). All viral constructs used were designed and produced by GeneDetect, New Zealand (http://www.genedetect.com). For surgery, mice were anesthetized with isoflurane, installed in a stereotactic frame and injected with 0.5 µl of either AAV-SLC6A15 or AAV-Null/Empty (titers: 1.3×10^{12} genomic particles/ml) bilaterally in the dorsal hippocampus at 0.06 µl/min by glass capillaries with tip resistance of 2–4 MΩ. The following coordinates were used: 1.9 mm posterior to bregma, 1.3 mm lateral from midline, and 1.3/1.8 mm below the surface of the skull, targeting the CA1 and dentate gyrus (DG) regions of the dorsal hippocampus, respectively (George Paxinos, 2012). Successful overexpression of SLC6A15 was verified by *in situ* hybridization.

3.5 Behavioral tests

3.5.1 Elevated Plus Maze (EPM)

For the CSDS the EPM was conducted on day 16 of the stress procedure. The device consisted of a plus-shaped platform with two opposing open arms (length: 30 cm × width: 5 cm × height: 0.5 cm) and two opposing enclosed arms (length: 30 cm × width: 5 cm × height: 15 cm), made of grey polyvinyl chloride (PVC), which were connected by a central area (5 cm × 5 cm). The whole device was elevated 50 cm above the floor. The illumination was 25 lx in the open arms and less than 10 lx in the closed arms. Testing duration was 5 min and all mice

were placed into the centre zone facing one of the closed arms at the start of the test. The time spent in the open arms compared to the total arm time was analysed.

3.5.2 Open Field (OF)

Testing (on day 17 of the CSDS procedure) was carried out in an empty open-field arena (50 cm × 50 cm × 50 cm) made of grey PVC, which was evenly illuminated with 15 lx. Testing time was 15 min, split into 3 bouts, 5 min each. Parameters of interest were the distance travelled in the centre as well as the inner zone time (inner zone size 25 cm × 25 cm).

3.5.3 Forced swim stress (FST)

For the FST (on day 19 of the CSDS), each mouse was put into a 2 l glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 6 min. The parameters time floating, time swimming, time struggling and latency to 1st floating were scored by an experienced observer, blind to genotype or condition of the animals.

3.6 Plasma corticosterone

The forced swim stress (FST) also served as an acute challenge in order to determine the stress response by measuring plasma corticosterone (CORT) concentrations. Blood samples were taken by tail cut 30 min (stress response) and 90 min (stress recovery) after the onset of the FST (Fluttert et al., 2000). For basal blood samples, trunk blood was collected on the day of the sacrifice. Samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled microcentrifuge tubes and stored at −20 °C until further processing. The determination of corticosterone was performed by radioimmunoassay (MP Biomedicals Inc.; sensitivity 6.25 ng/ml).

3.7 Sampling procedure

All animals were sacrificed 48 h after the last defeat, or the last behavioral test, during the circadian nadir by decapitation following quick anesthesia by isoflurane. Brains were removed, snap-frozen in isopentane at −40 °C, and stored at −80 °C for *in situ* hybridization. Adrenal glands and thymus were removed, dissected from fat and weighed.

3.8 *In situ* hybridization

Frozen brains were sectioned at −20 °C in a cryostat microtome at 18 µm at the level of the dorsal hippocampus, thaw mounted on Super Frost Plus slides, dried and stored at −80 °C. In situ hybridization using ^{35}S UTP labelled ribonucleotide probes (GluR1, slc6a15) was performed as described previously (Schmidt et al., 2007). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes were transcribed from a linearized plasmid. Tissue sections were saturated with 100 µl of hybridization buffer containing approximately 3–5 × 10^6 cpm ^{35}S labelled riboprobe. Brain sections were coverslipped and incubated overnight (14 h) at 55 °C. The following day, the sections were rinsed in 2× SSC (standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1× SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were scanned, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software (http://rsbweb.nih.gov/ij/). We performed two measurements (left and right for the hippocampus) for each brain slice and assessed two brain slices per animal. The data was analysed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest.

3.9 Statistical analysis

All results are shown as mean ± SEM and were analyzed by the software SPSS 16.0. For body weight and fur status progression as well as for the time-dependent behavioral parameters assessed in the OF, 3-factorial analysis of variance (ANOVA) with repeated measures was performed. Thereby, genotype (SLC-WT vs SLC-KO) and condition (CTRL vs CSDS) were two between-subjects factors and time the within-subjects factor. Two-way ANOVAs were employed for thymus, adrenal gland weight, neuroendocrine, and other behavioral variables as well as for gene expression contrasts. For the overexpression study, two-tailed t-test where used. When the assumptions required for the parametric tests were violated, Mann-Whitney U tests were performed. Whenever significant main ($p < 0.05$) or interaction ($p < 0.1$) effects were found in the ANOVAs, univariate *F*-tests or tests with contrasts followed for specifying and locating simple effects. As a nominal level of significance $p < 0.05$ was accepted and corrected according to Bonferroni (univariate *F*-tests, test of simple effects or contrasts).

4. Results

4.1 Experiment 1

4.1.1 Body weight, fur status and organ weight after CSDS

Body weight and fur status were assessed throughout the CSDS procedure. Overall, body weight was significantly affected by the progression of the stress (ANOVA, $F_{(6,36)}$=10.824, p<0.05), but not by the genotype. Stressed groups showed a significant increase in body weight during the CSDS, whereas non-stressed mice showed no significant alteration in their body weight during the experiment (Figure 1A). Similarly, fur status was significantly affected by the stress progression (ANOVA, $F_{(6,36)}$=50.750, p<0.05), with stressed animals of both genotypes showing a worsening of the fur status (Figure 1B). Thymus and adrenal glands were collected and weighted revealing a significant effect of the stress (ANOVA, $F_{(3,37)}$=20.159, and $F_{(3,37)}$=48.357, both p<0.05, respectively) with significantly heavier adrenals and lighter thymus weights in stressed SLC-WT and SLC-KO animals compared to non-stressed controls (respectively Figures 1C and 1D).

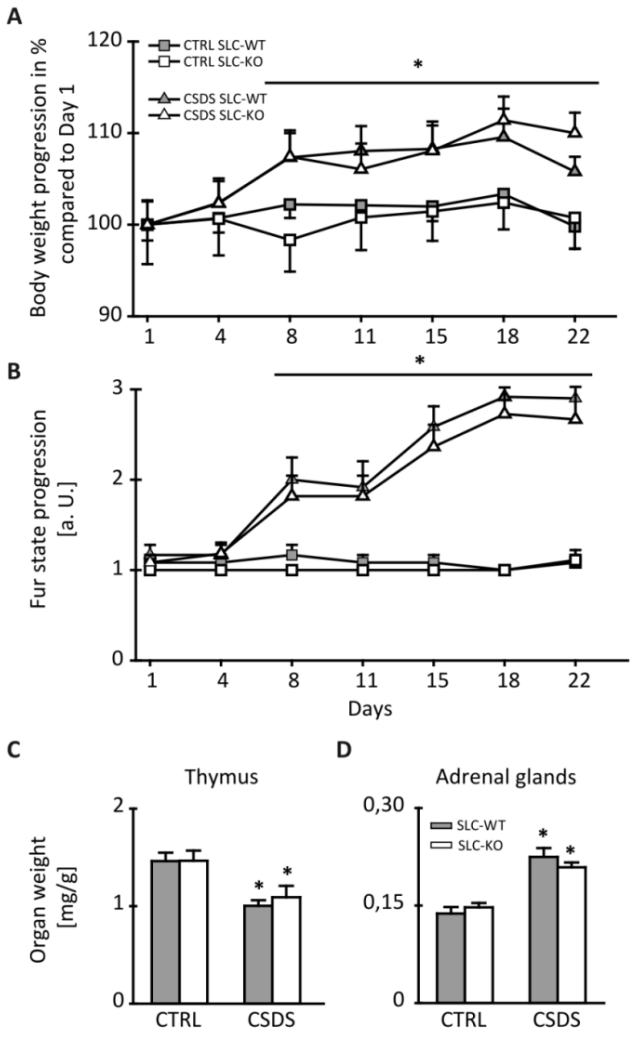

Figure 1: Effects of chronic social stress and genotype on organ and body weight. To assess the effectiveness of stress, general physiology indicators were measured. Body weight and fur status progression are significantly increased by CSDS, independently from genotype. Along this line, thymus and adrenal glands weights are not different between SLC-KOs and SLC-WTs, but are increased by CSDS. (Abbreviations: CSDS, chronic social defeat stress). Data are given as means ± SEM (* = p<0.05).

4.1.2 Corticosterone

Plasma corticosterone levels were assessed under basal conditions revealing a main effect of genotype (ANOVA, $F_{(1,35)}$=9,323, p<0,05) and condition ($F_{(1,35)}$=4,186, p<0,05) and an interaction effect of genotype * condition ($F_{(1,35)}$=4,229, p<0,05). Under non-stressed conditions, CORT levels of SLC-KO and SLC-WT mice were comparable. Following CSDS, SLC-KO mice had significantly higher basal CORT levels compared to stressed SLC-WT mice (Figure 2A). Corticosterone levels 30 minutes after an acute stressor (FST) were increased by CSDS in both SLC-WT and SLC-KO (ANOVA, $F_{(1,38)}$=21,547, p<0,05) compared to non-stressed animals, but did not differ between genotypes (Figure 2B). At recovery, assessed 90 minutes after the FST, CORT levels are still increased by the CSDS in both CSDS-WT and CSDS-KO compared to their respective controls (ANOVA $F_{(1,38)}$=21,547, p<0,05). A main genotype effect was found ($F_{(1,38)}$=5,482 p<0,05), with stressed SLC-KO showing lower CORT levels compared to SLC-WT. Similar to the situation under basal conditions, non-stressed SLC-WT and SLC-KO had comparable levels of CORT (Figure 2C).

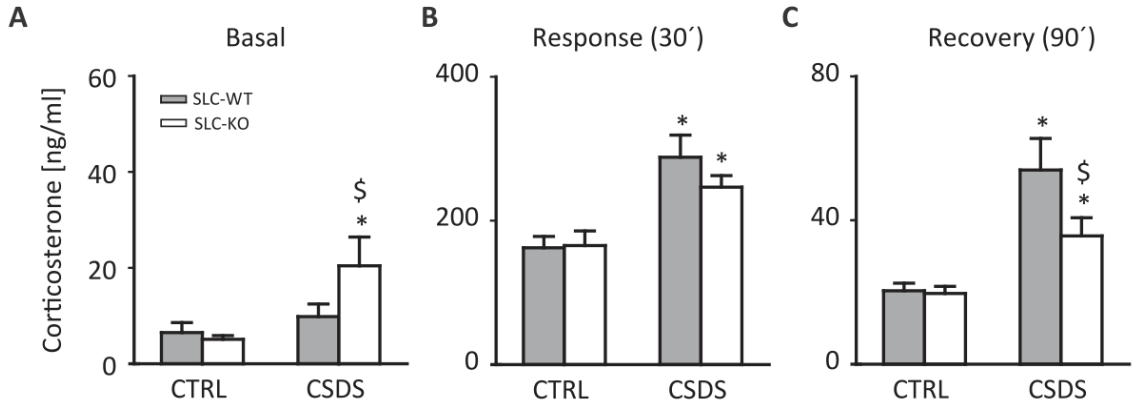

Figure 2: Corticosterone plasma levels after acute and chronic stress. Corticosterone levels were measured in the plasma under basal conditions, 30 minutes (response) and 90 minutes (recovery) after an acute stressor (FST). **(A)** Under basal condition, no differences were observed between SLC-WT and SLC-KO. After CSDS, basal corticosterone levels of SLC-KO were increased compared to SLC-KO and SLC-WT. **(B)** No genotype difference were observed 30 minutes after the stressor. **(C)** 90 minutes after SLC-KO return faster to basal levels compared to SLC-WT. Data are given as means ± SEM (*,$ = p<0.05, * = condition effect, $ = genotype effect).

4.1.3 Behavior after CSDS

For anxiety-related behavior, the EPM test revealed a main genotype effect in the % time spent in the open arms (ANOVA $F_{(1,35)}$=6,101, p<0,05). Post hoc analysis indicated that CTRL-KO spent significantly more time in the open arms compared to CTRL-WT. No statistical difference was found under chronic stress condition (Figure 3A). No significant effects were found in the number of entries in the open arms.

As additional measurement of anxiety, the OF test was used. Stress had a significant effect on the distance travelled in the center of the arena in the second and third interval of the test (ANOVA, $F_{(1,40)}$=9,795, and $F_{(1,40)}$=14,060, both p<0,05), and a significant interaction effect between condition * genotype was found (ANOVA, $F_{(1,40)}$=2,947, p<0,1). Post hoc analysis revealed that in both intervals, non-stressed groups were comparable, whereas after CSDS SLC-KO travelled significantly more compared to SLC-WT (Figure 3B). No effect in the time spent in the inner zone was found.

For stress-coping behavior, the FST revealed a significant main effect of the genotype in the latency to the first episode of floating (ANOVA, $F_{(1,36)}$=4,355, p<0,05), with no significant effects for the time spent struggling and floating. Post hoc analyzes revealed that under control conditions SLC-WT and SLC-KO are comparable, while after stress SLC-KO have a significantly higher latency to start floating compared to SLC-WT (Figure 3C).

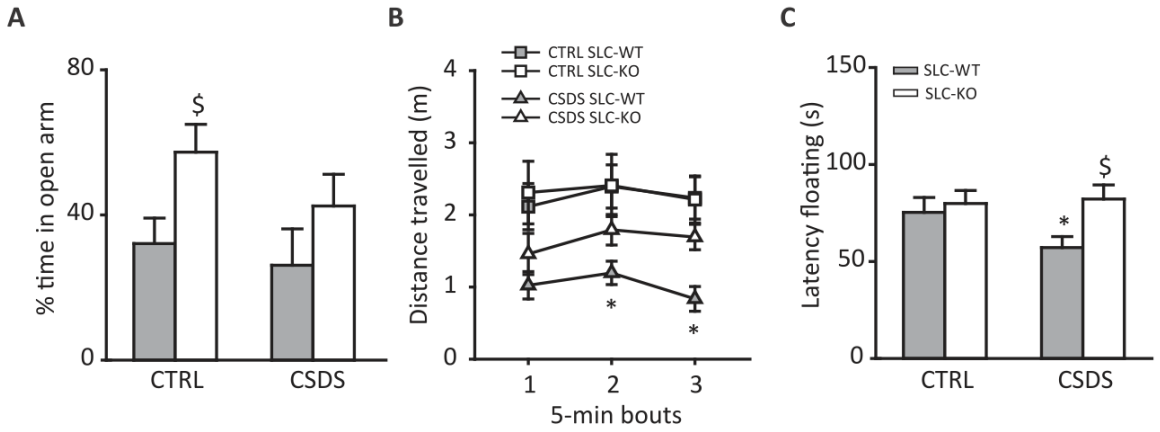

Figure 3: Behavioral profile after chronic stress. Alterations in emotional behavior were investigated in the elevated plus maze, open field and forced swim test. **(A)** SLC-KOs show reduced anxiety like behavior in the elevated plus maze compared to SLC-WTs under basal condition, but no genotype effect was reported under chronic social defeat. **(B)** In the open field test SLC-KOs and SLC-WTs travelled comparable distance under basal condition, whereas SLC-WTs travelled significantly less following chronic social defeat compared to SLC-KOs. **(C)** Depressive like behavior was assessed in the forced swim test, which revealed a significant difference between stressed SLC-WTs and control animals in latency to the first floating episode. Furthermore, stressed SLC-KOs showed a significantly higher latency time compared to SLC-WTs. Data are given as means ±SEM (*,$ = p<0.05, * = condition effect, $ = genotype effect).

4.2 Experiment 2:

4.2.1 Body weight and corticosterone levels

The body weight of the animals were assessed before the surgery (SLC-empty: 26,2 g ±0,33 and SLC-OE: 26,4 ±0,3) and at the end of the recovery phase (SLC-empty: 29,1 g ±0,50 and SLC-OE: 30,0 ±0,5) but no significant differences were found. For SLC-OE, the data of the CORT levels were not meeting the assumption for parametric testing (normal distribution); therefore non-parametric Mann-Whitney-U test was performed. Basal and recovery corticosterone levels were

not significantly affected by hippocampal SLC6A15 overexpression. Interestingly, response CORT levels are significantly reduced in SLC-OE compared to SLC-empty animals (U=56, p<0,05) (Figure 4A-C).

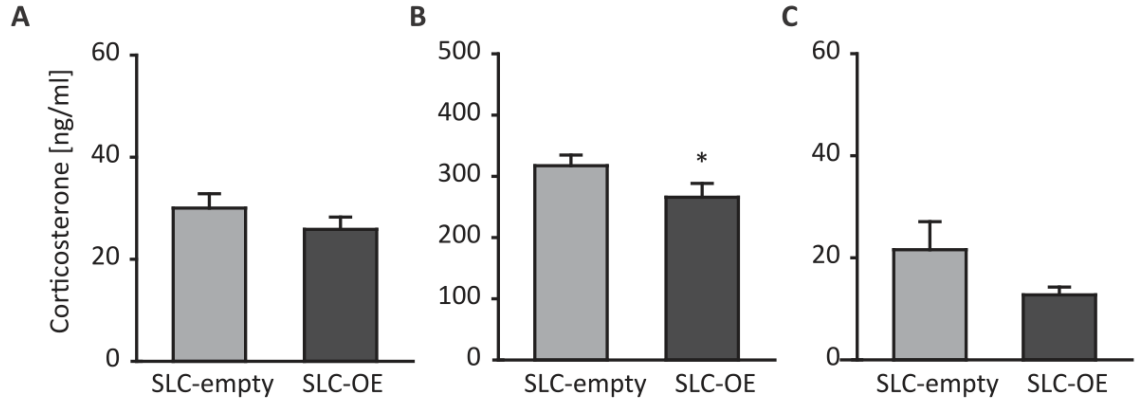

Figure 4: Corticosterone plasma levels after SLC6A15 overexpression. Corticosterone levels were measured in the plasma under basal conditions, 30 minutes (response) and 90 minutes (recovery) after an acute stressor (FST). **(A)** SLC-OE presented comparable profile of SLC-empty under basal conditions. **(B)** However, shortly after the FST, SLC-OE have reduced plasma corticosterone levels compared to SLC-empty. **(C)** No differences were observed 90 minutes following the stress exposure. Data are given as means ± SEM (* = p<0.05).

4.2.2 Behavior of SLC-OE animals

In the EPM, SLC-OE spent significantly less time in the open arms ($t_{(24)}$=2,414, p<0,05) compared to SLC-empty animals (Figure 5A), indicating an increased level of anxiety. Number of entries to the open arms was not affected by SLC6A15 overexpression. During the first interval of the OF, SLC-OE animals travelled significantly more than SLC-empty animals (U=31, p<0,05), whereas no difference was found during the other two intervals (Figure 5B). No difference in the time spent in the inner zone of the arena was found. The results of the FST test did not show significant differences in the behavior between SLC-empty and SLC-OE mice (Figure 5C).

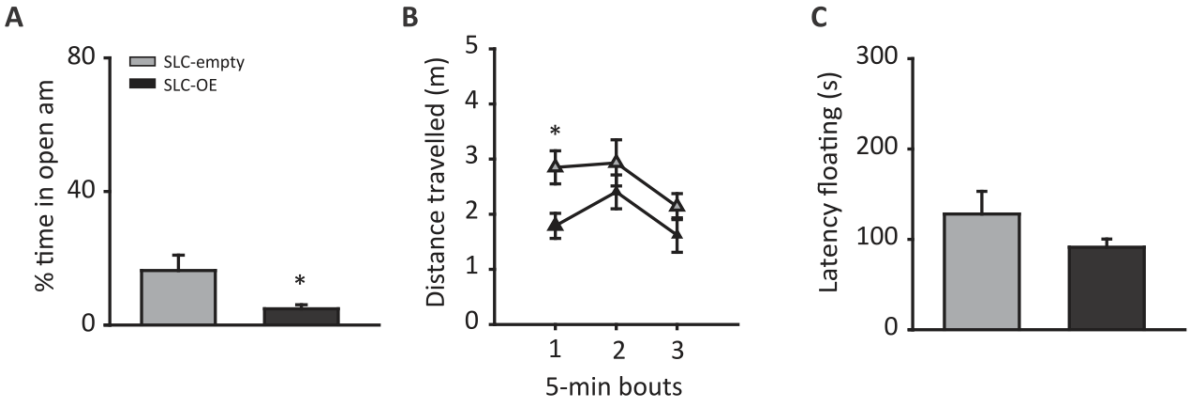

Figure 5: Behavioral profile after SLC6A15 overexpression. Alterations in emotional behavior were investigated in the elevated plus maze, open field and forced swim test. **(A)** Time spent in the open arm revealed in the elevated plus maze test that SLC-OE showed increased anxiety-like behavior compared to SLC-empty. **(B)** In addition, SLC-OE travelled less compared to SLC-WT in the open field test. **(C)** No difference was observed in the forced swim test. Data are given as means ± SEM (* = p<0.05).

4.3 *In situ* hybridization

For the experiment 1, GluR1 mRNA expression levels were significantly affected in the CA1 and CA3 area by the stress condition (ANOVA, CA1: $F_{(1,34)}$=6,657, CA3: $F_{(1,34)}$=13,256, both p<0,05). Post hoc tests indicated that SLC-KO animals after CSDS express significantly higher levels of GluR1 compared to non-stressed SLC-KO, while expression levels of SLC-WT mice remain comparable with or without CSDS (Figure 6A-B). In the dentate gyrus (DG), a significant interaction effect of stress condition and SLC6A15 genotype was found (ANOVA, $F_{(1,34)}$=6,161, p<0,05). Knockout of SLC6A15 decreased GluR1 expression levels under basal conditions in the DG. This effect was not observed in chronically stressed animals (Figure 6C).

For experiment 2, mRNA expression levels in the hippocampus revealed that GluR1 is significantly increased in the DG of SLC-OE compared to SLC-empty mice ($t_{(27)}$=0,926, p<0,05), with no significant effects in the CA1 and CA3 areas (Figure 6D-F).

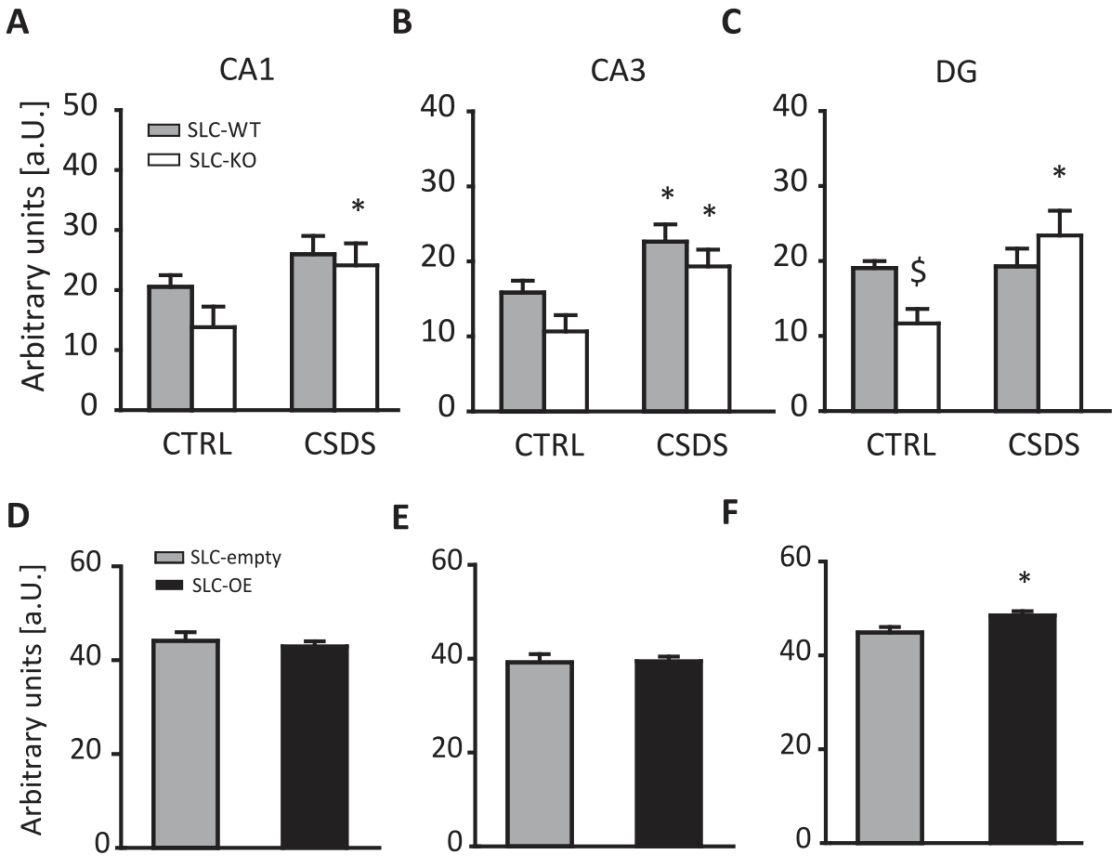

Figure 6: Gene expression. mRNA expression levels of GluR1 were measured in 3 subregions (CA1, CA3, DG) of the hippocampus. **(A)** In the CA1 chronic social defeat increased GluR1 levels of the SLC-KO. **(B)** A similar expression pattern was observed in the CA3, where after chronic social defeat mRNA levels are increased. **(C)** In the DG, levels of GluR1 were increased in SLC-KOs after chronic social stress, and decreased in SLC-KOs compared to SLC-WTs under control condition. **(D-E)** No significant GluR1 expression differences were observed in CA1 and CA3 between SLC-OE and SLC-empty animals. **(F)** SLC-OE have significantly increased levels of GluR1 in the DG. Data are given as means ± SEM (*,$ = p<0.05, * = condition effect, $ = genotype effect).

5. Discussion

Major depression is a multifactorial disease in which both adverse environmental conditions and genetic predisposition play a crucial role. Recently, SLC6A15, a transporter for neutral amino acids, has been correlated with higher incidence of depression and impairments in memory and cognition (Kohli et al., 2011; Schuhmacher et al., 2013). The main aim of this study was to unravel the relationship between SLC6A15 and stress vulnerability, in correlation with alterations in the glutamatergic system. We here show that SLC6A15 deletion dampens HPA axis activation following CSDS and provides resilience against behavioral alterations due to chronic social stress, whereas SLC6A15 overexpression in the hippocampus mimics stress effects by increasing anxiety- and depressive-like behaviors. Furthermore, expression levels of GluR1 in the hippocampus, especially in the dentate gyrus, are significantly affected by the levels of SLC6A15.

First, we addressed the interaction between SLC6A15 deletion and vulnerability to chronic stress. Chronic social defeat stress induced alteration in physiology (body weight, fur status, adrenal glands and thymus weight), which were similar in SLC-KO and SLC-WT, demonstrating the similar effectiveness of the stress procedure in the two groups. Interestingly, the regulation of the HPA axis end product CORT was differentially affected by chronic stress exposure in SLC-KO and SLC-WT mice. While the basal tone of HPA axis activity seems to be increased in stressed SLC6A15 deficient mice, these animals also displayed a more efficient negative feedback regulation of CORT levels following an acute challenge. On the other hand, in SLC-OE animals CORT levels were significantly reduced shortly after an acute stressor. This suggests a direct connection between HPA axis activation and regulation and SLC6A15 expression, but the reason for this relationship remains unresolved.

Also the behavioral effects following SLC6A15 manipulations were investigated. While no significant differences were found comparing emotional behavior (using the elevated plus maze, open field and forced swim tests) of SLC-KO and SLC-WT mice under basal conditions, chronic stress exposure significantly increased emotionality in wild type, but not in knockout mice. This suggests that a reduction of SLC6A15 expression levels provides protection against the negative effects of chronic stress on those behavioral domains. Conversely, an increased expression of SLC6A15 could mimic the stress-induced behavioral phenotype of increased anxiety and more passive stress-coping behavior. This is in line with the observation that lower SLC6A15 expression correlates with lower glutamate levels in the hippocampus, which have been proposed to mediate some of the stress effects in this region (Kohli et al., 2011). However, these data are in contrast to the conclusions drawn from the human data, where reduced SLC6A15 expression levels were

associated with the polymorphism conveying increased risk for depression (Kohli et al., 2011). As those findings were mainly based on mRNA expression levels of brain biopsies from treatment-resistant epileptic patients, the association of the human risk allele with SLC6A15 expression status requires further investigation, especially since it is established that epilepsy and seizures are associated with alterations in tissue proline content, one of the main substrates of SLC6A15 (Delwing et al., 2003, Di Rosa et al 2008).

Recently it has been shown that SLC6A15 levels correlate to glutamate content in the hippocampus (chapter 4 of this thesis), a key neurotransmitter for stress response. Therefore, we hypothesized that manipulations of SLC6A15 levels may also lead to altered gene expression of glutamatergic receptors. In addition, previous reports indicated that ionotropic glutamatergic receptors, in particular GluR1 subunits, are involved in stress response and adaptation (Schmidt et al., 2010). In line with our hypothesis, we found that GluR1 mRNA levels in the hippocampus are significantly reduced in SLC-KOs compared to SLC-WTs in basal condition, and significantly increased by chronic stress. Conversely, SLC6A15 overexpression resulted in increased GluR1 mRNA levels in the DG compared to controls, suggesting that there is a positive relationship between SLC6A15 expression levels and GluR1 subunit expression. One could therefore hypothesize that GluR1 levels are contributing to the observed differences in behavioral changes in SLC6A15 knockout and overexpression animals, supporting the theory that SLC6A15 regulates emotional behavior through its influence on the glutamatergic system. Moreover, it could be speculated that the alterations in GluR1 expression following SLC6A15 up or downregulation are due to structural and molecular interactions between GluR1 and SLC6A15, however this awaits experimental confirmation.

The current study has also a number of limitations to be kept in mind when interpreting the results. Only male mice were used in both of the experiments, in order to uniform our results across studies. However, it has to be kept in mind that women are more affected by depression and stress, likely due to a different regulation of stress response. In addition, SLC6A15 knockout are constitutional knockout animals and therefore possible developmental effects may have interfered with the adult phenotype. Further, we phenotyped SLC6A15 overexpressing animals under basal condition, but the effects of stress on the overexpression were not assessed in this study.

In summary, we here show that SLC6A15 controls HPA axis activation under chronic stress, regulates anxiety- and depressive-like behaviors and influences expression levels of GluR1 in the hippocampus. In addition, we also provide evidence that increase of SLC6A15 levels in the

hippocampus exerts behavioral and molecular effects similar to those seen following chronic stress exposure, already under basal conditions. Our data provide additional support for the involvement of SLC6A15 in mood disorders and position SLC6A15 as a promising novel target for the development of potential antidepressant drugs.

6. Acknowledgments

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General discussion

1. Nurture: environmental triggers to depression

Chronic or acute traumatic stress is currently regarded as one of the main environmental risk factors contributing to the risk for psychiatric disorders, in particular for depression. However, it is not stress *per se* that leads to depression, but rather a combination of genetic predisposition and epigenetic programming based on previous experiences that is thought to lead to a poor coping response to the aversive event. The mechanisms underlying the increased risk for psychopathology following stress have been extensively investigated, leading to the formulation of different theories about the relationship between stress and psychopathology.

1.1 Multiple stressors: triggering depression

The “wear and tear” theory of psychopathology is one of the first models which have been proposed. The “wear and tear” theory states that the intense use of a resource over time will lead to its exhaustion and damage early on. In the case of stress, repeated exposure to aversive experiences, especially during early stages of life, may exhaust the capacity of the physiological stress response systems (e.g. the HPA axis) to maintain homeostasis when facing stressors during adulthood, increasing the probability to develop psychiatric diseases (Juster, McEwen, & Lupien, 2010). In humans, increased HPA axis and autonomic system responsiveness at adulthood are observed following early childhood trauma compared with non-stressed controls (C Heim et al., 2000; Christine Heim, Mletzko, Purselle, Musselman, & Nemeroff, 2008), which correlates with low socioeconomic status during childhood (Evans & English, 2002).

Those adverse experiences during childhood are thought to chronically increase the load on the HPA axis and the cost of maintaining homeostasis is proposed to take its toll on later adult mental health (Charles, Piazza, Mogle, Sliwinski, & Almeida, 2013). Therefore, impaired response of the HPA axis to adult stressors, due to a history of repeated childhood adversities, is one of the mechanisms associated with higher risk of developing depression (Christine Heim, Newport, et al., 2008). Also animal studies reported evidence in support of the "wear and tear" theory. Repeated exposure to stress during puberty induced depressive-like and anxiety-like behaviors at adulthood and under basal conditions and after re-exposition to an acute stressor (Avital & Richter-Levin, 2005; Tsoory, Cohen, & Richter-Levin, 2007), as well as puberty-stressed animals showed impaired coping strategies (Eiland et al., 2012). Preclinical models have also given insight into molecular and cellular modifications leading from chronic stress exposure to increased vulnerability to psychopathology. Early maternal stress increases maternal GC release, which reaches the pups through the milk, causing long-term increased HPA axis reactivity in the

offspring (Cadet, Pradier, Dalle, & Delost, 1986; Koehl et al., 1999). At adulthood, GRs and MRs expression levels in the hippocampus of poorly reared animals are reduced, affecting the responsiveness of the HPA axis to stressors (Kosten & Kehoe, 2010), as well as increased pituitary-adrenal responsiveness that correlates with reduced hippocampal structural plasticity (Mirescu et al., 2004), which results in higher behavioral susceptibility to stress at adulthood (Ladd, Thrivikraman, Huot, & Plotsky, 2005).

1.2 Multiple stressors: promoting coping skills

However, evidence suggests that exposure to negative events does not necessarily induce negative effects. For example, memory and learning performances can be promoted by elevated levels of corticosterone, such as after stress exposure, and repeated stress experiences improve spatial memory (Avital & Richter-Levin, 2005). Moreover, some types of memory, such as contextual memory, have been reported to be enhanced under a high-stress environment only in individuals exposed to early life adversities (Oomen et al., 2010). Corticosterone levels are thought to affect memory performances in an inverted U-shape dose-response relationship, with better performance associated with intermediated levels of corticosterone, whereas both low and high levels are associated with memory impairments (Roozendaal, 2000; Sandi, Loscertales, & Guaza, 1997).

Emotional behavior may also be fostered by mild stress exposure. Predictable aversive experiences during adolescence have revealed reduced depressive and anxiety-like behaviors following chronic unpredictable stress at adulthood (Suo et al., 2013), along with increased hippocampal neurogenesis (Parihar, Hattiangady, Kuruba, Shuai, & Shetty, 2011). Also, evidence from clinical studies supports that experience of mild, controllable negative experiences could lower the activation of the HPA axis following stress (Lovallo, 2013). Lower cortisol levels after stress are associated with lower rates of psychopathology, suggesting that early stress exposure may also result in improved response to stress, and therefore in resilience against stress-induced psychopathology (Fisher & Stoolmiller, 2008). In other words, the lack of adaptation to stress results in psychopathology, which might be the result of ineffective coping skills rather than from early negative experiences itself. According to the match/mismatch hypothesis, the early life environment prepares the individual to face adversities during adulthood, consequently a mismatch between the rearing and the adult environment increases vulnerability to psychopathology. One possible explanation for the discrepancies in the literature could be that most of the previously published studies observing the effects of early life adversities have been involuntarily comparing mismatched individuals with animals that experienced neutral

environments. This thesis has explored the effects of different rearing environments in terms of HPA axis activity, emotional behavior and coping skills under different social contexts and in adaptation to different stressors.

Early life enrichment is thought to increase resilience toward adult stress, reducing corticosterone and ACTH levels (Babb, Masini, Day, & Campeau, 2014; Belz, Kennell, Czambel, Rubin, & Rhodes, 2003). However, some reports also suggest that early life enrichment strategies may be ineffective (Kohl et al., 2002) and criticisms have been raised as to which domains are benefited from environmental enrichment (Widman, Abrahamsen, & Rosellini, 1992). In *chapter 1*, we reported that early life enrichment has no effect on corticosterone levels after chronic physical stress, while it buffers the basal corticosterone increase due to chronic social stress. This suggests that the adult HPA axis reacts to adult stressors according to the individual early experiences. Our data may be beneficial to predict the effects of environmental enrichment, suggesting that early life enrichment has a significant effect only when early positive experiences resemble the features of adult stressors. The tight relationship between the stressor source and the source of enrichment might also explain the discrepancies in the scientific literature regarding the effects of environmental enrichment on stress response. Positive rearing is not the only experience that shapes HPA axis responsiveness to stress. It was previously shown in different studies that early life adversities as well as enrichment affect the HPA axis. However, there are still discrepancies in the literature about the long term effects of early life experiences on stress response. We therefore directly compared the effects of opposite rearing experiences (mice reared in a positive environment versus mice reared in an adverse one), on adult stress response. We found that acute adult stress, like forced swim stress, elicited similar corticosterone elevation in animals reared in positive or negative environments, whereas chronic stress corticosterone elevation was buffered by early life adversity compared to early life enrichment. After three weeks of chronic stress not only corticosterone levels, but also adrenal gland hypertrophy was reduced by negative rearing experiences when compared to positive rearing, suggesting a more profound difference in the HPA axis response to chronic stress between individuals reared in opposite environments. However, this difference emerged only after chronic stress and not after an acute challenge (*Chapter 1*).

The HPA axis is not the only system to be shaped by early life experiences as emotional behavior is also defined by rearing quality. Positive rearing is known to reduce anxiety-like phenotype and anhedonia under basal conditions (Branchi, D'Andrea, Cirulli, Lipp, & Alleva, 2010b; Francesca Cirulli et al., 2010). In this thesis, we have reported for the first time that positive rearing buffered stress-induced anhedonia only in the case of chronic social stress, but

not of physical chronic stress (*Chapter 1*). These findings, along with the endocrine results, suggest that adaptation to an adult chronic social stress is specifically promoted by positive rearing. Therefore, the response to adult social stressors, in terms of corticosterone levels and emotional behavior can be predicted by the features of the early life environment (*Chapter 1*, *2* and *3*).

Social skills are also shaped by the quality of the rearing experiences (Veenema & Neumann, 2009) and interaction with a non-aggressive conspecific is impaired by rearing in an environment with limited nesting resources (Raineki, Cortés, Belnoue, & Sullivan, 2012), although no data are available regarding the effects of the interaction of early life environment with adult stress on social skills. When we compared matched with mismatched animals, no significant differences were observed if the animals were reared in a positive environment. Interestingly, limited nesting offspring showed significantly divergent behavior in the sociability test, according to the adult life condition, suggesting that different adult environments could modulate long term early life effects. Negatively matched animals (individuals that were raised in negative environment and exposed to chronic social stress at adulthood) showed reduced interaction time with a conspecific when compared to mismatched animals with early life adversities. Interestingly, when we looked into agonistic behavior, rearing experiences exerted a different profile. Positive reared mice did not show differences in social avoidance, whereas when we looked at animals reared in an aversive environment, the adult environmental condition induced opposite phenotypes. Animals housed with a female conspecific, i.e. mismatched animals with early life adversities, scored the highest time spent in the interaction zone compared to all other groups, indicating lower fear levels or increased aggressiveness. On the other hand, negatively matched animals reported the lowest interaction time, a measure of higher levels of social avoidance. Therefore, responsiveness to a potential aversive situation, like a social competitor, is augmented in individuals exposed to early life adversities, but the direction of the response is dictated by the actual environmental condition (*Chapter 3*). Early life experiences have already shown to increase sensitivity to context (Lewis & Rudolph, 2014), and our data suggest that the adult environment could guide the direction of this change. In *chapters 1*, *2* and *3*, we reported that positive life experiences have long term consequences, according to timing and features. We showed that positive rearing and housing with a positive social stimulus contrasts the effects of chronic stressors in different ways, in accordance with reports that environmental enrichment could have significant effects only if performed during specific time windows and that differences in the enrichment protocol employed may change the effects of the enrichment itself (Hubrecht, 1993). Finally, these findings also support the evolutionary view that maternal experiences prepare the

offspring so that they will be best fitted to an environment resembling early life, in the attempt to improve fitness and survival rate, especially under challenging situations like high intra-specific social competition (Bateson et al., 2004). Overall, we conclude that early life stress does not always result in negative outcomes, rather the long term effects of aversive experiences are dependent on the actual living context.

1.3 The role of gender and hormonal regulation in stress vulnerability

Gender is another key factor, often neglected, influencing stress vulnerability. Epidemiological investigation revealed that women are more prone to develop psychiatric afflictions, in particular depression (Kuehner, 2003; Weich, Sloggett, & Lewis, 2001) and twin studies revealed that heritability of major depression is significantly higher in women than in men (Kenneth S Kendler, Gatz, Gardner, & Pedersen, 2006). In *chapter 2* we specifically addressed the role of gender and of estrous phases on emotional behavior. We firstly showed that the effects of early life and adult life stressors on anxiety and depressive-like behaviors are not-additive and moreover that the estrous cycle can further affect long term effects of stressful events. Similar behavioral profiles were observed between animals constantly exposed to adversities or to nurturing environments (negative versus positive matched animals), whereas alternation in the environment quality induced the strongest differences (mismatched animals). Moreover, when taking the estrous cycle into account phenotype-specific differences emerged: mismatched life trajectories affected anxiety-related and social behaviors mainly during the estrous phase, while locomotion and stress coping behavior were altered during the non-estrous phases.

Overall, lower anxiety and higher social interactions may represent an adaptive strategy for survival, especially during critical phases - e.g. estrous stage during mating periods, and potentially induce an advantage over other competitors in case of limited resources. The basis of these differences is likely to rely on the different hormonal levels during each of the estrous stages. It has been already shown that sexual hormones have a great impact on HPA axis regulation (Handa, Burgess, Kerr, & O'Keefe, 1994) and that increased levels of estrogens magnify the effects of stressful experiences, leaving more profound and long lasting effects (Kajantie & Phillips, 2006). One of the possible explanations for the interaction between HPA axis and sexual hormones is that progesterone, one of the most abundant female sexual hormones, is known to act as a glucocorticoid antagonist (Kudielka & Kirschbaum, 2005), suggesting that the HPA axis feedback of females is further regulated by gonadal steroids (Young, Altemus, Parkison, & Shastry, 2001). Therefore, it is crucial to design experimental procedures taking into account gender discrepancies and interpret the results in the light of hormonal differences.

However, gonadal steroids are not solely responsible for the gender differences in stress response observed and some researchers have also proposed a different theory to explain these gender differences. Generally, reaction to stress is described with the "fight or flight" response model. For females, due to their different social niche and survival needs, the theory of "tend and befriend" seems to be more relevant. "Tending" refers to the tendency of dams to protect the offspring in case of stress, instead of fighting back the intruder or fleeing, whereas "befriending" to seeking out the social group for mutual defense. In humans it has been proven that in case of stress women are more prone to look for social support rather than men (S E Taylor et al., 2000) and behavioral strategies of stress response, like avoidance coping styles, significantly differ between women and men (Matud, 2004). One possible explanation is that the female stress response does not appear to be mediated by the sympathetic arousal induced by testosterone, which is implicated in fight responses for males (Handa & Weiser, 2014). Furthermore, the female stress response appears to be tied less to sympathetic arousal than males, being instead more social in nature, as also emerged in this thesis.

1.4 Translational aspects of the match/mismatch hypothesis

Although our experiments are performed in mice, our findings are of high translational value in terms of promoting resilience and identifying alternative ways of providing treatment to patients. Typically, individuals facing stressful events who have experienced negative episodes early in life are considered to be at the highest risk to develop psychiatric disorders (American Psychiatric Association, 2013). However, in this thesis we have provided evidence that for such individuals (i.e. negatively matched) at least moderate levels of adversity do not necessarily have detrimental effects but can promote stress resilience later in life, especially regarding the HPA axis functionality and emotional behavior. In the evidence-based practice of psychology, a treatment based on an analogous principle has been formulated. The stress inoculation therapy is a psychotherapic technique intended to train patients to be prepared to cope with stressful events. It consists in teaching the patients about stress nature and its possible negative consequences and training how to anticipate the possible negative outcomes. This strategy is aimed to prepare individuals who have not experienced aversive events (i.e. mismatched individuals) to "rehearse" negative experiences, in order to learn how to develop effective coping skills.

In addition, our results also revealed that early social enrichment provides resilience at adulthood against chronic social stress. Our findings also strengthen the concept that individuals constantly living in harsh environments could benefit from receiving additional social support, in

the attempt to overcome the negative effects of the environment (Arnberg, Hultman, Michel, & Lundin, 2012).

2. Nature: genetic vulnerability to depression

2.1 Genetic contribution to psychiatric disorders

Genetic heritability accounts for about 30-50% of the risk of developing depression (World Health Organization, 1992). Consequently, a family history of depression increases vulnerability to the disease, with an increased risk for children of depressed patients to psychopathology compared to children of non-depressed parents (Weissman et al., 2005). Since the year 2000, when the entire human genome was sequenced, scientists have tried to find genes associated with vulnerability to psychiatric disorders using unbiased approaches. Many bioinformatic tools have been developed to screen the entire genome, such as genome wide association studies (GWAS), which are among the most successful methods to tackle the genetic component of complex disorders. To detect the most frequent variants associated with the higher risk for a disease, GWAS correlate which single nucleotide polymorphisms (SNPs) are more often reported within the patients in a large human sample group. GWAS reported significant and reproducible associations of SNPs and disease status for different psychiatric disorders, like CACNA1C and ANK3 for schizophrenia (Sklar & et al, 2011). Candidate genes for major depression vulnerability were also identified, like GRM7 (Shyn et al., 2011) or SP4 (Shi et al., 2011), however experimental validation of those candidates is still not exhaustive.

2.2 Pitfalls of simple genetic models

Although GWAS represent a powerful tool to detect genetic variances associated with psychiatric diseases, some criticisms have been raised. Recently, a mega-analysis of genome wide association studies including more than 18000 individuals was performed, revealing that no locus selected using classical GWAS reached genome-wide significance for major depression (Ripke et al., 2013; M. D. Sullivan et al., 2013; Wray et al., 2012). While this study was the largest yet performed, accurate statistical estimations indicate that the sample is still too small to identify robust targets for complex diseases like major depression. In the light of those findings some concerns about using association studies to tackle genetic heritability of major depression have been raised. It has been proposed that major depression is present in a number of different sub-pathologies, rather than a unique disease with a simple genetic background, that may weaken the effects of single gene mutations in large samples. For example, specific subtypes of major depression, like recurrent depression or early onset depression, although sharing a similar phenotype, may have very different genetic backgrounds, causing a dilution effect when analyzed together in large genomic screening studies (Levinson, 2006; P. F. Sullivan, Neale, & Kendler,

2000). In addition, single SNP analysis has limited power to explain the polygenic roots of depression. Therefore, instead of single genes, it is now considered a more effective strategy to investigate the dysregulation of entire pathways to understand the molecular mechanisms underlying the pathophysiology of major depression. Dysregulation of synaptic transmission (e.g. glutamatergic system) is among the most promising targets to understand depression. For example, gene sets associated with glutamatergic neurotransmission and synaptic plasticity are often significantly altered in major depression patients (Lee et al., 2012). This multigenic approach has already reported interesting results for other complex psychiatric disorders, like schizophrenia (Lips et al., 2012), bipolar disorder (Holmans et al., 2009) and autism (Glessner et al., 2009).

2.3 SLC6A15 as promising target for depression

In 2011, Kohli and colleagues identified a SNP in a regulatory region of the SLC6A15 gene that is robustly associated with higher incidence of major depression. SLC6A15 encodes for a neutral amino acid transporter mainly expressed in the brain, particularly within the hippocampus. Homozygote carriers of the risk allele of this SNP had a 1.42-fold-higher risk to suffer from depression compared to non-risk allele carriers. Furthermore, healthy risk allele carriers showed reduced gray matter in the hippocampus, one of the pathological signs of depression, and altered levels of NAA, a marker of glutamatergic metabolism (Kohli et al., 2011). These findings fit well with the recent hypothesis that correlates glutamatergic function in the hippocampus with stress vulnerability and major depression suggesting a key role for SLC6A15 as a regulator of the hippocampal glutamatergic system. In this thesis we have provided strong evidence in support of the involvement of SLC6A15 in hippocampal neurotransmission and in stress adaptation, in particular regulating the glutamatergic system in response to different environmental conditions.

2.3.1 SLC6A15 regulates hippocampal neurochemistry

We started by investigating availability of the primary substrates of SLC6A15, proline and other amino acids, in the hippocampus. Previous reports have indicated that SLC6A15 deletion significantly reduced amino acid uptake in synaptosomes (Bröer et al., 2006; Drgonova et al., 2007). We here showed that abundance of hippocampal amino acids, especially proline, is significantly reduced in absence of SLC6A15, likely due to a lack of sufficient amino acid uptake in the cells from the transporter. Among the amino acids, proline is the only one containing a distinctive cyclic structure, which confers higher conformational rigidity to peptides forming proline-rich motifs (e.g. SH3 and EVH1 domains) critical for intracellular signaling pathways.

Proline is also classified as a neuroactive amino acid, as it acts as weak agonist of the glycine receptor and ionotropic glutamatergic receptors (Henzi et al., 1992) and is able to induce behavioral and neurochemical alterations in animal models relevant for mental disorders (Baxter et al., 1985; Cherkin et al., 1976; S. M. Cohen & Nadler, 1997; Moreira et al., 1989; Roussos et al., 2009). In addition, there is growing evidence that proline could represent one of the *de novo* synthesis pools of glutamate, as it could be converted to glutamate via a mitochondrial pathway (Pérez-Arellano et al., 2010). However, when we overexpressed SLC6A15 in the hippocampus, no significant modification in proline content was observed. Although apparently in contradiction with the results obtained from the SLC6A15 knockout, it has to be noted that an increase in the expression levels of the transporter has no role in regulating the extracellular levels of proline, the most abundant pool of for this amino acid, which therefore will not affect the total amount of proline available in the hippocampus. One of the most striking differences associated with SLC6A15 identified by Kohli and colleagues' was the difference in glutamate content in the hippocampus of the risk allele carriers. With different experimental approaches and in a different batch of animals we were able to robustly induce a significant decrease of glutamate levels in the hippocampus following SLC6A15 deletion, and vice versa, to induce an increase in SLC6A15 expression levels associated with an increase in glutamate content (Figure 1). The glutamatergic phenotype, one of the hallmarks of depression in humans, is a key readout that suggests a high translational value of these studies, especially considering these results robustly reproduced the core alteration reported in depressed patients.

Although we have already highlighted differences following SLC6A15 deletion under basal conditions, to appropriately model major depression, gene*environment interactions needed to be taken into account. Therefore, we addressed the interaction between chronic social stress, an environmental manipulation known to induce persistent neurobehavioral modifications relevant to human depression, and SLC6A15 deletion. Due to the strong alteration observed in glutamate levels, we hypothesized that glutamatergic receptor expression levels may also be altered after SLC6A15 manipulation. It has already been shown that ionotropic glutamatergic receptors, especially the GluR1 and GluR2 subunits of the AMPARs, are linked to stress vulnerability (Schmidt et al., 2010). We reported for the first time that the deletion of SLC6A15 is associated with a hippocampal reduction of GluR1 mRNA levels along with an increased GluR1 expression following chronic social defeat.

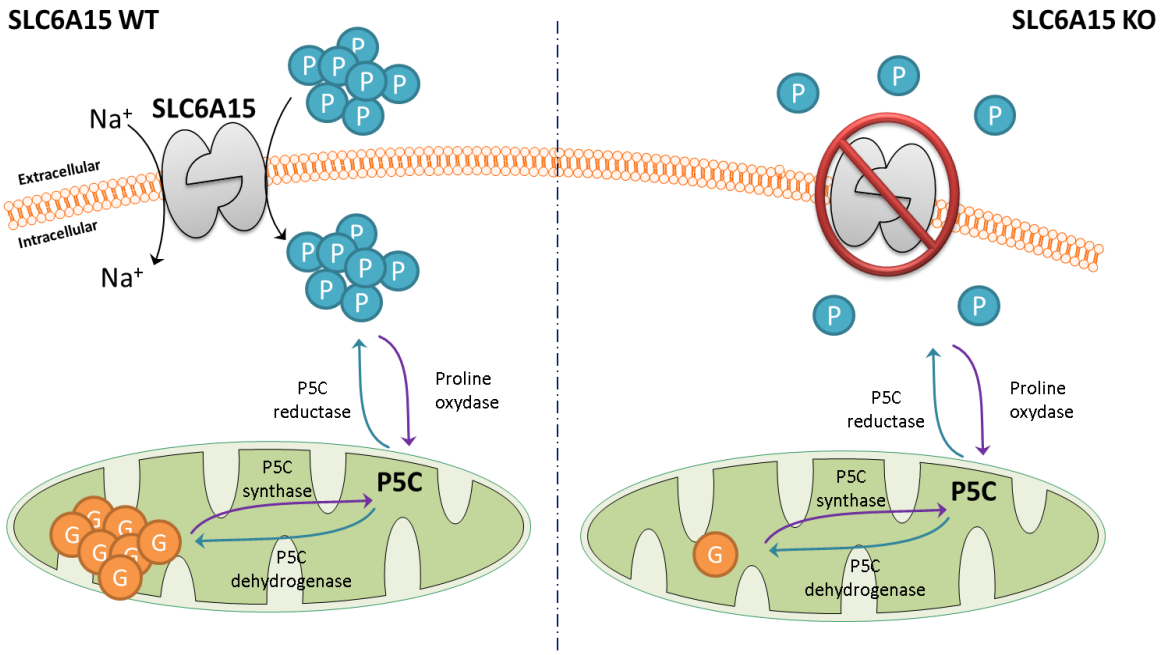

Figure 1: Overview of the role of SLC6A15. As transporter of proline, SLC6A15 regulate the amount of tissue proline content, which via a mitochondrial pathway is transformed in glutamate. P= Proline, P5C=1-Pyrroline-5-carboxylic acid, G= Glutamate.

Moreover, SLC6A15 overexpression increased GluR1 subunit mRNA expression, similar to the increase observed following chronic social stress. Different GluR subunits induce different calcium influx, resulting in a functional change in the excitability of the postsynaptic membrane, suggesting an altered postsynaptic transmission in glutamatergic synapses due to an alteration of AMPARs sensitivity, one of the cellular markers of stress vulnerability. Some reports suggested that individual vulnerability to stress may even be predicted by a different GluR1/GluR2 expression ratio in the hippocampus (Schmidt et al., 2010), therefore we investigated differences in stress response in more detail following SLC6A15 manipulation under basal conditions and chronic stress.

2.3.2 SLC6A15 regulates endocrine functions and behavior

Previous reports indicated that chronic social defeat paradigm significantly altered corticosterone plasma levels and adrenal glands size, suggesting a profound alteration of the HPA axis caused by repeated exposure to an unpredictable aversive experience (Wagner et al., 2012). In our study, chronic social defeat stress induced comparable alteration in SLC6A15 knock out and wild type mice regarding body weight and fur status, physiological indicators of stress effectiveness, suggesting comparable effects of the environmental manipulation on the two experimental groups. Along the same line, adrenal gland hypertrophy and thymus shrinkage had a similar effect size between the genotypes. However, neuroendocrine parameters diverged significantly, plasma corticosterone levels were increased after chronic social defeat, and deletion

of SLC6A15 further increased corticosterone basal levels whereas a quicker negative feedback regulation was observed after an acute challenge in absence of SLC6A15. This suggests a functional interference between SLC6A15 and HPA axis regulation, probably linked to the glutamatergic differences observed in the hippocampus, but the nature of this relationship requires further investigation.

Previous reports have also suggested that emotional behavior might be affected by SLC6A15 expression, especially following acute stress (Drgonova et al., 2007). Thus, we initially investigated the behavioral phenotype under basal condition, taking into account the molecular and endocrine profile observed following SLC6A15 deletion, tackling the domains that could be potentially affected by an alteration in the hippocampal proline and glutamate content. In other studies focusing on proline metabolism, which directly affected proline content in the brain, a direct correlation between sensorimotor gating and amino acid tissue content was observed (Gogos et al., 1999). Therefore, we investigated sensorimotor gating and startle responses in SLC6A15 knockout mice, which are robust behavioral readouts used both in humans and animals (Reynolds et al., 2005) to reveal anomalies in sensorial input filtering and motor integration. Intriguingly, SLC6A15 deletion increased the acoustic startle response already under basal condition, likely as a result of the reduced proline levels in the hippocampus.

Initial reports indicate that SLC6A15 knockout mice have an altered anxiety-like behavior compared to wild type littermates (Drgonova et al., 2007). Along with sensorimotor gating impairments, we also observed a significant reduction in anxiety-like behavior following SLC6A15 deletion, whereas hippocampal overexpression of SLC6A15 induced the opposite phenotype. Other animal models of psychiatric disorders have also reported increased startle response in combination with reduced anxiety-like behavior (Yen et al., 2013). The authors of the study suggested that overall impairments in gating and sensorimotor integration may indicate a lack of adaptation to the testing environment (resulting in increased startle reflex and increased time spent in the open arm) due to dysfunctions in the hippocampus, a key region in the environmental habituation process and where SLC6A15 is expressed endogenously (Sarantis, Antoniou, Matsokis, & Angelatou, 2012).

The initial behavioral screening of SLC6A15 knockout mice and the effects on HPA axis activation following chronic social stress suggested that after chronic social stress SLC6A15 could affect emotional behavior. Interestingly, the effects of chronic social stress, such as locomotion increase in an open field arena and passive stress-coping behavior in the forced swim test, were less pronounced in absence of SLC6A15. In addition, when we increased SLC6A15 expression

levels in the hippocampus we observed behavioral alterations that resembled the typical stress-induced behavioral changes. We can therefore conclude that there is a strong relationship between SLC6A15 expression levels and behavioral modification in response to stress, where low levels of SLC6A15 expression are correlated with reduced anxiety-like and depressive-like behavior. The molecular changes underlying those altered behavioral adaptations are likely to be connected to the alterations observed in the hippocampal glutamatergic system following manipulations of SLC6A15. Our hypothesis is supported by a number of previous findings that correlated stress-induced behavioral phenotypes with increased glutamatergic tone resulting in excitotoxicity (Choi, 1994). Moreover, the results obtained following reduction of SLC6A15 levels resemble the profiles of GluR1 knockout animals, suggesting a shared etiology underlying the common behavioral and cellular phenotypes (Wiedholz et al., 2008) reinforcing our hypothesis that SLC6A15 levels affect behavior through the glutamatergic system. The subcellular localization of SLC6A15 might suggest that the alterations in GluR1 observed following changes in SLC6A15 expression levels may be due to structural or molecular interactions between the two proteins. However, there is only sparse evidence about SLC6A15 cellular interactors, therefore more experimental data are needed to support this hypothesis. The results obtained from this doctoral work substantiated the role of SLC6A15 as regulator of the glutamatergic system and involvement in stress vulnerability. This further supports the pivotal role of the glutamatergic system in vulnerability to psychiatric disorders, especially in relation to stress-induced psychopathologies. Our findings also substantiated the involvement of the SLC6A15 in hippocampal glutamatergic system, the crucial role of AMPARs composition and of the HPA axis activation in response to chronic stress.

2.3.3 SLC6A15 as novel drug target

Blockade of SLC6A15 represents a novel tool to specifically target glutamatergic dependent hippocampal functions, which are central alterations reported in many patients of psychiatric disorders, especially depression. For the restricted expression of SLC6A15 to the brain and in particular to the hippocampus, pharmacological blockade of SLC6A15 could represent a strategy to manipulate glutamatergic transmission in the hippocampus without affecting other brain areas, resulting in hippocampal-specific alteration in behavior and neurochemistry, mainly after chronic stress. Compounds that could interfere with SLC6A15 activity may therefore represent a novel category of drugs that would have a strong effect on relevant neurobehavioral alterations. Furthermore, due to the restricted neuroanatomical expression of SLC6A15, such compounds may avoid many of the side effects commonly observed with classical antidepressants, which target

neurotransmitter systems that are widely expressed both in the CNS and in the periphery (e.g. 5-HT) and require a high dosage to observe a significant effect. Currently, no compound is known to specifically target SLC6A15. In collaboration with the research group of Dr. Felix Hausch, a suitable inhibitor of SLC6A15 activity has been developed. Extensive characterization of this substance in *in vitro* assays has already revealed its suitability as a potential neuroactive drug, with low cellular toxicity and high target selectivity. Preliminary *in vitro* and *ex vivo* studies have shown a strong neurotrophic effect of this inhibitor, suggesting a potential neuroprotective effect of blockade of SLC6A15 *in vivo*, an outcome that is in line with the findings obtained with genetic ablation of SLC6A15 in this thesis. We have already performed *in vivo* preliminary trials to assess general tolerability and behavioral effects of the inhibitor. The preliminary experiments did not reveal any adverse effect of local administration of this compound, supporting its safety as a pharmacological treatment. Further experiments are currently ongoing to fully characterize the effects of the inhibitor in animal models of psychiatric disorders, and to elucidate the molecular mechanism linking SLC6A15, the glutamatergic system and behavior, which is necessary to understand the full potential of SLC6A15 inhibitors. Overall, SLC6A15 inhibitors could represent an innovative class of psychoactive compounds, with a novel mechanism of action and reduced side effects well suited for commercial applications (Figure 2).

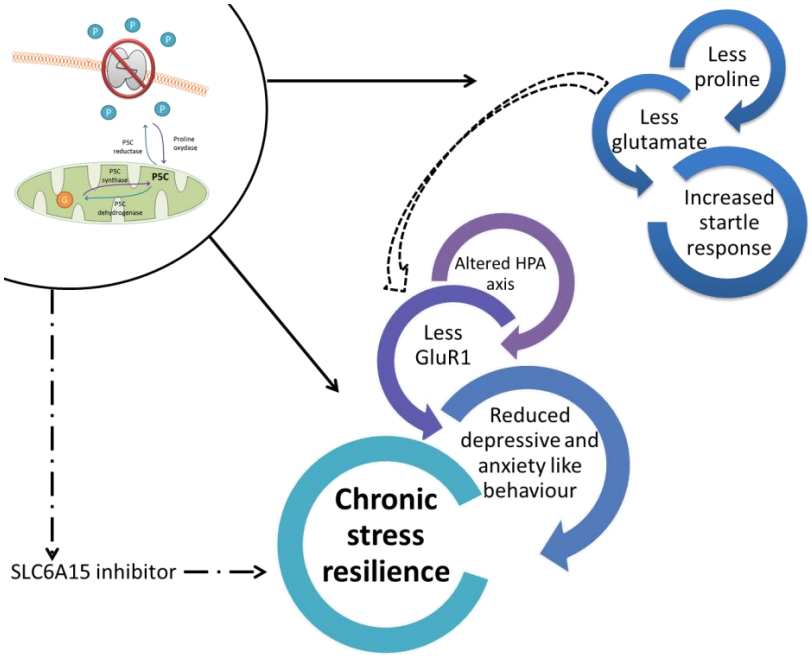

Figure 2: Overview of the involvement of SLC6A15 in neurobehavioral functions. In our first study we have demonstrated a direct correlation between expression levels of SLC6A15 and hippocampal neurochemistry and sensorimotor gating. Consequently, we showed that SLC6A15 deletion promotes resilience to chronic stress. Ultimately, promising preliminary findings suggest that treatment with an inhibitor of SLC6A15 may induce stress resilience. HPA, hypothalamus-pituitary-adrenal glands; GluR1, ionotropic glutamatergic receptor subunit 1.

2.3.4 Limitations

The current study also has limitations that should be kept in mind. As a preliminary study we focused only on adult male mice, to maintain consistency across different studies. We are aware of the higher incidence of psychiatric disorders in women, especially depression, and an analysis of the interaction gender*genotype is warranted. Furthermore, to study the effects of SLC6A15 deletion we used SLC6A15 constitutional knockout animals and potential developmental effects cannot be excluded at this stage. In the study from Kohli and colleagues in 2011, a significant interaction effect between genotype and depressive status was reported on hippocampal volume. However, we did not observe, at least under basal conditions, differences between genotypes on hippocampal volume. We are aware of the differences between a human polymorphism and an animal model with a genetic deletion and therefore all the conclusions should be considered taking these differences into account. Additional experiments are required to clarify the relationship between SLC6A15 expression levels and structural changes in the hippocampus and under which environmental condition this may become apparent. In our study, the deletion of the SLC6A15 gene resulted in resilience against chronic social defeat stress. On the other hand, in humans reduced SLC6A15 expression levels are associated with the gene variant conveying the higher risk for depression (Kohli et al., 2011). Among the possible reasons for this inconsistency, a detailed analysis of the human samples from which the SLC6A15 expression levels were obtained reveals that those brain biopsies originate from treatment-resistant epileptic patients. Epilepsy and seizures have been associated with alterations in proline tissue content (Delwing et al., 2003, Di Rosa et al 2008), which could induced an alteration in SLC6A15 expression levels.

3. Nurture interacts with nature that interacts with nurture

This thesis demonstrated with preclinical models that both environment and genetic factors play a key role in stress vulnerability. On one side, we reported that early life environments have a strong impact on adult stress coping strategies, and that early life negativity does not necessarily result in higher vulnerability to stress. On the other side, genetic predisposition determines adaptation to aversive environments, but in case of "neutral" environments no differences are observed due to genetic differences. It is worth noting that although in preclinical models it is possible to control and disentangle all the vulnerability factors discussed (the genetic makeup, the rearing environment and the adult environment), in clinical practice all those are always contributing and it is virtually impossible to define a "human control rearing environment" or a "control genotype", due to the vast variability of real life experiences and human genome heterogeneity. Therefore, the attempts to accurately model major depression should take all three factors into account simultaneously. Recently, preliminary reports of gene*early environment*adult environment interactions have been reported. It was previously reported that 5-HTTLPR risk allele carriers are at increased risk to develop depression following early life adversities (Caspi et al., 2003); Van der Doelen and colleagues have shown that this is true only in the case of a mismatched adult environment: in their study early life adversities provided improved resilience against stress later in life, specifically in individuals carrying the risk allele (van der Doelen et al., 2013) supporting the importance of the match/mismatched hypothesis to explain complex multifactorial diseases, like depression.

4. Conclusions

Since the 1980´s, depression has been classified as multifactorial disorder, in which gene by environment interactions increase disease vulnerability. These interactions have only explained a limited percentage of predictability for depression, probably also due to the difficulty in modeling genetic and environmental risk factors simultaneously (Maher, 2008). To date, the mechanisms underlying the missing heritability and the environmental contribution to depression are not fully understood. In this thesis we aimed to gain knowledge on the function of a novel risk factor, the gene SLC6A15, and to improve the validity of the models used to develop preclinical tools to tackle stress vulnerability and recapitulate depression. We used complementary animal models that incorporate different endophenotypes of depression. First, we investigated the characteristics of the rearing environment that provide adequate coping skills to adult social chronic stressors (*Chapter 1*). Afterwards, we extensively characterized the phenotype of mice reared in positive or negative environments following different adult environmental conditions. Interestingly, gender-specific factors in stress adaptation and coping skills development was observed, one of the most underestimated issues in preclinical studies (*Chapter 2-3*). Ultimately, we clarified the genetic contribution of SLC6A15 expression to hippocampal neurochemistry, in particular the glutamatergic system (*Chapter 4*) and on the behavioral and neuroendocrine functions of SLC6A15 after chronic stress (*Chapter 5*). Depression is a very heterogeneous disease and therefore the preclinical tools that attempt to model this disease should reflect the complexity of the disorder. In conclusion, rather than focusing on “vulnerability” factors it would be more appropriate to investigate the “sensitivity” elements, involved in higher or lower responsiveness to adversities, contributing to the complexity of depression and could be strategically manipulated to improve stress coping skills and reduce vulnerability to psychiatric disorders, in particular depression.

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Curriculum Vitae

Personal information

Name	Sara Santarelli
Date of birth	July 31st, 1986
Place of birth	Chieti, Italy
Nationality	Italian

Education

Current-2011	**PhD studies** In the department of 'Stress Neurobiology and Neurogenetics' at the Max Planck Institute of Psychiatry, Munich, Germany **PhD student** of the International Max Planck Research School of Life Sciences (IMPRS –LS)
2010-2008	**Master of Science in Neurobiology** from University of Rome "Sapienza", Rome, Italy, 110/110 *cum laude*
2008-2005	**Bachelor Degree in Biology** from University of Camerino (MC), Camerino, Italy, 110/110 *cum laude*

Professional affiliation

2011-2015	**Student Member** of European Brain and Behaviour Society (EBBS)
2013-2015	**Student Member** of European College of Neuropharmacology (ECNP) **Technical assistant** for the MaxLab, the student´s and visitors lab of the Max Planck for Neurobiology and Biochemistry

Awards and Scholarships

2014	**Young Investigator Award** for the European Winter Conference on Brain Research-EBBS School **Travel grant** for the FENS forum 2014 from the German Neuroscience Society **Travel grant** for the Society of Neuroscience 2014 from DAAD
2013	**Selected** participant for the ECNP Workshop on Neuropsychopharmacology for Young Scientists
2010	**Travel grant** from Societá Italiana di Etologia for European Conference on Behavioural Biology
2009	**"Wanted the best"** award from University of Rome "Sapienza" for students who obtained BSc with honors (*cum laude)*
2007	**Selected** participant for the International Exchange program form the University of Camerino with the University of Victoria, Canada

Publications

2014 **Santarelli S**, Lesuis SL, Wang XD, Wagner KV, Hartmann JH, Labermaier C, Scharf SH, Müller MB, Holsboer F, Schmidt MV. *Evidence supporting the match/mismatch hypothesis of psychiatric disorders in female mice.* Eur Neuropsychopharmacol. 2014 Jun;24(6):907-18.

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Assertion

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation

“The molecular and behavioral function of SLC6A15, a novel candidate gene for depression”

selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum oder annähernd übernommen worden sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

My personal contribution has regarded the following parts of the manuscripts:

Branchi I, Santarelli S, D'Andrea I, Alleva E. *Not all stressors are equal: early social enrichment favors resilience to social but not physical stress in male mice.* Horm Behav. 2013 Mar;63(3):503-9. doi: 10.1016/j.yhbeh.2013.01.003.

- Study design and planning: In collaboration with IB, EA
- Performing the experiments: In collaboration with ID
- Data analysis: In collaboration with IB, ID
- Manuscript preparation: In collaboration with IB, ID, EA

Santarelli S, Lesuis SL, Wang XD, Wagner KV, Hartmann JH, Labermaier C, Scharf SH, Müller MB, Holsboer F, Schmidt MV. *Evidence supporting the match/mismatch hypothesis of psychiatric disorders in female mice.* Eur Neuropsychopharmacol. 2014 Jun;24(6):907-18. doi: 10.1016/j.euroneuro.2014.02.002.

- Study design and planning: In collaboration with MVS, MBM, FH
- Performing the experiments: In collaboration with SLL, WXD, KWV, HJH, LC
- Data analysis: In collaboration with KWV, SHS
- Manuscript preparation: In collaboration with MVS

Santarelli S, Kalideris G, Lesuis SL, Uribe A, Dournes C, Balsevich G, Hartmann JH, Nadal MN, Schmidt MV. *The match/mismatch hypothesis of psychiatric disorders: evidence in male mice* (in preparation).

- Study design and planning: In collaboration with MVS

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- Data analysis: In collaboration with MMN
- Manuscript preparation: In collaboration with MVS

Santarelli S, Namendorf C, Gerlach T, Anderzhanova E, Bedenk B, Kaltwasser S, Wagner KV, Labermaier C, Drgonova J, Czisch M, Uhr M, Schmidt MV. *SLC6A15 is a regulator of hippocampal neurochemistry and behavior* (submitted, major revision)

- Study design and planning: In collaboration with MVS
- Performing the experiments: In collaboration with CN, TG, EA, BB, SK, KVW, CL, JD
- Data analysis: In collaboration with MC, MU
- Manuscript preparation: In collaboration with MVS

Santarelli S, Wagner KV, Labermaier C, Uribe A, Dournes C, Balsevich G, Hartmann JH, Nadal MN, Schmidt MV. *SLC6A15, a novel stress vulnerability candidate, regulates anxiety and depressive like behaviors through glutamatergic system.* (in preparation)

- Study design and planning: In collaboration with MVS
- Performing the experiments: In collaboration with KVW, CL, AU, CD, GB, HJH, MMN
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