# Identification of novel molecular factors involved in individual stress vulnerability

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# 1 Introduction

## 1.1 The concept of stress

One name is closely connected to the concept of biological stress: Hans Selye. Although not called stress yet, Selye described in 1936 in his letter to *Nature* the ''general adaption syndrome'' and backed up some fundamental concepts by a plethora of animal experiments (Selye, 1936). He insisted that the stress response is unspecific as well as independent of the type of stressor and extended the work of Walter Cannon, who described the catecholaminergic response to stress, by the influence of the hypothalamic-pituitary-adrenal (HPA) axis (Cannon, 1920).
Cannon coined the term ''homeostasis'', describing an optimal state of the organism with fixed set points. This equilibrium is under constant challenge from external stimuli, so-called ''stressors''. The homeostatic concept was extended by Sterling and Eyer in 1988 by the introduction of ''allostasis'' (Sterling and Eyer, 1988). Allostasis acknowledges changes in the environment and that optimal conditions for an organism can change over the course of a lifetime. Therefore it describes an adapted state based on internal and external needs. Stellar and McEwen further introduced ''allostatic load'', which represents the cost of allostatic adaption to the organism (McEwen and Stellar, 1993).
Also, much effort was put in the classification of different stressors. Many authors separate eustress, or ''positive'' stress, meaning that the following adaptive response is able to re-instate homeostasis, from ''distress'' resulting in pathological outcomes. Furthermore, stressors are often categorised into physical (also called reactive, interoceptive or systemic stressors), for example an immune challenge or cold conditions, or anticipatory stressors (also called predictive, exteroceptive, neurogenic, psychogenic or processive stressors) (Dayas et al., 2001; Pacák and Palkovits, 2001). Examples for the latter would be the anticipation of a predator by its smell. Interestingly, the different stressor types are transmitted via distinct pathways. While physical stressors mainly involve somatic, visceral or circumventricular pathways, anticipatory stressors are primarily processed in the limbic system, including the hippocampus, amygdala and prefrontal cortex (Engelmann et al., 2004; Pacák and Palkovits, 2001; Chrousos and Gold, 1992). This insight attenuated Selye's postulation of a completely unspecific response to all stressors.

## 1.2 The stress response

The stress response is mainly orchestrated by two systems in interplay: The sympathetic nervous system (SNS) and the HPA axis. The SNS is mostly responsible for the ''fight-or-flight'' reaction described by Cannon, resulting in the release of epinephrine or norepinephrine from the adrenal medulla. In contrast, activation of the HPA axis is characterised by release of different hormones, including cortisol (or corticosterone (CORT), depending on the organism), and is believed to mediate not only immediate stress effects, but also long-lasting changes.

### 1.2.1 The sympathetic nervous system

The general function of the SNS is to redirect energy to systems useful for direct survival, while energy-consuming functions not important for direct survival are downregulated (Goldstein, 1987).

The central command of the SNS is exerted at multiple levels. The spinal cord with its sympathetic preganglion neurons represents the lowest level followed by the medulla and the pons. One of the higher centres is for example the hypothalamus (Goldstein, 1987; Jansen et al., 1995; Jänig and McLachlan, 1992; Gilbey and Spyer, 1993).
Stress-induced activation of the SNS is manifested by increased cardiac output, which - in combination with vasoconstriction - leads to enhanced delivery of oxygen and glucose to skeletal muscles and glucose availability is further increased by stimulated glycogenesis and glycogenolysis in the liver, while insulin secretion from the pancreas is inhibited. In addition, SNS activation causes increased renal sodium retention expanding the blood volume. In contrast, domains like digestion or gonadal function are downregulated by the SNS stress response (Goldstein, 1987). Activation of the SNS also has profound effects on immunological parameters, however the interactions are complex and time-dependent (Molina, 2005).

### 1.2.2 The hypothalamic-pituitary-adrenal axis

The starting point of the HPA axis is the hypothalamic paraventricular nucleus (PVN). Upon a stressful stimulus, secretory neurons from the parvocellular part of the PVN release corticotropin-releasing hormone (CRH, also called corticotropin-releasing factor or short CRF) as well as arginine vasopressin (AVP). These neurons are connected to the hypophyseal portal system via the median eminence allowing the release of the hormones directly into the blood circulation. CRH and AVP work synergistically on the anterior pituitary to stimulate the synthesis of pro-opiomelanocortin (POMC), which is mainly processed to corticotropin (ACTH). In the adrenal glands, specifically in the *zona fasciculata* of the adrenal cortex, ACTH stimulates the release of corticosterone (or cortisol in humans) (de Kloet et al., 2005). Rodents are not able to produce cortisol, as they lack the necessary enzyme 17$\alpha$-hydroxylase in the adrenal glands (Laplante et al., 1964), although this view is controversial (Touitou et al., 1990). A schematic overview of the HPA axis can be found in Figure 1.

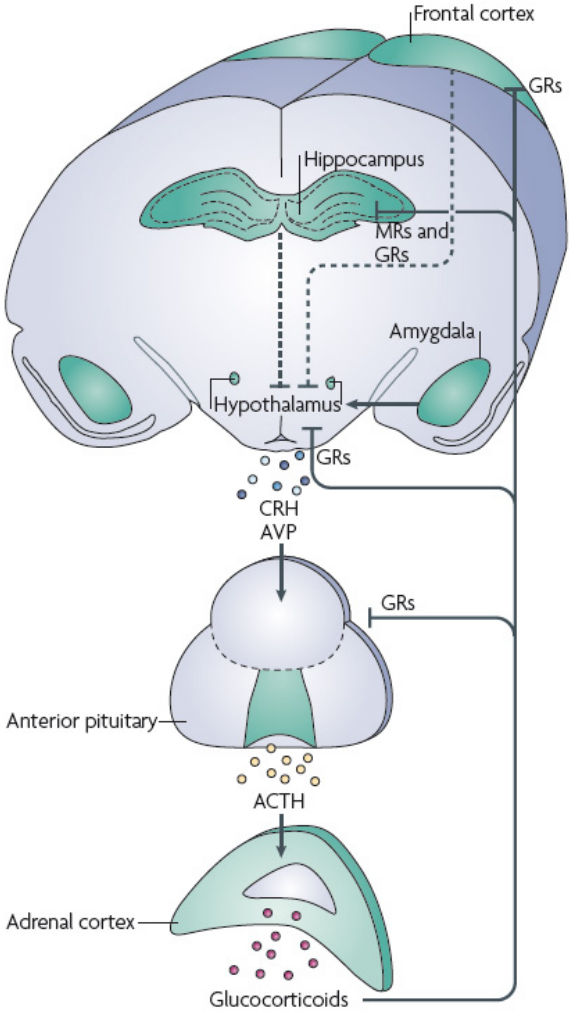


Figure 1: Schematic overview of the HPA axis. Following the perception of a stressful stimulus, CRH and AVP are secreted from the PVN and ultimately cause secretion of ACTH from the anterior pituitary. ACTH in turn stimulates the secretion of glucocorticoids in the adrenal cortex. In a simplified fashion, the feedback and modulation of the HPA axis are also depicted. Feedback works mainly via the GR, which shows inhibitory influences directly on the PVN and the anterior pituitary, but affects the hippocampus and the frontal cortex. In addition, the frontal cortex and the hippocampus with their function as inhibitory centres and the amygdala as activating influence are also illustrated. For a detailed dissection of the modulation, please refer to 1.3. Arrows with pointed ends present activating influence while arrows with blunted ends depict inhibitory influence. Adapted from Lupien *et al* 2009.

The effects of corticosterone are mediated via its two receptors, the mineralocorticoid receptor (MR, coded by the nuclear receptor subfamily 3, group C, member 2 or short Nr3c2 gene) and the glucocorticoid receptor (GR, coded by the Nr3c1 gene). These receptors not only strongly differ in their affinity to corticosterone, with a high affinity for the MR ($K_d \approx 0.5$ nM) and a ten-fold lower affinity for the GR ($K_d \approx 5.0$ nM), but also in their spatial distribution. While the GR is ubiquitously expressed, the MR is mainly found in limbic brain regions, as depicted in Figure 2 (Kloet et al., 1998; Kolber et al., 2008).
Upon ligand binding, the GR and the MR can either act as monomer, homodimer or heterodimer and regulate gene expression via glucocorticoid response elements (GRE). Due to the many different combinational possibilities, the system is able to code gene activation as well as repression (Schoneveld et al., 2004). As the MR is already occupied under basal levels, while the GR does need elevated corticosterone levels for activation, the classical hypothesis stated that the MR is involved in the maintenance of the basal stress system activity whereas the GR modulates the stress-induced changes, for example regulation of gene expression. However, recent data also show rapid, non-genomic effects of both the MR and the GR, mediated by a putative membrane-bound version of these receptors (Pasricha et al., 2011).

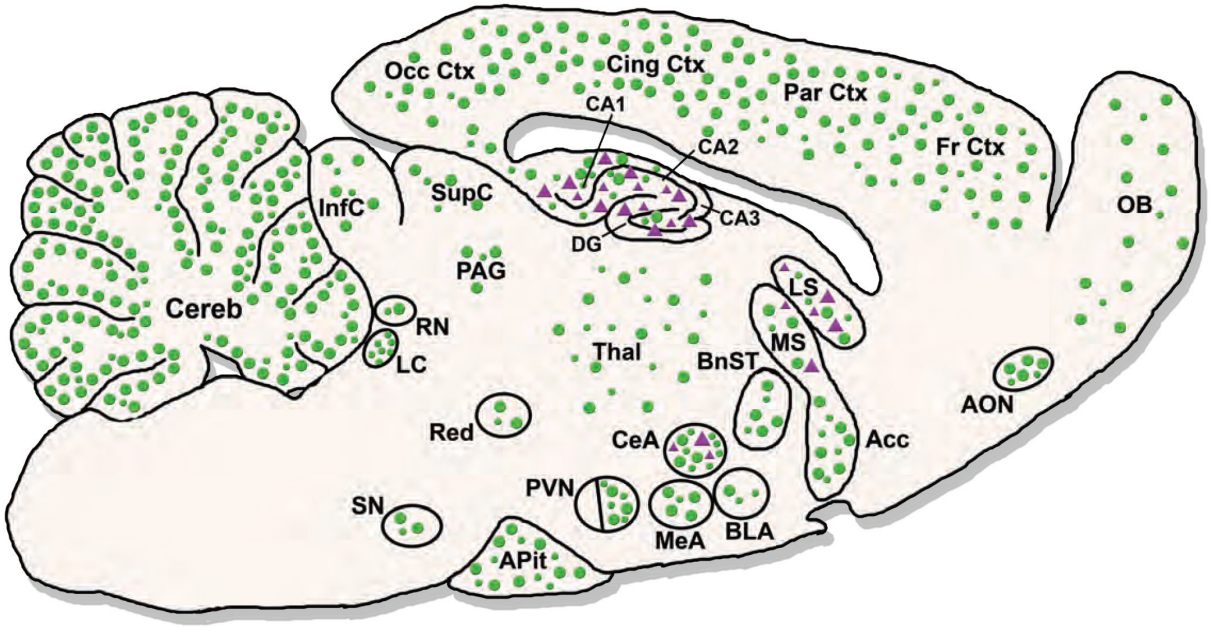


Figure 2: Schematic overview of the GR and MR distribution in the adult rodent brain. Green circles represent glucocorticoid receptors (GR) and purple triangles represent mineralocorticoid receptors (MR). Abundance of receptors is given by the relative density of circles or triangles in an area. Acc nucleus accumbens; AON anterior olfactory nucleus; APit anterior pituitary gland; BLA basolateral nucleus of the amygdala; BnST bed nucleus of the stria terminalis; CA1, CA2, CA3 hippocampal areas CA1 to CA3; CeA central nucleus of the amygdala; Cereb cerebellum; Cing Ctx cingulate cortex; DG dentate gyrus; Fr Ctx frontal cortex; InfC inferior colliculus; LC locus coeruleus; LS lateral septum; MeA medial nucleus of the amygdala; MS medial septum; OB olfactory bulb; Occ Ctx occipital cortex; PAG periaqueductal gray; Par Ctx parietal cortex; PVN paraventricular hypothalamic nucleus; Red red nucleus; RN raphe nuclei; SupC superior colliculus; SN substantia nigra; Thal thalamus. Adapted from Kolber *et al* 2008.

## 1.3 Modulation of the HPA axis

### 1.3.1 The road to regulation: connectivity of the PVN

Before one can delve deeper into the modulation of the HPA axis, with its initial centre, the PVN, it is important to understand the various connections of the relevant areas to the PVN. Two distinct types of influence emerge: directly and indirectly connected types. Directly connected influences include the *nucleus tractus solitarius* (NTS) and - to a lesser extent - cholinergic and serotonergic afferents from the brainstem (Sawchenko and Swanson, 1983; Sawchenko et al., 1983). Most limbic structures need to address the PVN indirectly. These converge in the bed nucleus of the stria terminalis (BNST) and the peri-PVN region (pPVN) (DiMicco, 2002; Choi et al., 2007). However, a majority of the limbic structures not only innervate these integrative centres, but are also interconnected with each other. Regions important for HPA axis regulation that innervate these centres include the ventral subiculum (vSUB), the ventral part of the medial prefrontal cortex (mPFCv), the ventral lateral septum (vLS), the medial amygdala (MeA), the suprachiasmatic nucleus (SCN) and the ventromedial hypothalamic nucleus (VMH) (Ulrich-Lai and Herman, 2009).
It has been shown that the majority of neurons in the pPVN are GABAergic ($\gamma$-aminobutyric acid), providing inhibitory influence on the PVN (Roland and Sawchenko, 1993; Boudaba et al., 1996; Cole and Sawchenko, 2002; Park et al., 2009). Due to multiple inputs, signals are probably modulated and integrated. Moreover, the inhibitory neurons allow a change in the sign of the signal. Excitatory glutamatergic input form the vSUB for example is transformed into an inhibitory signal for the PVN. Data about connectivity and modulatory influences was very nicely reviewed by Ulrich-Lai and Herman in 2009 (Ulrich-Lai and Herman, 2009).

### 1.3.2 Activating influences

Various brain regions are involved in the activation of the PVN. Catecholaminergic projections from the brainstem, specifically from the NTS as well as the C1 to C3 regions, provide activation following systemic stressors. Selective lesion studies of ascending (to the PVN) epinephrine- and norepinephrine-containing neurons were able to block the effects of glucoprivation, but not of forced swim stress (Ritter et al., 2003). Notably, the circadian secretion pattern of corticosterone was not affected. However, some non-catecholaminergic cells seem to convey information about both systemic as well as neurogenic stressors (Kinzig et al., 2003). Activating influence also derives from serotonergic cells in the median raphe nuclei, possibly via multiple subtypes of the serotonin receptor (Jørgensen et al., 1998).
Another important centre in the regulation of the HPA axis is the dorsomedial hypothalamus (DMH), which is believed to mainly process neurogenic stimuli (DiMicco, 2002; Thrivikraman, 2000). A study in rats has shown that microinjection of muscimol (a $GABA_A$ receptor agonist inhibiting neuronal activity) into the DMH was able to abolish the effects of a 10 minutes air puff (while restrained) (Stotz-Potter et al., 1996).
The situation is more complex for the amygdala, for which 3 main regions can be differentiated: the central amygala (CeA), the medial amygdala and the basolateral amygdala (BLA). Lesion studies in the CeA have shown that the stress response is decreased after an immune challenge

(Xu et al., 1999), but increased after an episode of restraint (Carter et al., 2004) in lesioned animals. Another study could show cell activation in the CeA after systemic stressors, with little effect on the MeA while neurogenic stressors caused a profound increase in c-fos positive cells in the MeA, with little effect in the CeA (Dayas et al., 2001). Also, the HPA response to predator odor is weakened in animals with MeA lesions, but not in animals with CeA lesions (Masini et al., 2009). Lesions in the MeA caused an attenuated response in corticosterone levels following acute restraint, but not chronic stress (Solomon et al., 2010). For the BLA, lesions caused a decrease in the acute response to restraint, however it should be noted that inactivation of the BLA by muscimol did not reproduce this effect (Bhatnagar et al., 2004). Taken together, the main body of studies show an activating influence of the amygdala, with stressor-specific integration in the different nuclei, but some conflicting evidence remains.
The medial prefrontal cortex (mPFC) has been implicated in HPA axis regulation for some time. However, recent data show different effects of the subregions. Lesions in the mPFCv (comparable with the infralimbic fields) caused a decrease in stress-induced effects following restraint, demonstrating an activating influence of the mPFCv (Radley et al., 2006).
As most of the named limbic structures do not have direct connections to the PVN, the signal must be conveyed via an indirect route, which is most possibly the BNST. The BNST has a complex structure, but activating influence has been localised to the anteroventral nucleus (avBNST) (Choi et al., 2008). In an interesting study by Burow *et al.* rats were subjected to loud noise levels and cell activation was measured via c-fos induction. The authors showed c-fos induction in the anterior BNST, among other regions, pointing at an activating influence (Burow et al., 2005; Choi et al., 2007).

### 1.3.3 Inhibitory influences and feedback

An important aspect of the stress response is its proper termination. The system does not only possess a direct negative feedback loop, but also many other brain regions can inhibit the stress response. The negative feedback mainly works at the level of the PVN and the anterior pituitary via activation of the GR. This direct feedback is known since the 1940s, but more recent experiments have shown that this effect is non-genomic and requires the cannabinoid receptor CB1 (Evanson et al., 2010; Di et al., 2003).
One important region for inhibitory influence on the HPA axis is the hippocampus. A great deal of studies have investigated this connection, nicely reviewed by Jacobson and Sapolsky (Jacobson and Sapolsky, 1991). Further studies have shown that hippocampectomy of the dorsal, but not the ventral hippocampus, was able to reduce the suppressing effect of dexamethasone (Feldman and Conforti, 1980). This is especially interesting as the ventral hippocampus has been implicated with emotion and affect, while the dorsal part seems to be reserved purely for memory function (Fanselow and Dong, 2010).
It has also been proposed that the ventral part of the DMH exerts inhibitory influence, although data is sparse (Cullinan et al., 1996).
The same ambiguity has been found in the mPFC. While, as described above, the mPFCv seems to have an activating influence, the dorsal part of the mPFC (mPFCd, comparable to the prelimbic

field) has been shown to act in an inhibitory fashion (Figueiredo et al., 2003; Radley et al., 2008, 2006; Diorio et al., 1993).
Nevertheless, also the inhibitory influence from the limbic sites needs to traverse the integrating centres like the BNST and the pPVN. While in the pPVN, with its diffuse architecture, the transgression of the inhibition seems straightforward (glutamatergic synapses activate GABAergic projections to the PVN or GABAergic projections inhibit glutamatergic activation), the BNST has specific nuclei with inhibitory function. Lesion studies have located the inhibitory neurons in the posterior (principal nucleus) or anteroventral (dorsomedial/fusiform nucleus) part of the BNST (Choi et al., 2007).
Another direct GABAergic input to the PVN comes from the medial preoptic area (mPOA). Lesion in the mPOA caused a significant reduction in the response to neurogenic restraint stress (Viau and Meaney, 1996).

### 1.3.4 Circadian rhythm and pulsatility

Adrenal activity and corticosterone secretion are not a static process. During the activity phase (the light phase for humans and the dark phase for nocturnal animals like most rodents), basal levels of corticosterone peak. A study in 1972 showed that this circadian rhythm is most probably generated by the SCN, as a lesion of the latter completely abolished corticosterone rhythmicity (Moore and Eichler, 1972). However, the majority of projections do not directly reach the PVN, but end in the subparaventricular area, a part of the pPVN (Vrang et al., 1995; Watts et al., 1987; Watts and Swanson, 1987), giving rise to the assumption that the SCN signal can be influenced by input (or is influencing the input) from limbic sites. And indeed it has been found that a lesion of the SCN changed the response of corticosterone and ACTH to a novel environment in rats (Buijs, 1997).
In recent years, it has been found that the different levels of corticosterone are not the result of constant secretion, but the integrated function of distinct pulses of different frequency and amplitude (Windle, 1998; Lightman et al., 2008).

## 1.4 Acute versus chronic stress

The response to stress *per se* is beneficial. As discussed above, it is meant to re-establish homeostasis and adapt the organism to changes in the environment. However, if the system is active over a prolonged period of time or the regulation is impaired, maladaptive consequences can arise. Longer periods of continuous stress are generally referred to as chronic stress.
Three prominent changes have already been described by Hans Seyle. First, chronically stressed animals show increased adrenal glands and more recent studies could show that this effect stems from hyperplasia as well as hypertrophy in the *zona fasciculata*, the site of glucocorticoid production (Ulrich-Lai et al., 2006). Furthermore, animals show involution of the thymus gland, possibly caused by hypercortisolism, as it has been shown that high levels of glucocorticoids are toxic for the thymus (Brewer et al., 2002). Finally, Selye and others described the high incidence of gastrointestinal ulceration in stressed animals (Caso et al., 2008).
In addition, molecular as well as structural parameters are altered in the brain. Diverse studies

have found that chronic stress (or chronic treatment with glucocorticoids) decrease branching and length of apical dendrites in CA3 pyramidal cells (Magarinos and Mcewen, 1995; Woolley et al., 1990) as well as in the infralimbic mPCF (Goldwater et al., 2009). It has also been shown that the distribution and expression of the GR and MR were found decreased after chronic stress exposure in the hippocampus (Schmidt et al., 2007; Sterlemann et al., 2008). Also, the expression and functionality of different serotonin receptor subtypes was found to be decreased following chronic stress in the hippocampus (Wang et al., 2009). Studies have shown that chronic stress decreased long-term potentiation (LTP) in the hippocampus, which is believed to play an important part in the formation of memory (Pavlides et al., 2002; Joëls et al., 2004) and it has also been shown that chronic stress exposure can decrease plasticity in the hippocampus (McCormick et al., 2010; Xu et al., 2009; Yun et al., 2010).
Concomitant with the molecular effects, differences in behaviour and cognition have been found. Chronic stress caused cognitive impairments in domains like spatial memory (Wang et al., 2011b; Sterlemann et al., 2010; McCormick et al., 2010; Xu et al., 2009) and fear learning (Yun et al., 2010; Hoffman et al., 2010). In addition, anxiety-related behaviour as well as depression-like behaviour were found to be increased following chronic stress (Schmidt et al., 2007; Sterlemann et al., 2008). Another common finding is the increase of anhedonic behaviour (Pohl et al., 2007; Strekalova et al., 2004; Wang et al., 2008; Ushijima et al., 2006).

## 1.5 Stress as a risk factor for disease

Another deleterious outcome of chronic stress exposure is the increase in risk for disease. While chronic stress alone does not present a causal factor for disease, the exposure can cause a drastic rise in the vulnerability to certain disorders (de Kloet et al., 2005). This has been shown for - not exclusively - cardiovascular diseases (Rosengren et al., 2004), metabolic disturbances (Abraham et al., 2007) as well as affective disorders (Charney, 2004; de Kloet et al., 2005). In many cases, this is coupled to genetic risk loci. In a widely known study, Caspi and colleagues were able to show that early trauma did only elicit negative outcomes in carriers of a risk allel in the serotonin transporter gene (Caspi, 2003). By now, a multitude of risk alleles for affective disturbances have been identified, ranging from neurotrophins (Kohli et al., 2010) over ion channels (Green et al., 2010) to still unknown genetic loci (McMahon et al., 2010). However, the validation of these single loci is often treacherous and therefore newer hypotheses, backed up by some experimental data, favour genetic risk profiles of many combined genetic risk factors rather than single genes (Demirkan et al., 2010; Ising et al., 2009).
Interestingly, chronic stress exposure not only increases the risk for affective disorders but also shows similar phenotypes. For example, depressed patients often show hypercortisolism (Checkley, 1996), which also is a hallmark of chronic stress exposure. Moreover, patients suffering from Cushing's Syndrome, a disease characterised by increased levels of glucocorticoids mostly caused by adrenal carcinoma, often show depressive symptoms as comorbidity . Intriguingly, if the cause of hypercortisolism is treated in those patients (for example tumor surgery), the depressive states tend to disappear (Pereira et al., 2010). More similarities like this have been shown for HPA axis reactivity, sleep disturbances or anxiety-like behaviour (Chrousos and Kino, 2009).

## 1.6 Important time windows for stress exposure and development

The results of stress exposure differ vastly depending on the developmental stage of the organism and the developmental stage of the stress system. In general, 5 important stages can be differentiated: (1) The prenatal period, describing the time of gestation from conception to birth, (2) the postnatal period, (3) adolescence, describing the time until the end of puberty, (4) adulthood and finally (5) the aged individual. A good review of this field was provided by Lupien and colleagues recently (Lupien et al., 2009). It should be noted that the following chapters present only an excerpt of the literature. Effects on domains like substance abuse were deliberately omitted as they are not the focus of the present thesis. The interested reader may be directed to extensive reviews of the respective fields, for example in the case of substance abuse (Sinha, 2008).

### 1.6.1 The prenatal period

The prenatal period is an important time in the formation of the central nervous system, which is to a certain degree conserved between mammalian species (investigated on the example of rat and man in Bayer et al. (1993)). Interfering in this delicate process can cause detrimental effects in the future life. Normally, the foetus is protected from high levels of corticosteroids via the foeto-placental barrier, in which the enzyme 11$\beta$-hydroxysteroid dehydrogenase 2 (11$\beta$-HSD 2) is active (Waddell et al., 1998). However, if corticosterone levels in the mother reach high levels - for example during stressful conditions - this barrier is not sufficient any more. Moreover, it has been shown that prenatal stress (PNS) causes a drastic drop in the expression as well as the enzymatic activity of 11$\beta$-HSD 2 in the placenta, further weakening the protective barrier (Mairesse et al., 2007).

These high levels of corticosterone in the developing foetus have been shown to cause maladaptions in various parameters. It has been shown in rats that PNS causes impaired feedback to an acute stressor in adult offspring (basal levels and peak levels were not affected), which nicely fits the concomitant reduction in GR, but not MR, binding (Barbazanges et al., 1996). Interestingly, this HPA axis responsiveness fluctuates over time. While at post-natal day (PND) 3 and PND 21 the response to restraint was elevated in offspring of PNS rats, no effect on stress-induced CORT increase was found in adult (PND 90) and middle-aged (16 months) rats. However in the latter two groups, the feedback was impaired (Darnaudéry and Maccari (2008) nicely combined the data of Henry et al. (1994) and Vallée et al. (1999)). It should be noted here that elevated CORT levels at PND 3 fall into the so-called stress-hyporesponsive period (SHRP), which will be discussed in the next chapter, and might exert strong downstream effects. Furthermore, studies have shown that neurogenesis was decreased in hippocampi of male, but not female, offspring and a closer examination could pin those effects to the ventral, but not the dorsal part (Zuena et al., 2008). However, it should be noted that the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus was elevated in male PNS rats in this study. This decrease in neurogenesis was shown to be present at least until 22 months of age (Lemaire et al., 2000).

The manifold molecular changes are also reflected in behaviour and cognition. Several studies have shown that PNS caused a decrease in the time spent in the open arm of the elevated plus maze (EPM), a measure of anxiety-like behaviour (Barros et al., 2006; Wakshlak and Weinstock, 1990; Vallée et al., 1999; Zohar and Weinstock, 2011; Fan et al., 2009), however these effects seem

to be predominant in male offspring and a strong sex effect has been suggested (Zuena et al., 2008). Also, a decrease in hedonic behaviour (saccharin consumption) has been reported in PNS rats (Keshet and Weinstock, 1995; Mueller and Bale, 2008). Furthermore, PNS rats seem to favour passive coping strategies in the tail suspension test (TST) (Mueller and Bale, 2008) and the forced swim test (FST) (Kjaer et al., 2010; Smith et al., 2004). Multiple studies were also able to show a decrease in spatial memory in PNS rats (Lemaire et al., 2000; Lui et al., 2011; Markham et al., 2010).
PNS not only directly affects the offspring, but also the dams and thereby maternal behaviour (Smith et al., 2004; Champagne and Meaney, 2006). These findings have prompted some authors to speak of perinatal instead of prenatal stress and this notion is backed up by cross-fostering studies (or a general improvement of the environment, for example environmental enrichment), showing that the PNS effects could be attenuated or completely abolished by these procedures (Smith et al., 2004; Champagne and Meaney, 2006).

### 1.6.2 The postnatal period

The postnatal period is also an important part in the development of the pups. Environmental factors show a strong influence and this period includes the SHRP, an important time window in the development of the stress system. The SHRP, which was first described in rats (Schapiro et al., 1962), was also described for the mouse and seems to last from pnd 1 to roughly pnd 12 (Schmidt et al., 2003). During this time, basal levels of CORT are low and no corticosterone response to mild stressors is mounted (Schmidt et al., 2003).
Prominent postnatal or early life stress (ELS) paradigms exclusive to this period include maternal separation (MS) or restriction of nesting materials (causing reduced maternal care) (Ivy et al., 2008; Millstein and Holmes, 2007). It has been shown that ELS produced alterations quite similar to the effects of PNS. Regarding molecular effects, downregulation of the GR has been described in (early) adult rodents following ELS (Maniam and Morris, 2010b; Navailles et al., 2010), while the MR seems to be unaffected (Navailles et al., 2010). Other studies have found an upregulation of the MR and no effect on the GR in the hippocampus in adult MS rats (Ladd et al., 2004). Single studies aside, the consensus seems to be that the MR/GR ratio is shifted towards the MR. Reduction in gene expression in the hippocampus in adult ELS animals also include the 5-hydroxytryptamin (serotonin) receptor 1a (5-HT1A) or BDNF (Maniam and Morris, 2010b; Llorente et al., 2011).
The shift in MR/GR ratio might also be a contributing factor to the findings regarding basal and stress induced levels of glucocorticoids. In theory, the shift towards the MR would imply no differences in maintenance of basal levels but inadequate feedback regulation. And indeed, most studies in adult ELS animals have shown that basal corticosterone levels are not altered, while the feedback following diverse acute stress challenges seems to be impaired (Uchida et al., 2010).
Stress during the postnatal period also affects behaviour. Studies have shown that MS in mice caused impaired spatial and reversal learning in adult mice (Wang et al., 2011a; Ivy et al., 2010). The literature on anxiety-like behaviour is heterogeneous. While most studies show an increase of anxiety-like behaviour in adult ELS animals (Huot et al., 2001; Maniam and Morris, 2010a; Lee et al., 2007; Uchida et al., 2010; Luán et al., 2008; Veenema et al., 2008), others find no effect

(Wang et al., 2011a; Millstein and Holmes, 2007) or even a decrease in anxiety-like behaviour (Parfitt et al., 2007). Possible explanations include differences in sex, strain, methodology, age of testing and the stress levels of the dams (McIntosh et al., 1999; Millstein and Holmes, 2007; Weiss et al., 2011). Further studies have shown more passive coping strategies in ELS animals (Uchida et al., 2010; Hui et al., 2011) as well as increased anhedonia (Huot et al., 2001; Hui et al., 2011).

### 1.6.3 Adolescence

The development of the stress system is still in progress during adolescence (Romeo et al., 2006; McCormick and Mathews, 2010). However, the adolescent period is probably the least investigated developmental period regarding stress exposure and its effects.
Studies in our group and others have shown that the GR as well as the MR were downregulated in the hippocampus of adult animals that underwent stress during adolescence (Schmidt et al., 2007). Some of these effects were even shown 12 months after the stress procedure in aged mice (Sterlemann et al., 2008). In addition, stress during adolescence was shown to decrease hippocampal cell proliferation (McCormick et al., 2010) as well as a decrease in levels of synaptophysin and BDNF (Sterlemann et al., 2010) pointing at decreased synaptic plasticity. In the PVN, stress exposure caused an increase in CRH mRNA (McCormick et al., 2007)
Corticosterone levels were shown to be persistently increased in animals stressed during the adolescence (Schmidt et al., 2007; Gu et al., 2009; Lepsch et al., 2005), in some cases up to the age of 15 months in mice (Sterlemann et al., 2008). Concomitant with the rise in basal corticosterone levels, increased adrenal glands as well as involution of the thymus are common findings (Schmidt et al., 2007).
Behavioural effects of the stress exposure in adult animals include an increase in anxiety-like behaviour (Schmidt et al., 2007; Wright et al., 2008; McCormick et al., 2008; Pohl et al., 2007) and a lasting impairment of cognitive function in animals stressed during adolescence (McCormick et al., 2010; Sterlemann et al., 2010). Sucrose consumption as a indicator of hedonic behaviour has been found to be decreased in animals that underwent stress during adolescence (Pohl et al., 2007).

### 1.6.4 Adulthood

Many studies investigating the effects of chronic stress exposure in adult animals have been performed. In general, studies show that the effects of chronic stress exposure in adult animals are not as pronounced or long-lasting as when performed during development. A study in rats showed that animals that underwent chronic mild stress (CMS) showed an attenuated response of CORT and ACTH to a novel environment - although this effect was only shown 4 d and 7 d after the stress and could not be detected any more 30 d after the stress. This was also reflected in adrenal gland weight: while stressed rats showed enlarged adrenals 16 h, 4 d and 7 d after stress, adrenal weight normalised at 30 d. Moreover increased levels of CRH in the PVN were only present 16 h after stress (Ostrander et al., 2006). The elevated levels of CRH are a common finding after stress exposure in the PVN (Pournajafi-Nazarloo et al., 2009; Romeo et al., 2007; McCormick et al., 2007; Pinnock and Herbert, 2001) as well as the CeA (Merali et al., 2008; McCormick et al., 2007). Also, increased adrenal glands and involution of the thymus are commonly found (Reber

et al., 2006).
Another common finding are increased basal levels of CORT (Bartolomucci et al., 2004; Ushijima et al., 2006), however these seem to normalise over time. Anxiety-like behaviour has been found to be increased in stressed animals (Kinsey et al., 2007) and the habituation to a novel environment seems to be impaired (Park et al., 2001; D'Aquila et al., 2000). As also seen during development, chronic stress in adult animals caused a marked decrease in sucrose consumption, which was stable for at least one week (Strekalova et al., 2004; Wang et al., 2008; Ushijima et al., 2006). Regarding the FST, more passive coping strategies have been found via the FST in animals that underwent chronic stress (Park et al., 2001).

### 1.6.5 Aging

The long-term effects of chronic stress are hard to investigate in aged individuals due to the obvious pitfalls. One of the few studies investigating chronic stress in aged rats found that while the response to the stress was similar to young rats, the feedback in the aged animals was impaired (Odio and Brodish, 1989). This impaired termination of the HPA axis was also found in various other studies (Scaccianoce et al., 1995; Sapolsky et al., 1986a). Furthermore, it has been hypothesised that chronic stress during the lifetime accelerates and exaggerates the effects of aging (Sapolsky et al., 1986b).

## 1.7 The persistence of stress effects

As described in the previous chapter, some effects of stress exposure can endure over the whole lifetime of an organism. The question remains how exactly these long-term changes are realised. One might hypothesise that it is at least partly based upon differences of gene expression. For example, the sensitivity of the GR can be regulated via different co-factors, including Fk506 binding protein (Fkbp5). So if the gene expression of Fkbp5 is lastingly affected, long-term changes in GR sensitivity might be achieved. A recent publication showed that knockout (KO) of Fkbp5 modulated the response to chronic social defeat (Hartmann et al., 2012). When investigating the mechanisms of the lasting changes in gene expression, much consideration has been given to epigenetics. Epigenetic changes describe covalent modification of the DNA or histones without altering the genomic sequence (Tsankova et al., 2007). One of the pioneering studies in the field of stress-related epigenetics showed that the quality of maternal care influences DNA methylation patterns of the GR promoter and that this change in methylation patterns influences the stress response in later life (Weaver et al., 2004). Since then, the field of stress-related epigenetics has been burgeoning. It has also been shown that the stress-induced decrease in BDNF levels was regulated via histone acetylation (Tsankova et al., 2006) supporting the influence of epigenetic modification in stress-induced gene expression changes.

## 1.8 The concept of vulnerability and resilience

Much has been said in the previous chapters about the effects of stress. Interestingly however, these effects almost never affect the whole population to the same extent. While some individuals show a strong stressed phentotype, other parts of the population are only mildly affected by

the experience or not affected at all. Studies from our group have shown that when a cohort of heterogeneous outbred mice are subjected to chronic social stress, some animals were able to cope with the siutation, while others showed long-term stress effects (Schmidt et al., 2010a). This difference in susceptibility is also reflected in the fact that stress is a risk factor, but not a causal factor, for disease. When elaborating about stress vulnerability and resilience, the terms and definitions are important and need to be clear. So when in the following chapters stress vulnerability or resilience is addressed *in general*, the terms vulnerability, resilience or susceptibility describe one and the same thing - the *concept* of vulnerability and resilience. For example the statement ''Gene X influences stress vulnerability'' does not imply a direction of the regulation. It states the exact same as ''Gene X influences stress resilience''. Stating here every single time ''Gene X influences vulnerability and resilience depending on the regulation'' seems futile and will be omitted. In contrast, when the statement is imbued with a *direction*, the terms are used in their naturalistic meaning. So the statement ''Knockout of gene X causes increased stress vulnerability'' describes a rise in stress vulnerability while ''Knockout of gene X causes increased stress resilience'' describes a decrease in stress vulnerability.

## 1.9 Models of vulnerability and resilience

The section on models of vulnerability and resilience has been published in advance (Scharf and Schmidt, 2012). When modelling gene × environment interaction, different approaches are feasible. One possibility is to modify the genome of animals, resulting in knockout or transgenic (knock-in) animals specific for selected candidate genes. For this approach, murine models are favoured in comparison to other species due to technical advantages. Another possibility is the selection of subpopulations within the whole cohort of animals based on molecular or behavioural parameters, which would also reflect genetically or epigenetically defined populations. Finally, subpopulation selection can be used as a differentiating factor for selective breeding to putatively enhance the differences in the subgroups and isolate the genetic component transferred over the germ line. The following sections provide an overview of the different approaches, including examples of recent studies focusing on stress vulnerability. Therefore, it does not provide and should not be seen as a complete overview of all available models, but rather as an illustration of the concepts.

### 1.9.1 Transgenic models

With the refinement of techniques for directed genetic mutations or gene targeting, transgenic animal models have become more and more useful for the broad scientific community. In addition, as many transgenic lines are commercially available, research is no longer limited to institutions with specific transgenic facilities. In recent years, various genetic animal lines have been published, highlighting the influence of single genes on stress vulnerability or resilience. Recent studies range from genes known to be involved in the stress response to novel candidate genes. For example, studies from our group with a pituitary-specific KO of the glucocorticoid receptor showed that KO animals were protected from stress induced elevated basal levels of corticosterone, but not from a stronger response to acute stress. In addition, stress exposure increased anxiety-like behaviour in wild-type (WT), but not in KO animals (Wagner et al., 2011). Interesting findings were also

revealed by studying Fkbp5, a co-chaperone of the glucocorticoid receptor. Here, animals that underwent chronic social defeat showed elevated basal levels of CORT and a stronger response to acute stress, all of which was blocked by a conventional Fkbp5 KO. In addition, KO animals also showed a blunted response to the combined dexamethasone/corticotropin-releasing hormone response test. No gene × environment interaction was found for anxiety-like behaviour, but stressed KO animals showed stronger active coping strategies in the FST (Hartmann et al., 2012; O'Leary et al., 2011). Other interesting examples are models with a modulated glutamate transmission, which also has been implicated in depression (Sanacora et al., 2012). VGLUT1 (vesicular glutamate transporter 1) KO mice, for example, show under stress a stronger anhedonic phenotype in the sucrose preference test, more immobility in the FST, and higher ambulation than their WT littermates. Interestingly, anxiety-like behaviour as well as object recognition memory were modulated via stress in a genotype-independent manner. It should be noted that the heterozygous KO of VGLUT1 caused a (potentially compensatory) increase in VGLUT2 levels in the frontal cortex and the hippocampus (Garcia-Garcia et al., 2009). Deficiency in neural cell adhesion molecule has also been proposed as a genetic model of stress vulnerability. Mice with a forebrain-specific KO of the neural cell adhesion molecule showed more passive coping strategies in the tail suspension test and cognitive impairment in the Morris water maze following a mild stress paradigm that did not produce a phenotype in WT animals. However, the model is very new, and characterization is still ongoing (Bisaz and Sandi, 2012). Using transgenic animal models has different advantages and limitations. These characteristics are mainly dependent on the type of KO. Conventional KO animals can provide information about the general function of the investigated gene, but in most cases, this approach ignores the fact that genes often have different and potentially even opposite functions in different tissues or cell types (Refojo et al., 2011), which can make interpretation of the results challenging. With the possibility of conditional KO, transgenic animals can reach specificity ranging from selective KO of a gene in the central nervous system or single brain regions to specific cell types. The same holds true for the time frame during which gene expression is modulated. The maturation of the stress system in different stages of development is a vital influence on stress vulnerability in adult life (Lupien et al., 2009), and a KO of a specific gene can have completely different results in different developmental stages. Therefore, the most sophisticated are inducible KO systems that can be activated in a previously determined period (Andersson et al., 2010). The optimal approach obviously is the combination of conditional and inducible KO; however, the more complex the KO system, the more time consuming the breeding becomes. Thus, when using transgenic models, the planning of experiments should in the best case include consideration of how much specificity, be it spatial or temporal, is needed for each research question. Here it should be noted that improvements in molecular techniques such as viral vectors and optogenetics open up the possibility also to modify genes in a precise and time-specific manner.

### 1.9.2 Selection of subpopulations

Another approach is to use the intrinsic heterogeneity of whole populations. Hereby, one or preferably more characteristics are investigated and the animals are grouped into subpopulations based on, for example, performance in a behavioural assay. For example, Bergström and colleagues

(Bergström et al., 2008) used CMS to elicit an anhedonic phenotype in rats. However, not all rats developed this phenotype, so the cohort was split into CMS-resilient and CMS-susceptible animals, allowing the identification of molecular changes in these subphenotypes. One of the main findings was that BDNF levels were higher in the hippocampus of resilient rats (Bergström et al., 2008). This model was also supplemented with a thorough investigation of genetic differences (Bergström et al., 2007; Christensen et al., 2011) as well as noninvasive imaging techniques (Delgado y Palacios et al., 2011). Despite the extensive investigations, only single potential candidate genes for stress resilience remain, and the authors themselves state that the resilient phenotype is most likely caused by a combination of many different factors. It also has been shown that when animals are selected based upon their CORT levels after recovery from chronic stress, vulnerable animals show decreased levels of the glutamate receptor 1 (GluR1) and increased levels of GluR2 in the hippocampus (Schmidt et al., 2010b). Interestingly, the opposite situation was found for the nucleus accumbens (NAc) in animals divided by their antisocial behaviour following social defeat stress. Here susceptible animals show higher levels of GluR1 and decreased levels of GluR2. In addition, it was shown that GluR2 overexpression was able to reverse the susceptible phenotype, fluoxetine treatment increased GluR2 levels in the NAc, intra-NAc infusions of an AMPA receptor antagonist increased vulnerability and the NAc of postmortem human depressed brain tissue had lower levels of GluR2, but not GluR1. At least for the NAc, this is probably mediated via $\Delta$Fosb and upstream via serum response factor (Vialou et al., 2010a,b). Extensive studies in the same model focused mostly on the ventral tegmental area, the NAc, and the periaqueductal gray and showed increased levels of BDNF in the NAc following stress (Berton et al., 2006). In addition, it has been shown that only susceptible animals develop an anhedonic phenotype (shown in the sucrose preference test) as well as differences in thermoregulation, while other parameters, such as anxiety-like behaviour (elevated plus maze) or elevated CORT levels, were a general effect of the stress exposure. No effects were found in both the FST and the tail suspension test. This study also showed that the increased levels of BDNF in the NAc were only present in the susceptible subgroup, concomitant with increased levels of Akt (Krishnan et al., 2008), glycogen synthase kinase-3$\beta$, and extracellular signalregulated kinase (ERK)1/ERK2 (downstream molecules of BDNF signaling). Infusion of recombinant BDNF increased susceptibility, while overexpression of ERK decreased susceptibility (Krishnan et al., 2008). In addition, it has been shown that firing rates of ventral tegmental area dopamine neurons are higher specifically in susceptible animals ex vivo (Krishnan et al., 2007), as well as in in vivo studies (can be prevented by chronic antidepressant treatment) (Cao et al., 2010). Working with subpopulations offers several benefits but is not without caveats. This approach can lay excellent groundwork for unbiased approaches such as whole-genome, transcriptome, proteome, epigenome, or metabolome studies. Due to the selection of a phenotype in contrast to a single gene, selecting subpopulations can help in identifying novel targets as well as networks. In addition, this approach is an excellent choice when the expected effect sizes are low, as in the field of affective disorders. Therefore, selection of extremes (and thereby omission of unaffected individuals) can help detect effects that would otherwise not be detectable due to a low signal-to-noise ratio. Some authors also argue that so-called population validity should be provided. This states that when a human pathology only affects a certain percentage of the population, this should be reflected in the animal

model (Schmidt, 2011). On the other hand, finding a meaningful parameter of selection can be a challenging task, as described previously. Furthermore, the subpopulations are often characterized after the stress exposure, which prevents clear assertions about the causality of the phenotypic variations on stress vulnerability. In any case, selecting a subpopulation provides a solid basis for gene × environment studies, as responders and nonresponders show distinct enrichment of specific genetic traits (Schmidt et al., 2010b), which in turn can then be investigated under different environmental conditions.

### 1.9.3 Selective breeding

Another viable approach is the selection of a specific phenotype followed by selective breeding, aiming at enhancing and stabilizing the phenotype. After some generations, the phenotype of the breeding lines diverges and allows further characterization and investigation of the underlying molecular principles or the use as a disease model. For example, rats were bred for high or low levels of exploratory behaviour (termed high responders and low responders). Following 4 weeks of CMS, it was shown that low-responder rats developed anhedonic symptoms (sucrose preference test) much faster and more strongly. The same was true for the novelty-induced suppression of feeding test (anxiety-like behaviour), in which stressed low-responder rats took significantly longer times to approach as well as consume the palatable snack (Stedenfeld et al., 2011). Another example would be animals that were bred for low and high short-term memory. Here it was shown that low short-term memory animals, with increased levels of GluR2, are significantly more affected by stress exposure, which was blocked by treatment with an AMPA (2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid) receptor potentiator (Schmidt et al., 2010b). Another quite thoroughly characterized model is the stress reactivity mouse line (Touma et al., 2008). In this model, animals were bred based on high or low CORT response to an acute stressor. High-response animals show, among other parameters, disturbances in sleep patterns, cognitive deficits, and decreased levels of hippocampal BDNF (Fenzl et al., 2011; Knapman et al., 2010b,a). The same advantages as seen with subpopulation selection apply here as well, with one major addition. As the phenotypic differences here are present before the stress exposure, careful study design allows the identification of causal factors. Nevertheless, selective breeding is a costly and time-consuming approach. In addition, the possibility of a genetic drift  potentially causing a shift in the phenotype  can be minimized but can never be completely prevented. Using selective breeding can be an excellent option to study gene × environment interaction and investigate the underlying genetic predisposition of vulnerability. For example, it has been used successfully to associate a specific sequence deletion in the promoter region of the vasopressin gene with extremes in anxiety behaviour (Bunck et al., 2009).

## 1.10 The evolutionary perspective of vulnerability

At first glance, the existence of vulnerability genes seems counterintuitive. Life as we know it today is the result of billions of years of evolution, favouring - as Darwin stated - the ability to adapt. So how is it possible that we find genes that increase the negative impact or impair the recovery from a stressful event? Some authors explain this via plasticity. Belsky and colleagues argued that what we see are plasticity genes and only our one-sided perspective labels them

as vulnerability genes (Belsky et al., 2009). In short, the authors state that individuals with a certain risk allele suffer more from a negative environment - however, when exposed to a positive environment, these individuals benefit more from it. So what we find is a genotype defining the general response to environmental stimuli - be they beneficial or harmful. In a study investigating different single-nucleotide polymorphisms (SNPs) in the serotonin transporter gene promoter region, a region strongly implicated in the vulnerability to depression or other affective disorders (for example see (McHugh et al., 2010; Cervilla et al., 2006)), it was found that while the s/s carriers showed a higher risk to depression under high risk environment conditions, individuals with the same genotype show lower rates of depression compared to l/l carriers under low environmental risk conditions (Eley et al., 2004).
Closely related is the mismatch hypothesis of disease (Schmidt, 2011). Here, vulnerability originates from dissociations in the environment. It states that during development, individuals are optimally prepared for their current environments and that pathologies arise if the adult environment strongly differs from the developmental conditions. For such a case, vulnerability gene variants seem useful, as they could provide individuals better adaption to specific environments. However, if the adult environment changes, those adaptive changes can easily lose function and even become maladaptive. This has been proposed for affective disorders like depression (Schmidt, 2011) as well as other areas like metabolic disturbances (Gluckman et al., 2008).

## 1.11 The hippocampus as a prime target in stress research

The hippocampus is of grave importance due to its strong connections (described in 1.3.1) and its well described inhibitory effects (see 1.3.3) to the HPA axis. This goes in hand with the strong expression of both the MR and the GR. The hippocampus is one of the few regions in the brain that stay in a relatively plastic state even in the fully matured brain (Kelsch et al., 2010) and it has been shown that this plasticity is necessary to convey some stress-induced features, for example social avoidance behaviour (Lagace et al., 2010).
As described before, changes in the hippocampus are frequently found after chronic stress exposure, like dendritic remodelling of CA3 neurons or differences in gene expression of the GR and the MR, and these alterations surely contribute to the resulting phenotypes. Therefore, the hippocampus is an interesting target for investigating the long-term effects of stress.

## 1.12 Mouse models in psychiatric research

The many similarities of chronic stress exposure and diverse affective disorders as well as the number of studies showing increased risk for pathologies after stress exposure led to the development of animal models of affective disorders via various stressful experiences. But creating meaningful mouse models is not an easy task, especially in the setting of psychiatric research this is challenging. The following chapter mainly addresses the mouse as model organism. This should in no way indicate that this is the ‘‘best’’ model organism, but it has various advantages in some areas, while other model organisms like rats, tree shrews, primates, *Caenorhabditis elegans*, yeast or *Drosophila*, just to name a few, show different strengths and weaknessess.
The main advantages of the mouse are its easy husbandry coupled with short generation time and the many ways to modulate the genome. Not only is the mouse genome completely sequenced since

2001, it also shares strong similarities, up to 99 %, with the human genome (Paigen, 2003). In addition, genetic manipulation including (conventional or conditional) gene knockdown, knockout or overexpression are easier to perform in mice than in most other organisms. This led to the development of a plethora of different genetic mouse lines, most of which are commercially available and thereby easily accessible.
The first step in selecting (or creating) an appropriate mouse model is the choice of a suitable mouse strain. Generally, one can distinguish inbred mouse lines and outbred colonies. Inbred mouse lines have been subjected to sibling mating for at least 20 generations and include lines like DBA or C57Bl/6. These lines are bred to be genetically identical and thence perfectly suited for the study of the influence of single genes or single mutations. In contrast, outbred stocks of mice are kept below 1 % of inbreeding and show genetic heterogenity. These animals can be used to investigate population-based effects and more complex traits.
The next step is the selection of a paradigm, be it genetic (for example a specific knockout) or environmental (for example exposure to a specific form of chronic stress) (Schmidt et al., 2011b; Schmidt, 2011; Nestler and Hyman, 2010). Willner has formulated three important validity criteria that should - in the best case - always be fulfilled: constructive, face and predictive validity (Willner, 1984, 1997). Construct validity describes the need that the modelled disease is based upon the same molecular rationale, which can be challenging in the case of psychiatric disorders, as information about the molecular basics are scarce. One successful example is the insertion of disease-causing alleles for familial Alzheimer's disease (Götz and Ittner, 2008), which caused plaque formation in both humans as well as animals. Face validity states that the model organism should display the same symptoms as the human patient suffering from the disease. This also is characterised by several caveats in the case of psychiatric research, as some symptoms just cannot be modelled in animals. For example one of the criteria for depression in the *Diagnostic and Statistical Manual of Mental Disorders, $4^{th}$ edition* (DSM-IV) is ''Recurrent thoughts of death or suicide'', which cannot be modelled in the mouse. However, criteria like anhedonia or sleep disturbances can be investigated and have been reported for some models. Predictive validity is fulfilled if pharmacological treatment (succesful in humans) is also able to improve symptoms in the animals. One example is the effects of anxiolytic drugs on an animal model of anxiety, based on selective breeding (Liebsch, 1998).

## 1.13 Activity-regulated cytoskeleton-associated protein

One gene thoroughly investigated in this thesis is the activity-regulated cytoskeleton-associated protein (Arc, also known as Arg3.1) gene. First described in 1995, it was named both for its ability to be induced via synaptic activity as well as its co-sedimentation with F-actin, suggesting an association with the cytoskeleton (Lyford et al., 1995). The gene was described in an independent lab in the same year (Link et al., 1995), hence the two different names. Arc belongs to the class of immediate-early genes (IEG) and was found to be enriched in dendrites. An overview of the processes described in the following section can be found in Figure 3.

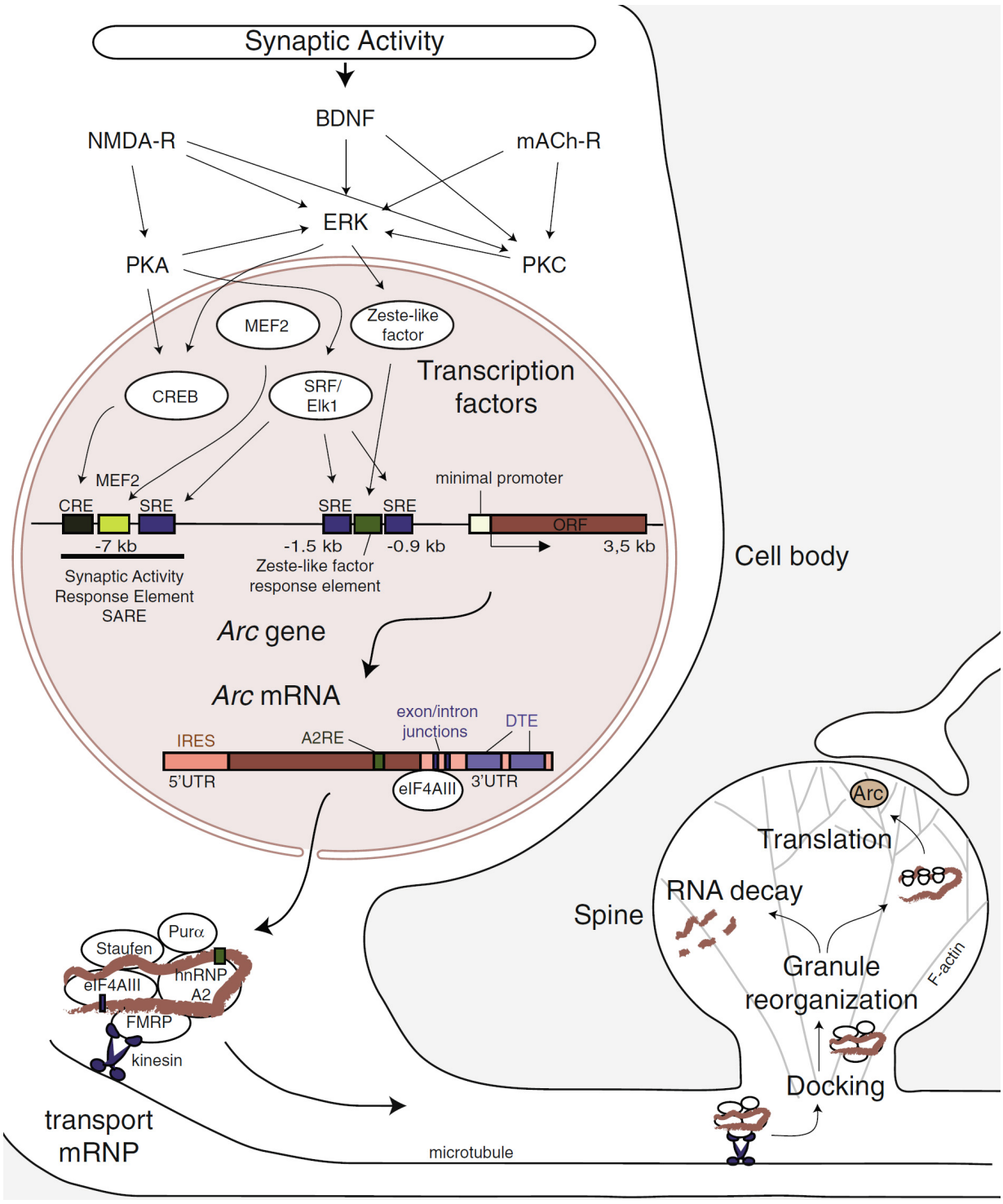


Figure 3: The different stages of the Arc gene. Systems are described in detail in 1.13. Adapted from Bramham et al 2010.

### 1.13.1 Induction of the Arc gene

By now, a plethora of factors able to induce Arc expression have been identified. Environmental factors include free spatial exploration (Ramírez-Amaya et al., 2005; Vazdarjanova et al., 2006), learning (Montag-Sallaz and Montag, 2003; Guzowski et al., 2001; Daberkow et al., 2007), seizures (Vazdarjanova et al., 2006; Lyford et al., 1995) or acute social defeat (Coppens et al., 2011). However, as Arc expression seems to be induced by generic synaptic activity, this list is surely not exclusive. Increases in Arc mRNA can be found as fast as five minutes after long-term potentiation (LTP) (Messaoudi et al., 2007). The pathways used in these environmental factors have been narrowed down and investigated in greater detail in multiple molecular studies, but not yet fully understood. A small overview of the so-far identified pathways will be given in a top-down manner. In general, 3 prominent molecules have been implicated in Arc activation: the neurotransmitters glutamate and acetylcholine (ACh) as well as BDNF. The involvement of glutamate in the induction of a synaptic activity-dependent gene comes as no surprise, given its role as most abundant excitatory neurotransmitter in the brain. It has for example been shown that blockade of the ionotropic N-Methyl-D-Aspartate (NMDA) receptor subtype decreased basal expression levels of Arc (Lyford et al., 1995) and blocked increases in Arc expression via LTP (Steward and Worley, 2001) or via activation of the 5-$HT_2$ receptor (Pei et al., 2004). Both of the latter studies also showed a similar effect with AMPAR antagonist treatment. It should be noted however, that other studies have also found an inhibiting influence of the AMPAR (Rao et al., 2006). Other studies focused on the metabolic glutamate receptor (mGluR) class. Treatment with dihydroxyphenylglycine (DHPG, an agonist of the group I mGluRs including mGluR1 as well as mGluR5) caused increased Arc expression (Brackmann et al., 2004; Waung et al., 2008; Park et al., 2008). Furthermore, pharmacological stimulation of the muscarinic acetylcholine receptor (mAChR) induced a robust upregulation of Arc, which was prevented by antagonist treatment (Teber et al., 2004). Stimulation with BDNF also caused induction of Arc (Ying et al., 2002; Pintchovski et al., 2009). Interestingly, despite the initial widespread nature of activating factors, the downstream pathways seem to converge strongly. Most of the pathways are dependent on ERK or - to a lesser extent - on protein kinase A (PKA) (Bramham et al., 2010). Modulatory functions of these pathways via protein kinase C (PKC) have also been described (Bramham et al., 2010). ERK in turn recruits and phosphorylates multiple adaptor proteins, like for example serum response factor (SRF) to finally target regulatory sequences in the Arc gene.

### 1.13.2 Structure and binding motifs of the Arc gene

The Arc gene itself is roughly 3050 base pairs (bp) long (exact number depending on the source) in the mouse genome, consists of 3 exons and is located on chromosome 15. Interestingly, only 1091 bps of exon 1 form the coding region. Regulation of the Arc gene is believed to be mainly controlled via a synaptic activity response element (SARE, roughly 6.5 kbp 5' of the start sequence) and a zeste-like factor response element (ZRE, roughly 1.5 kbp 5' of the starting sequence) (Pintchovski et al., 2009). The SARE consists of a serum response element (SRE), a myocite-enhance factor-2 (MEF2) site and a cAMP response element (CRE), however the latter two are not yet thoroughly described. In addition, the region of the SARE was found to be responsive to PKA, but no binding site has been identified yet. The same holds true for the 2 putative repressing sites found on the

Arc gene.

### 1.13.3 Arc mRNA transport and post-transcriptional modifications

One of the most intriguing aspects of Arc is its characteristics in the mRNA stage. After induction, Arc mRNA is transported out of the nucleus, however not transcribed to protein in the soma, but is transported to synaptic spines in the dendrites and transcribed locally under strict control. For transportation, the mRNA is packed together with co-factors into so-called messenger ribonucleoprotein particles (mRNP). Essential for the transport process to the dendritic targets is binding of heterogeneous nuclear ribonucleoprotein (hnRNP) A2 (Gao et al., 2008). The 3' untranslated region (UTR) of the Arc gene holds two dendritic targeting elements (DTE) also involved in the targeting (Kobayashi et al., 2005), however, when the 3' UTR was removed in the Gao study, targeting was not impaired. The authors explain this phenomenon by the hypothesis that the DTEs are responsible for steady-state transport, while the A2 pathway is used for activity-dependent transport. Other important co-factors in the Arc-mRNP complex include for example fragile-X mental retardation protein (FMRP), which inhibits mRNA translation during the transport (Zalfa et al., 2003) and can also link the complex to kinesin (Davidovic et al., 2007). Arc mRNA also associates with Kif5, which gives another hint of kinesin as an active transporter (Kanai et al., 2004). Arc is found at dendritic synapses that were recently active. Interestingly, this targeting process requires actin polymerisation: when this was blocked via rho kinase inhibitors or an NMDAR antagonist, Arc targeting was impaired. The same study also shows that ERK phosphorylation is necessary for Arc targeting, strengthening the role of ERK as a central molecule in Arc regulation (Huang et al., 2007).

### 1.13.4 Translational control of Arc

Intriguingly, ERK seems to be also important for the translation of Arc mRNA to protein, acting through a series of downstream molecules (Panja et al., 2009). In addition, activation of the NMDAR and G-coupled receptors can increase Arc translation (Bloomer et al., 2008). But not only the translation, but also the decay of Arc protein is tightly controlled. One of the major controlling systems seem to be nonsense-mediated mRNA decay (NMD, very nicely explained for Arc in (Giorgi et al., 2007)). In short, a exon-junction complex (EJC) is added to the pre-spliced mRNA in the nucleus. This EJC complex is normally removed by the ribosome upon the first (pioneer) round of translation. However, if a stop codon is present before the EJC, the mRNA is tagged for degradation. In the case of the Arc mRNA, the two exon junctions (introns 1 and 2) - binding sites of the EJC - are both situated in the 3' UTR after the open reading frame (ORF), thereby after the stop codon. This strongly suggests that Arc mRNA is only used for a single round of translation, adding a system of tight regulation and an additional layer of control that can potentially be modulated. This might also explain the relatively short half-life of the Arc protein at roughly 47 min (Rao et al., 2006). However, not all of the transcripts are degraded after the pioneer round. The Arc protein also includes a PEST sequence at the c-terminus, which is a potential target for proteasome-mediated degradation and it has been shown that the proteasome is involved in the Arc degradation (Rao et al., 2006), possibly via the recruitment of UBE3A (Greer et al., 2010).

### 1.13.5 Functional relevance of Arc

#### *1.13.5.1 Electrophysiology*

First hints that Arc is involved in LTP came from early studies showing the induction of Arc via LTP (Lyford et al., 1995; Link et al., 1995). By now studies with Arc KO mice ($Arc^{-/-}$) have shown that KO of the gene caused increased early LTP, but completely abolished late-phase LTP, after stimulation of both the schaffer collateral (SC) as well as the perforant path (PP) pathways. Also for NMDAR-mediated long-term depression (LTD), Arc seems to be necessary. In addition, baseline NMDAR and AMPAR currents as well as release probabilities (paired-pulse facilitation) were unaltered in the KO animals (Plath et al., 2006). Similar results (knockdown of Arc blocks maintenance, but not induction of LTP) have also been achieved via antisense oligodeoxynucleotide (AS-ODN) infusion in the hippocampus (Guzowski et al., 2000). Another study with these AS-ODNs showed that LTP is quickly reversed when the AS-ODNs are given 2 h after LTP induction, but not after 4 h. In contrast to the previous study, infusion of Arc AS-ODNs before high-frequency stimulation prevented LTP induction, but not late-phase LTP in this study. The authors were also able to link F-actin stabilisation to Arc expression and prevent the stabilisation via Arc AS-ODN infusion. Interestingly, this link to actin stabilisation seems to be of critical importance, because when F-actin was stabilised pharmacologically, the efficacy of the Arc AS-ODNs to prevent LTP consolidation was completely abolished. Messaoudi et al. argue here that sustained expression of Arc is necessary for functional LTP (Messaoudi et al., 2007). Arc is also involved in LTD, but while the data on NMDAR-mediated LTD is still inconclusive, Arc seens to be most important for mGluR-LTD (Park et al., 2008). It has been found that Arc modulates mGluR-LTD via AMPAR endocytosis (Chowdhury et al., 2006): when Arc was knocked down via viral-delivered short-hairpin RNA against Arc (shARC), the expression of surface AMPAR was increased (the opposite effect was found after Arc OE) concomitant with an increase in basal synpatic function. In addition, it was shown that the normally seen internalisation of AMPARs after mGluR activation can be prevented via Arc knockdown (Waung et al., 2008). Due to its reciprocal characteristics (for example: Arc can regulate AMPAR, but at the same time AMPAR activation regulates Arc expression) some hypotheses of feedback-regulated systems have been generated. It seems for example possible that Arc forms a positive feedback loop in the synapse to facilitate LTP (Bramham et al., 2010) or functions as a regulator of neural circuit homeostasis (Shepherd and Bear, 2011).

#### *1.13.5.2 Learning and memory*

Due to its strong involvement in LTP and LTD, which is thought to be the molecular underpinnings of learning and memory, an involvement of Arc in the latter does not come as a surprise. Many of the studies investigating electrophysiological effects often also showed an effect of learning and memory. For example, KO of the Arc gene caused disruptions in learning in the Morris water maze (MWM), novel object exploration as well as fear conditioning (Plath et al., 2006). Consolidation of memory was blocked via infusion of AS-ODNs in the MWM as well as - when injected in the amygdala - fear conditioning (Guzowski et al., 2000; Maddox and Schafe, 2011; Ploski et al., 2008). Interestingly, short-term memory was left intact.

#### *1.13.5.3 Stress and stress vulnerability*

Not much data is available about the role of Arc in stress and stress vulnerability. Multiple studies have shown upregulation of Arc following acute stress exposure, however it is hard to dissect the specific effects of the stress itself and the activity aspect. For stress vulnerability, the situation is even more unclear. One interesting study showed that in the mPFC of stress vulnerable animals, Arc was decreased while resilient animals showed no difference from controls. In addition, the authors presented data that Arc is also reduced in the ACC of depressed individuals in post-mortem brain tissue (Covington et al., 2010).

## 1.14 Aims

The specific hypothesis upon which the experiments are based are included in detail in the discussion. This was done to explain to the reader how the hypotheses changed with the availability of new data and were modified for the next stages of experiments. But as a general overview the main aims are as follows:

- The identification of novel, stress-regulated genes in the murine brain.
- The identification of genes involved in individual stress vulnerability in the murine brain.
- Finding biomarkers in peripheral blood to determine individual stress vulnerability.
- Thorough investigation of Arc, a candidate gene for individual stress vulnerability.

# 2 Materials & methods

## 2.1 Animals

The experiments were carried out with male C57BL/6N or CD1 mice from Charles River Laboratories (Maastricht, the Netherlands). Housing consisted of Plexiglas cages (45 x 25 x 20 cm) with bedding and additional nesting material under controlled conditions, with a regular 12L:12D light cycle (lights on at 6:00 am) as well as constant temperature (23 ± 2 °C) and humidity (55 ± 5 %). Standard mouse chow (Altromin 1324, Altromin GmbH, Germany) and tap water were provided *ad libitum*.
Experiments were carried out in the animal facilities of the Max-Planck-Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

## 2.2 Experimental design

### 2.2.1 Experiment 1: Long-term effects of stress exposure on gene transcription in the hippocampus

#### *2.2.1.1 Experiment 1a: Long-term effects following chronic social stress exposure*

For this experiment, male CD1 mice were used. Animals were 28 days old (PND28) at the beginning of the experiment. Upon arrival, animals were housed in groups of 4 animals per cage and were randomly assigned to either the stress or control condition. The animals from the stress condition were subjected to 7 weeks of chronic social stress (CSS), described in detail in 2.3.1. In short, stress animals' cage mates were changed twice a week during the whole stress paradigm, whereas control animals stayed in the same combination of animals over the whole 7 weeks. Directly after cessation of the stress phase, all animals were single-housed and tail blood was collected. Animals were allowed to recover from the stress and were sacrificed 5 weeks after stress. Whole brains of the animals were extracted, snap-frozen and stored until further processing (for all sampling procedures see 2.4). The animals for the microarray study (CON $n = 9$; STR $n = 9$) were part of a larger cohort of animals (CON $n = 24$; STR $n = 24$) used for multiple experiments not described here and were selected randomly. This represents the discovery sample for experiment 1. An overview can be found in Figure 4.

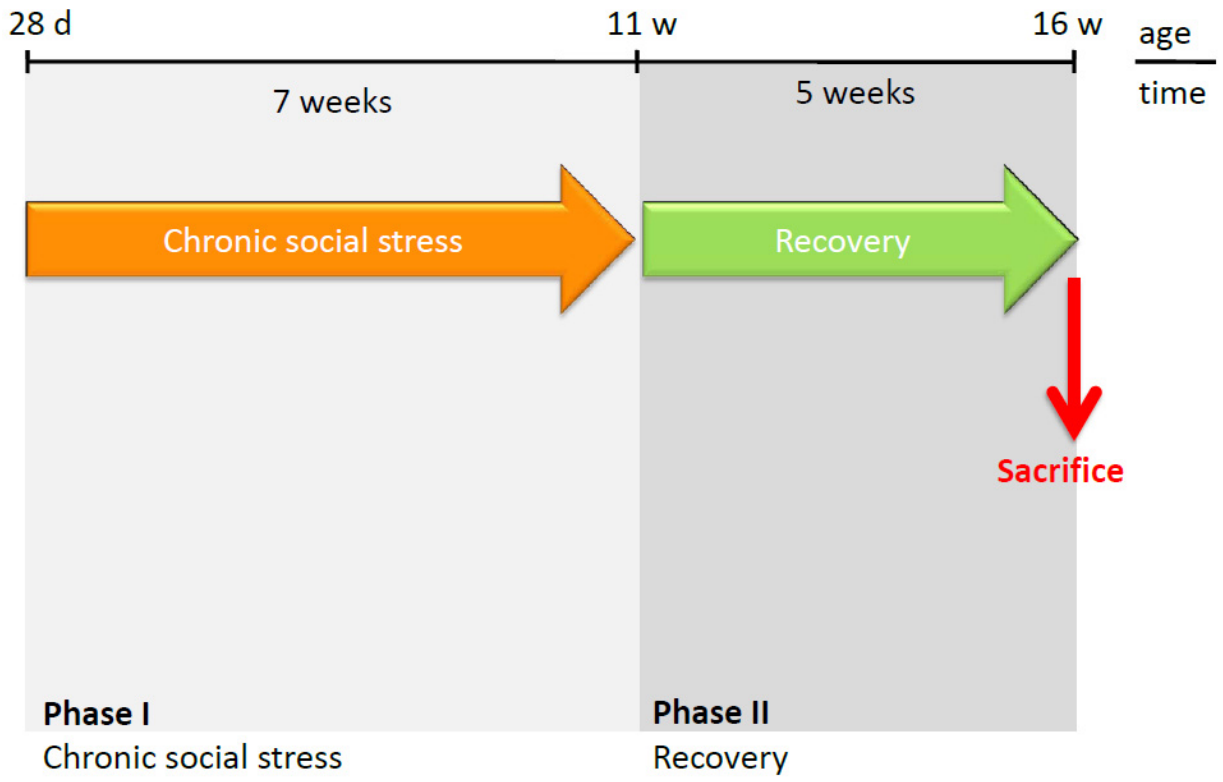


Figure 4: Experimental design.

#### 2.2.1.2 *Experiment 1b: The effect of acute stress exposure on selected candidate genes*

To study the effects of acute stress on the expression levels of selected genes, two different stress paradigms were performed. A total of 24 male C57Bl/6N mice (11 weeks of age) were used and randomly either left undisturbed as a control group (CON $n = 8$), underwent 30 minutes of restraint stress (RES $n = 8$) or were subjected to 24 hours of food deprivation (FD $n = 8$), described in detail in 2.3.3 and 2.3.4, respectively. Animals were sacrificed between 0700 h and 1200 h. Animals of the restraint stressed group were sacrificed 4 hours after onset of the restraint stress. Trunk blood and whole brains were collected, as described in 2.4. An overview can be found in Figure 5.

#### 2.2.1.3 *Experiment 1c: Influence of the GR on selected genes via pharmacological activation*

As the effects of stress can be conveyed via a plethora of mechanisms, male C57Bl/6N mice were treated with the exogenous GR agonist dexamethasone, to test specifically the influence of GR activation on gene expression. A total of 48 animals (11 weeks of age) were subcutaneously (s.c.) injected between 0800 h and 1000 h with either 130 $\mu$l of 10 mg/kg body weight dexamethasone (Ratiopharm, Ulm, Germany)(DEX $n = 24$) or 0.9 % saline as vehicle-injected control group (VEH $n = 24$). The dexamethasone concentration was based on publications that proved this dosage sufficient to induce effects in the brain (Barbany and Persson, 1992; Lee, 2005). Animals of both treatment groups were sacrificed 1 hour, 4 hours, 8 hours and 24 hours after injection with six animals per group (VEH $n = 6$; DEX $n = 6$ for each time point, see Figure 6). Trunk

blood and whole brains were collected, as described in 2.4.

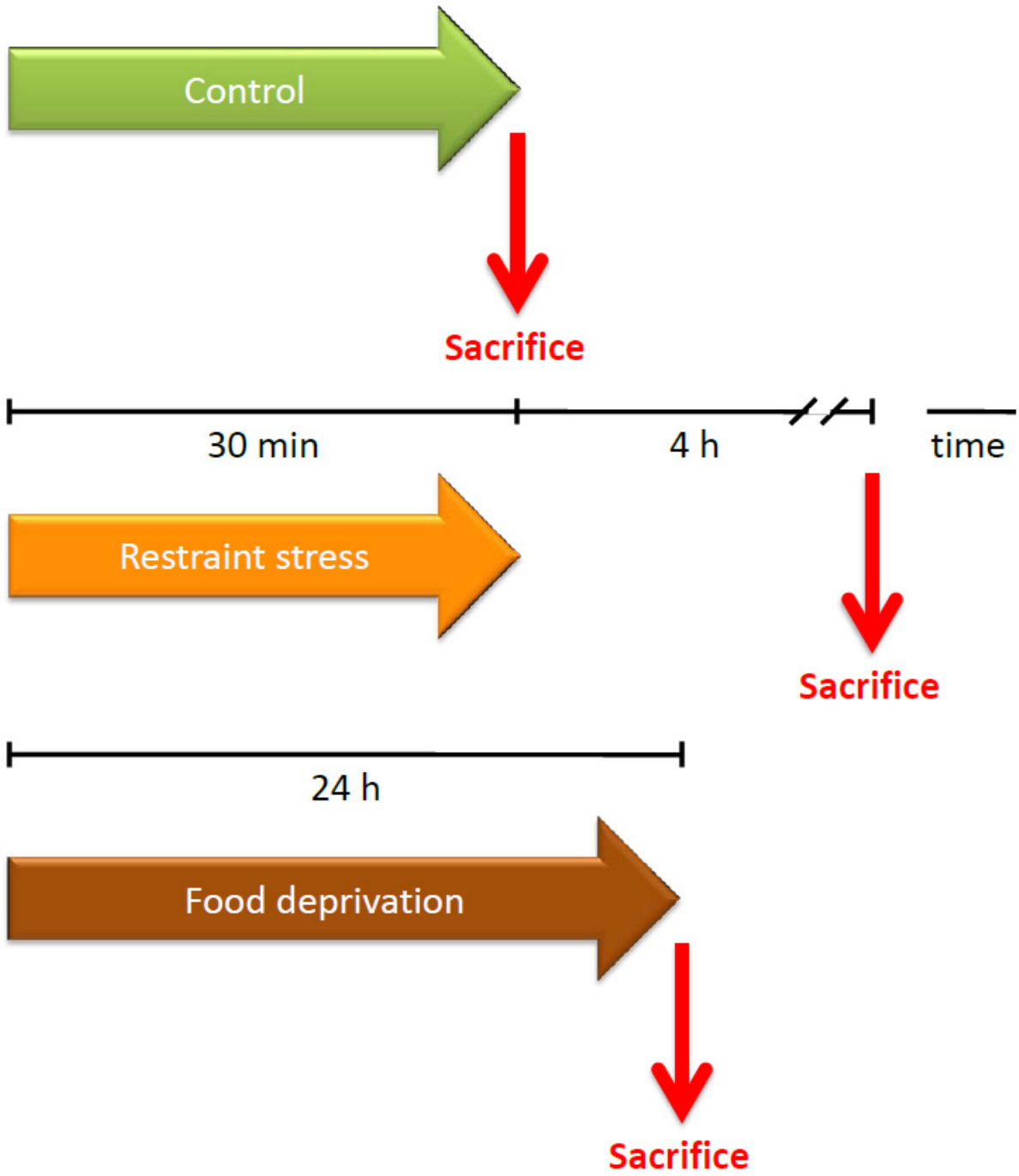


Figure 5: Experimental design.

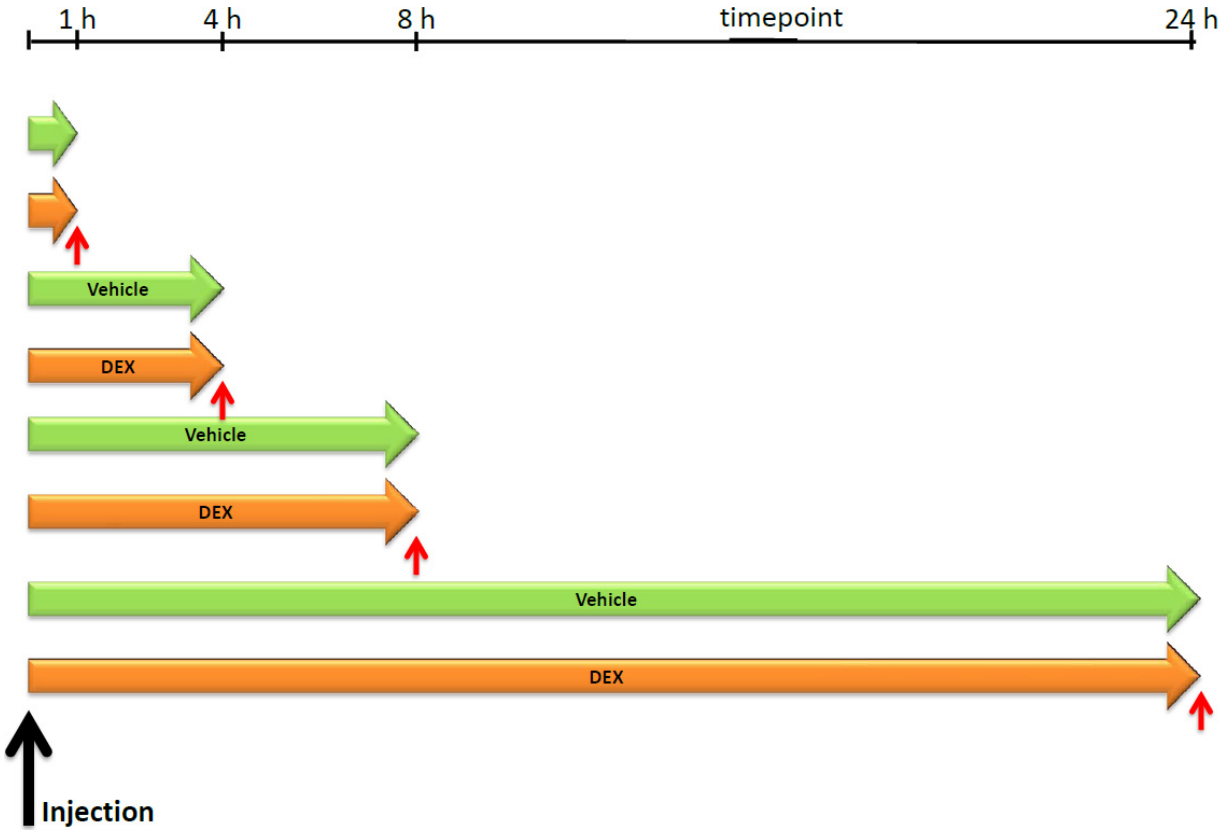


Figure 6: Experimental design.

### 2.2.2 Experiment 2: Transcriptome differences between stress-vulnerable and stress-resilient animals

#### *2.2.2.1 Experiment 2a: Vulnerability genes in the hippocampus*

For this experiment, male CD1 mice were used. Animals were 28 days old (post-natal day 28, pnd28) at the beginning of the experiment. Upon arrival, animals were housed in groups of 4 animals per cage and were randomly assigned to either the stress or control condition. The animals from the stress condition were subjected to 7 weeks of CSS, described in detail in 2.3.1. In short, stress animals' cage mates were changed twice a week during the whole stress paradigm, whereas control animals stayed in the same combination of animals over the whole 7 weeks. Directly after cessation of the stress phase, all animals were single-housed and tail blood was collected. Animals were allowed to recover from the stress and were sacrificed 5 weeks after stress. Whole brains of the animals were extracted, snap-frozen and stored until further processing (for all sampling procedures see 2.4). From all stress animals (STR $n = 160$), the extremes in corticosterone levels 5 weeks after stress were selected. Animals with high levels of CORT were defined as stress-vulnerable (top 20 %; VUL $n = 32$) while animals with low levels of CORT were termed stress-resilient (bottom 20 %; RES $n = 32$). The animals for the microarray study (RES $n = 5$; VUL $n = 5$) were part of this cohort and were selected randomly. This represents the discovery sample for the brain gene expression from experiment 2.

#### *2.2.2.2 Experiment 2b: Vulnerability genes in peripheral lymphocytes*

For this experiment, male CD1 mice were used. Animals were 28 days old (post-natal day 28, pnd28) at the beginning of the experiment. Upon arrival, animals were housed in groups of 4 animals per cage and were randomly assigned to either the stress (STR $n = 96$) or control condition (CON $n = 24$). The animals from the stress condition were subjected to 7 weeks of chronic social stress (CSS), described in detail in 2.3.1. In short, stress animals' cage mates were changed twice a week during the whole stress paradigm, whereas control animals stayed in the same combination of animals over the whole 7 weeks. Directly after cessation of the stress phase, all animals were single-housed and tail blood was collected. Animals were allowed to recover from the stress and were sacrificed 5 weeks after stress. Whole brains of the animals were extracted, the hippocampal formation was dissected, snap-frozen and stored until further processing (for all sampling procedures see 2.4). In addition, blood was taken during the sacrifice and was used for RNA extraction as well as hormone measurements. From all stress animals, the extremes in corticosterone levels 5 weeks after stress were selected. Animals with high levels of CORT were defined as stress-vulnerable (top 20 %; VUL $n = 20$) while animals with low levels of CORT were termed stress-resilient (bottom 20 %; RES $n = 20$). The animals for the miroarray study (RES $n = 12$; VUL $n = 12$) were part of this cohort and were selected based on promising CORT levels and RNA quality. This represents the discovery sample for the blood gene expression from experiment 2 and was also used as replication sample for the brain gene expression from experiment 2. In addition, samples from this experiment (CON $n = 15$; STR $n = 15$) were used as replication sample for experiment 1.

### 2.2.3 Experiment 3: Causal influence of Arc on stress vulnerability

#### *2.2.3.1 Experiment 3a: Efficacy of the Arc shRNA knockdown*

In this experiment, the efficacy of the Arc shRNA (also referred to as shARC in short) was investigated in 12 week old C57BL6/N animals. Animals were then allowed to recover for 4 to 5 weeks before sacrifice. Animals were sacrificed either under standard isoflurane anaesthesia or via cardiac perfusion. Arc expression was investigated via western blot ($n = 2$), in-situ hybridisation ($n = 2$) and immunofluoresence ($n = 2$).

#### *2.2.3.2 Experiment 3b: Influence of Arc shRNA knockdown in the hippocampal CA1 region on stress vulnerability*

This experiment was performed with C57Bl/6N mice at the age of 16 weeks. An adeno-associated virus (AAV) expressing a shRNA construct of Arc was bilaterally injected in the hippocampal region of the animals (shARC $n = 22$). Another cohort of animals was injected with a virus expressing a scrambled construct (SCR $n = 22$). The animals were then allowed to recover for 4 weeks to ensure sufficient transfection of the cells as well as expression of the shRNA. After the recovery, animals were subjected to 3 weeks of chronic social defeat (SD) stress, in which animals are exposed to a larger dominant mouse until physical defeat followed by sensory contact only (described in detail in 2.3.2). During the last week of SD, the behavioural tests were performed. The behavioural testing battery included the open field test (OF), the object recognition test (OR), the sociability test and the FST. The FST did include the sampling of blood to determine corticosterone levels. Basal samples for the stress response were taken roughly 48 hours after cessation of the defeat paradigm in the morning (5 days after the FST). Directly after the social defeat, all animals were single housed and screened with the sucrose preference test for 12 consecutive days. In total 4 weeks after the end of the social defeat procedure, behavioural testing was repeated using the same tests in the same order. Basal sample for the stress response were taken 4 days after the FST. Finally, animals were sacrificed via perfusion and whole brains as well as adrenal glands were collected. Body weight was assessed regularly over the course of the experiment with intervals depending on the stage of the experiment (once per week after surgery until the beginning of the stress paradigm; twice per week during the stress and in the following recovery; two additional timepoints were measured: directly before and directly after the social defeat). Beginning from the social defeat phase, fur state was assessed twice per week. Single procedures are described in detail in the following sections. An overview can be found in Figure 7.

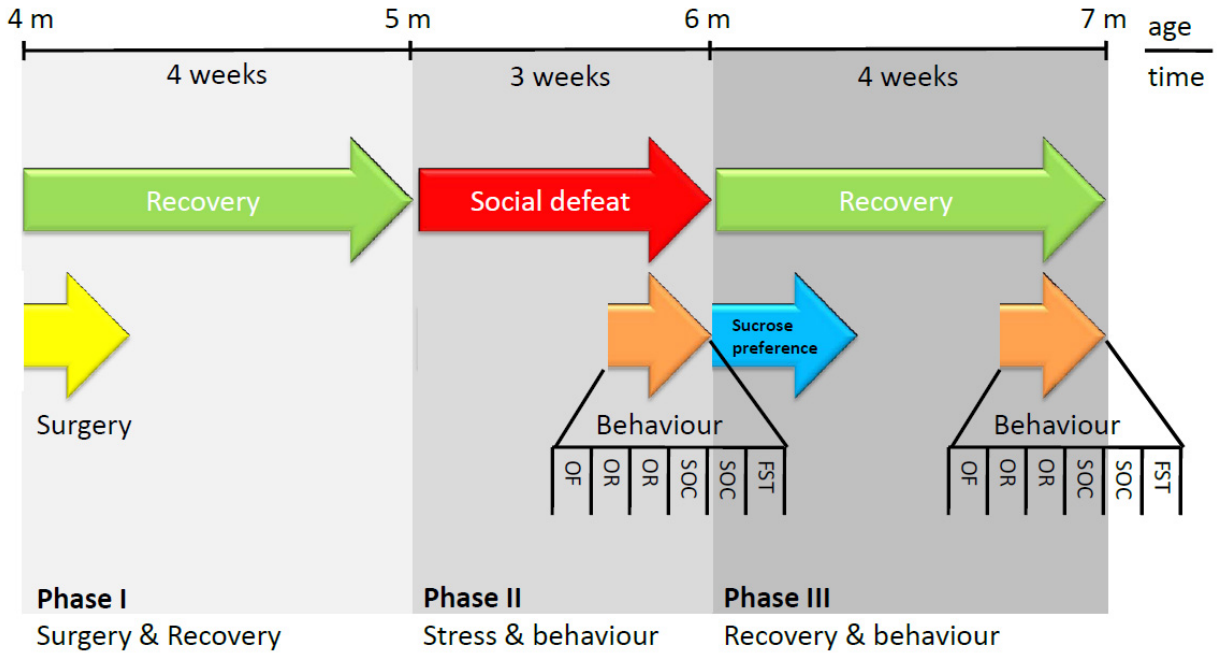


Figure 7: Experimental design.

## 2.3 Stress paradigms

### 2.3.1 Chronic social stress

The CSS procedure was performed according to Schmidt et al. in the adolescence period, during the age of 4 to 12 weeks (Schmidt et al., 2007). The mice were housed in groups of 4 and group composition in each cage was changed twice per week for 7 weeks in a way that always 4 mice from different cages were put together in a new, clean cage. The rotation schedule was planned to eliminate the chance of a repeated encounter of the same mice throughout the experiment. After 7 weeks of chronic social stress procedure, all mice were single housed. Although single housing represents a stressor itself in many species, in male mice it has been shown that single housing does not affect main immunoendocrine parameters under basal conditions (Bartolomucci, 2003; Arndt et al., 2009).

### 2.3.2 Chronic social defeat

Chronic social defeat was performed following the literature (Wagner et al., 2011). The procedure lasted for 21 days in total and each day, the experimental animals were placed into a cage containing a larger resident mouse (CD1). Following physical defeat, the mice were separated via a mesh grid to ensure continuous sensory contact to the resident mouse. Mice remained in the social defeat cages the whole time until the next defeat and were not brought back to their home cages in between defeats. Social defeat was performed in the afternoon between 1200 h and 1600 h.

### 2.3.3 Restraint stress

Restraint stress presents an inescapable, moderately stressful situation for the animals (Jørgensen et al., 1998). Mice were placed in a 50 ml Falcon tube, which allowed only minimal movements, for 30 minutes. The Falcon tube was perforated for sufficient ventilation.

### 2.3.4 Food deprivation

Food deprivation presents a very strong stressor for small rodents (Kiss et al., 1994) and includes a metabolic component. During this stress paradigm, the animals had no access to food for 24 hours. Starting between 0900 and 1200 h the animals' access to standard chow was restricted for 24 hours, while the animals had access to water at all times. Cages were changed to avoid potential remaining food leftovers in the bedding. The animals of the control group had access to food and water *ad libitum*.

## 2.4 Sampling procedure

Trunk or tail blood was collected using 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). Tail blood was collected without anaesthesia as described previously (Fluttert et al., 2000) by a small incision in the dorsal tail vein using a razorblade. For trunk blood collection, animals were anaesthetised using isoflurane and decapitated. All blood samples were immediately put on ice and centrifuged for 15 minutes at 8000 rpm at 4 °C. Plasma was transferred to clean 1.5 ml microcentrifuge tubes and stored at -20 °C. In every case, the time between the disturbance of the animals and the blood sampling was less than 3 minutes.
Adrenal glands as well as thymus glands were removed by experienced assistants and transferred to microcentrifuge tubes. Later on, organs were freed from fat and other residues and the organ weight was determined.
Whole brains were removed by experienced researchers and in case of experiment 2b, the hippocampal formation was extracted on ice. Whole brains or hippocampi were immediatly snap frozen in pre-cooled 2-methylbutane and stored at -80 °C.

## 2.5 Cardiac perfusion

Before the perfusion, animals were anaesthesised with pentobarbital (Narcoren). To access the heart, the ribcage was cut open and after a small incision of the left ventricle, the blunted needle was inserted into the aorta and fixed. The system was then flushed with saline (containing heparin) and a small incision in the right atrium ensured a congestion-free flow until the system was devoid of blood. Subsequently, saline was replaced with 4 % PFA and the mouse was perfused for 5 minutes. Finally, the animals were sacrificed via decapitation and whole brains were collected. The brains were post-fixed over night in 4 % PFA at 4 °C and transferred to a 20 % saccharose solution. Here, the brains stayed for about 3 days at 4 °C until fully dehydrated. In the end, brains were carefully freed from surplus saccharose and frozen at -80 °C until further processing.

## 2.6 Radioimmunoassay

To determine the concentrations of corticosterone in the plasma of the animals, a radioimmunoassay (RIA) was performed. The RIA is a competitive binding assay utilising the fact that corticosterone from the plasma samples and radioactively labelled corticosterone compete for a limited number of a specific antibody. As the antibody does not distinguish between labelled and non-labelled hormone and the binding happens by chance, a change in the ratio of labelled to non-labelled hormone is reflected by a change in the amount of antibody-bound radioactive corticosterone. If the concentration of corticosterone in the sample is higher, the amount of radioactively labelled corticosterone bound to antibodies will decrease. If the antibody-bound and non antibody-bound fractions are separated and one or both of these fractions are analysed, the percentage of antibody-bound radioactive corticosterone can be measured. The resulting value can be compared to a standard curve created by known concentrations of corticosterone thereby revealing the absolute concentration of corticosterone in the investigated sample. For the analysis of the tail-cut as well as the trunk-blood samples, commercially available kits were used (ImmunoChem Double Antibody Corticosterone 125I RIA Kit, MP Biomedicals, LLC, Orangeburg, NY) with a detection limit of 7.7 ng/ml, following the included protocols. Various dilutions were used depending on the expected ranges of corticosterone (1:200 - 1:50), as the expected values would therefore be in the more exact linear span of the assay. Furthermore, an additional low standard was used to extend the lower range of detection.

## 2.7 Behavioural tests

In this study, multiple behavioural tests were performed. Tests were performed in a separate room in which the animals were housed during the course of the testing. Animals were allowed to habituate to the new room for at least 4 days before the actual testing started. Housing conditions were the same as described for standard housing (see 2.1). The tests were always performed in the morning (latest test ended at 1321 h; lights on at 0800 h), with the exception of the afternoon session of the OF test. All tests were recorded and analysed (both automatically and for some parameters manually) with the help of the AnyMaze software (Version 4.20 from Stoelting).

### 2.7.1 Open field test

The open field test gives information about the basic locomotion of the animals and - depending on the lighting conditions - about anxiety-like behaviour. In brightly lit arenas ($> 300$ lux), the centre of the arena is aversive to the animals not only by its open space, but also due to the bright light (Zueger et al., 2005; Kinsey et al., 2007). Main readout parameters in the OF test are the travelled distance of the animals as well as the preference of the centre. In our setup, the centre was illuminated with roughly 100 lux, which should provide a slightly to medium aversive atmosphere. We calculated travelled distance as well as preference of the centre zone (measured via entries to and time spent in the zone) with the help of AnyMaze auto-tracking. Animals were put into the open field box ($50 \times 50 \times 50$ cm) near the middle of the wall and were allowed to explore the arena for 15 min before being returned to their home cage. The inner zone was defined as an area of $20 \times 20$ cm in the centre of the arena. Between each animal, the OF arena

was freed of faeces and urine, cleaned with tap water and quickly dried with a tissue. The test was performed in the morning and was repeated in the afternoon on the same day. The second OF test also served as habituation trial for the spatial object recognition the next day. Data was analysed once in total and the same dataset was also split in three segments of 5 minutes each and the segments were analysed separately.

### 2.7.2 Object recognition test

In the object recognition test, animals either have to remember the nature or the position of an inanimate object over a distinct amount of time. In our setup, animals had to remember the spatial position of an aluminium cube, therefore it was used to assess hippocampus-dependent spatial memory. The OR test was performed with the same arenas used for the OF test the day before, which meant that the animals were already familiar with the setup. Animals were put into the open field box ($50 \times 50 \times 50$ cm) near the middle of the wall and were allowed to explore the arena containing two aluminium cubes for 10 min before being returned to their home cage (Acquisition I). After an inter-trial interval (ITI) of 15 min, the animals were placed into the OF box again under the same conditions for another 10 min (Acquisition II). Following another 30 min ITI, the animals were returned to the OF arena, but this time one of the two aluminium cubes was displaced. The animals were allowed to explore the modified setting for 5 min (Retrieval). Between each animal, the OF arena was freed of faeces and urine, cleaned with tap water and quickly dried with a tissue. In addition, the objects were cleaned with tap water between each animal. As readout parameters we used both the time spent and entries into a 2 cm zone around the objects (via head tracking mode: this means that the head of the animal was used as reference for tracking and not the barycentre of the animal). To normalise for potential individual differences, the discrimination ratio was calculated following equation 1 (exemplarily for the time spent in the zone - entries were calculated in the same fashion). Animals which showed no exploration of the objects during the retrieval trial were excluded from the analysis. This test was performed under low- to medium light conditions (70 lux).

$$\text{discrimination ratio} = \frac{\text{time at displaced object}}{(\text{time at displaced object} + \text{time at undisplaced object})} \tag{1}$$

### 2.7.3 Sociability test

To test social behaviour, approach and avoidance, we performed the sociability test (Moy et al., 2004; O'Tuathaigh et al., 2008). The setup was a modified OF arena. The OF box was split in half with a plastic divider (resulting in a $25 \times 50$ cm arena), which then in turn was split in three additional parts. One central part ($12 \times 25 \times 40$ cm) and two mirrored compartments (interaction chambers; $19 \times 25 \times 40$ cm) on each side, connected via closable doors. For this test, animals were put in the centre zone of the apparatus for 5 min with no access to the interaction chambers for acclimatisation and reduction of initial anxiety. After these 5 minutes, the interaction chambers were opened and the animals were allowed to explore the whole setup for 10 additional minutes. One of the chambers contained a round wire cage with a so-called dummy mouse (toy mouse with the same hair colour as test mouse) while the other chamber held a wire cage with a live mouse (male C57Bl6/N, 2 months of age). Due to the wire cages only limited physical contact

was possible (snout touching), while retaining full sensory contact. The sociability test was taped and rescored manually to ensure that only definite social interaction behaviour was measured. The readout parameter was interaction with the object (dummy or live), which was measured via the number of interactions (number of key presses) as well as the total time of interaction (time key pressed). Again, a discrimination index was calculated similar to the object recognition test, but adapted to fit the parameters measured in the sociability test (see Equation 2). Animals which showed no exploration of the objects were excluded from the analysis. The floor of the apparatus was covered with sawdust bedding, so no cleaning was performed between the tests. Social animals were switched every 4 trials to prevent highly stressful conditions. Before the actual test day, social animals were habituated to the wire cages for at least 3 days (30 minutes per day) and the tested animals were habituated to the whole setup (containing the empty wire cages) the day before testing (for 10 minutes). This test was performed under low-light conditions (3 lux).

$$\text{discrimination ratio} = \frac{\text{interaction with live mouse}}{(\text{interaction with live mouse} + \text{interaction with dummy mouse})} \quad (2)$$

### 2.7.4 Forced swim test & stress response test

As the final test of the behavioural test battery, the forced swim test (FST) was performed. The FST was used at the same time as an acute stressor for the stress response test, therefore the stress response test will also be described here although it is not a behavioural readout. In this test, animals were put in a 2 l beaker filled with tap water (beaker was filled up to 1.5 l to ensure that the animals were not able to feel the bottom of the glass and were not able to reach the rim). The water was filled in the day before testing to reach room temperature (21 °C). The test lasted for 6 minutes and afterwards animals were shortly dried with a towel and returned to their home cages. After 24 minutes (30 minutes after FST onset, response sample) and 84 minutes (90 minutes after onset, recovery sample), animals were blood sampled via tail-cut as described in 2.4. Please note that blood samples for the basal timepoint were not taken directly before the test to not influence the FST, but were taken 4 - 5 days later under basal conditions in the morning. The FST was taped and scored manually, classifying the behaviour into either struggling, swimming or floating (both number and total time of each aspect served as readout here).

## 2.8 Brain processing

Frozen brains were cut in the coronal plane in a cryostat at -20 °C with a strength of 20 $\mu$m. Brains were cut at the level of the dorsal hippocampus and slices were thaw-mounted on either Superfrost Plus slides for *in-situ* hybridisation or on special membrane-coated slides for laser-microdissection. Membrane-coated slides were pre-treated by 2 hours of dry heat at 180 °C followed by 30 minutes of UV-light exposure at 254 nm to increase adhesive properties. For immunohistochemistry, perfused brains were cut in the coronal plane at -16 °C with a strength of 25 $\mu$m and were mounted on uncoated slides.

## 2.9 Laser-microdissection

To acquire RNA samples from the CA1 and DG region of the hippocampus, the laser-microdissection and pressure-catapulting (LMPC) technique was used. In this technique, brain slices mounted on membrane-coated slides are cut by a focused laser beam and catapulted via a short laser pulse below the actual cutting plane. LMPC is characterised by high precision, making it possible to even isolate single cells. As the cells are catapulted against gravity, contamination by floating particles is minimised. Furthermore, no physical contact with the sample is needed, due to the catapulting step. These characteristics ensure that LMPC is a very precise and clean procedure. Samples were transported on dry ice and put into the -20 °C freezer on site. After a short acclimatisation phase of approximately 5 minutes, slides were air-dried for 4 minutes followed by cresyl violet staining. The staining solutions were prepared fresh before the experiment and cresyl violet was autoclaved twice to ensure RNase free environment. Slides were dipped in 70 % ethanol for 1 minute then in $H_2O_{DEPC}$ for 5 minutes and finally for 90 seconds in cresyl violet solution. Afterwards, the slide was dipped 12 times in each of the solutions in the following order: 70 % ethanol, 95 % ethanol and 100 % ethanol. The samples were allowed to dry for 4 minutes under a fume hood.
Prepared slides were inserted in the LMPC microscope (P.A.L.M. Microlaser Technologies, Bernried, Germany) and desired regions for extraction were marked by hand with the software. The following settings, with minor modifications for the single slides, were used: RoboLPC, energy 66, focus 78, speed 100. For each captured area (CA1, DG) a new sticky cap tube was used. After successful capture of one area, samples were immediately dissolved in 100 $\mu$l TRIZOL and frozen on dry ice. One slide contained 8 brain sections and capture of one area took approximately 90 minutes. The marked region can be found in Figure 8 for the DG exemplarily. Samples were frozen on dry ice and stored at -80 °C until RNA isolation.

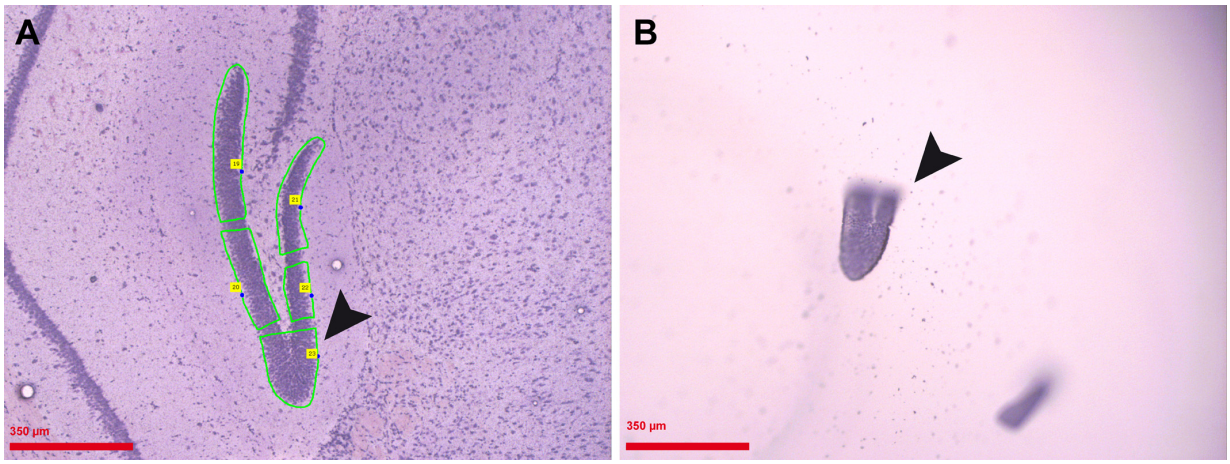


Figure 8: Region-selective dissection using LMPC. **(A)** The DG region of the hippocampus was marked for extraction. **(B)** The dissected pieces were found in the sticky cap of the collection tube.

## 2.10 RNA isolation

### 2.10.1 Whole hippocampi

RNA from the whole hippocampi was isolated using the TRIZOL reagent (Invitrogen) with a modified protocol. In short, 1 ml of cool TRIZOL was added to the frozen whole hippocampi, the mixture was homogenised with a tissue homogeniser (VWR international) and was allowed to incubate at room temperature (RT) for 5 minutes. Subsequently, 100 $\mu$l 1-Bromo-3-chloropropane (BCP, replacing the chloroform) were added, thoroughly mixed and incubated for 15 minutes at RT. After centrifugation at 4 °C with 12000 rpm for 15 minutes, the lipophobic upper phase containing the RNA was transferred to a clean microcentrifuge tube. For alcohol precipitation of the RNA, 500 $\mu$l of iso-propanol were added, the solution was mixed and incubated at RT for 10 minutes. The sample then was centrifuged (10 min, 12000 rpm, 4 °C) and the supernatant was carefully removed and discarded. Further washing steps followed: 250 $\mu$l of 80 % ethanol were added, the sample was centrifuged (10 min, 12000 rpm, 4 °C) and the supernatant was carefully removed and discarded. This was repeated once. After the final removal of the supernatant, the pellet was air dried for about 4 minutes and dissolved in 50 $\mu$l of $H_2O_{DEPC}$.

### 2.10.2 Laser-microdissection

The isolation was done similar in general, although with smaller amounts of reagents and some additional steps. Samples were stored in 100 $\mu$l TRIZOL. To this solution, 20 $\mu$l of chloroform were added to denature proteins and cell components. After 15 seconds of vortexing and 3 minutes of incubation at RT, the sample was centrifuged (15 min, 13000 rpm, 4 °C). The upper phenol phase, including the RNA, was transferred to a new tube, 5 $\mu$l of linear acrylamide (as coprecipitant because of low RNA content) and 50 $\mu$l iso-propanol were added, the sample was vortexed for 15 seconds and incubated for 10 minutes at RT for alcohol precipitation of the RNA. The sample then was centrifuged (10 min, 13000 rpm, 4 °C) and the supernatant was carefully removed and discarded. Further washing steps followed: 500 $\mu$l of 75 % ethanol were added, the sample was centrifuged (5 min, 9000 rpm, 4 °C) and the supernatant was carefully removed and discarded. This was repeated once. After the final removal of the supernatant, the pellet was air dried for about 10 minutes under a fume hood and dissolved in 12 $\mu$l of $H_2O_{DEPC}$. From this solution 1 $\mu$l was transferred to a new tube for quality control of the RNA sample. The remaining 11 $\mu$l were stored at -80 °C until RNA amplification.

## 2.11 RNA quality control

RNA quality and integrity is a vital facor for all down-stream applications. Therefore, micro gel-electrophoresis was conducted with the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Depending on the amount of RNA, RNA Pico LabChips or RNA Nano LabChips (Agilent Technologies, Inc., Waldbronn, Germany) were used. This method has a detection limit of 50-5000 pg / $\mu$l for total RNA. The measurements were done following the Agilent protocol. The bioanalyzer investigates, among other parameters, the 28S:18S ratio of the RNA bands, which should lie about 2.0. With this ratio and other features, the Agilent software

calculates the RNA integrity number (RIN) of the sample, which can be used to easily assess the quality of the present RNA (Schroeder et al., 2006).

## 2.12 RNA amplification

For amplification of the RNA samples for the microarray, the commercially available Illumina TotalPrep RNA Amplification Kit (Ambion Inc. Austin, Texas, USA) was used with minor modifications. In short, RNA samples are the templates for reverse transcription, creating cDNA. Thereafter, the second strand of the cDNA is synthesised by DNA polymerase while degrading remaining RNA using RNase H. Some purification steps utilising filter cartridges follow. The main step of the procedure is the *in vitro* transcription, creating multiple copies of cRNA, which are already biotinylated for binding on the microarray chips. After a final purification step, samples were stored at -80 °C. Before the microarray analysis, samples were concentrated to a final concentration of 1.5 $\mu$g RNA in 10 $\mu$l volume by evaporation using a vacuum centrifuge.

## 2.13 cDNA Transcription

The transcription of RNA to cDNA for the quantitave reverse-transcription polymerase chain reaction (qRT-PCR) was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the standard protocol. In short, a master mix was prepared on ice as described in table 1. To this master mix, 10 $\mu$l of total RNA were added, carefully mixed by

Table 1: Composition of the reverse transcription master mix.

| **component** | **volume / $\mu$l** |
|---|---|
| 10x RT buffer | 2 |
| 25x dNTP Mix (100mM) | 0.8 |
| 10x Random primers | 2 |
| MultiScribe Reverse Transkriptase | 1 |
| RNase Inhibitor | 1 |
| Nuclease-free water | 3.2 |
| **Total (per reaction)** | **10** |

pipetting and briefly centrifuged. Thereafter, samples were loaded into a thermal cycler, using the program defined in table 2. Finished product was stored at -80 °C until further processing.

Table 2: Program of the thermal cycler

| **step** | **temperature /°C** | **time /min** |
|---|---|---|
| Pre-incubtation | 25 | 10 |
| Amplification | 37 | 120 |
| Termination | 85 | 5 |
| Cooling | 4 | $\infty$ |

## 2.14 Microarray analysis

For transcriptome analysis in experiment 1a, MouseWG-6 v1.1 BeadChips (Illumina Inc.) were used, allowing the identification of about 48000 gene-sequences (50mer oligotides) with about 30 fold redundancy. In experiment 2b, the then available MouseWG-6 v2.0 BeadChips (Illumina Inc.) were used while experiment 2a was performed on MouseRef-8 v1.0 chips (Illumina Inc.). Preparing the samples for the microarray chips was done following the Illumina protocols. This method is based on measuring fluorescence of sites with specific oligomeric sequences. In short, the RNA samples are allowed to hybridize with the defined sequences on the chip and afterwards, various washing steps and a final blocking step minimize unspecific signal. For detection of the probes, the RNA, which was biotinylated during the amplification step, is incubated with streptavidin-Cy3, as streptavidin forms tight bonds with biotin molecules and Cy-3 is a fluorescent dye which can be detected in the Illumina BeadArray Reader (Illumina Inc.). Chips were analysed using the BEADARRAY package (www.bioconductor.org) with additional required packages. For experiment 1a and 2b, only biological replicates were used (experiment 1a: $n = 9$ per group and region; experiment 2a: $n = 5$ per group; experiment 2b: $n = 12$ per group). In experiment 2a, samples were pooled for each of the investigated regions and quadruple technical replicates were investigated on the chips.

## 2.15 Quantitave RT-PCR

For the analysis of gene expression differences in the samples, we used qRT-PCR. The RT in the name stands for reverse transcription, not for real-time as commonly believed, meaning that as a substrate complimentary DNA (cDNA) instead of RNA is used. The fact that it is also in real time is given by the use of the quantitative prefix. The difference between real-time and conventional PCR is that while in the conventional PCR, the end product is detected, the real-time PCR is monitored after each cycle. Therefore, quantification of the original sample requires extensive post-PCR handling steps (Guatelli et al., 1989), whereas the amplification curve can easily be extrapolated in case of the real-time PCR.
The PCR can be summarised in three principal steps in repeating cycles: (1) denaturation of the double-stranded DNA (dsDNA) at high temperatures (95 °C in our case), (2) primer annealing at moderate temperatures and (3) elongation at moderate temperatures. Our protocol, given in Table 3, has some additions due to the use of the LightCycler 2.0 (Roche) and the QuantiFast SYBR Green PCR Kit (Qiagen). The preincubation step is necessary as the Qiagen kit works with the HotStarTaq Plus DNA polymerase. The latter, named taq after its host, the bacterium *Thermus aquaticus*, is in an inactive state and needs to be activated by a 5 minute incubation at 95 °C. Furthermore, the kit is optimised for short cycling times by a special buffer system and molecules that increase DNA polymerase affinity, so that steps (2) and (3) can be combined in a single step. The final step is a melting curve analysis. The kit uses SYBR green I as a fluorescent dye, which emits a fluorescent signal only when bound to dsDNA (excitation at 494 nm, emission at 521 nm), grouping it to the non-specific, DNA-binding fluorophores. Therefore, specificity of the amplified product needs to be secured, due to the risk of primer-dimerisation which confounds the results. In a melting point analysis, the temperature is slowly increased, while the fluorescent output is constantly measured (in our case from 50 to 95 °C). Fluorescence decreases slightly at

higher temperatures, but valid products of a specific length break apart at a specific temperature ($T_M$) causing a steep decrease in fluorescence. The same holds true for primer dimers, however in that case the melting temperature is lower, as they are shorter. To easily detect the melting points, the first derivative of the fluorescence output is calculated ($\frac{dF}{dT}$) and plotted against the temperature. The resulting peaks show the melting temperatures (see Figure 9).

Table 3: Program of the thermal cycler

| step | cycles | target /°C | hold /s | slope /°C/s | aquisition mode |
|---|---|---|---|---|---|
| preincubation | 1 | 95 | 300 | 20 | none |
| amplification | 40 | | | | |
| denaturation | | 95 | 10 | 20 | none |
| annealing / elongation | | 60 | 30 | | single |
| melting curve | 1 | 95 | 0 | 20 | none |
| | | 50 | 10 | 20 | none |
| | | 95 | 0 | 0.1 | continuous |
| cooling | 1 | 42 | 30 | 20 | none |

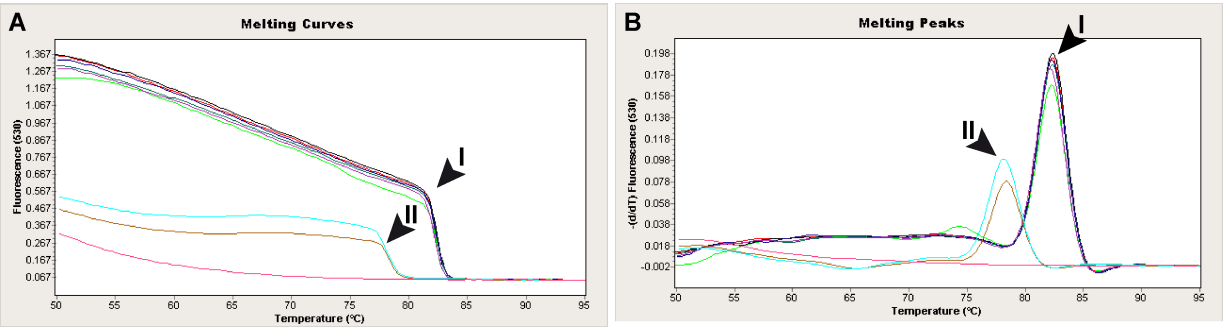


Figure 9: Melting curve analysis. **(A)** Melting curves show a steady decrease until the product (I) and the primer-dimer (II) denature. **(B)** The change in fluorescence over time plotted against the temperature makes it easy to distinguish temperatures with high change in fluorescence. Black arrowhead point at the denaturation curves/peaks of product (I) and primer-dimer (II). In addition, a water control with no signal is shown as negative control (pink line).

Reactions were prepared in LightCycler Capillaries (Roche) following the official Qiagen protocol. In short, a mastermix for all samples in one run was prepared on ice as depicted in Table 4. Master mix was pipetted into the capillaries, 2 $\mu$l of sample were added and the capillary was capped. The latter step was done at RT and took about 30 minutes for a full carousel. The carousel was centrifuged, placed in the apparatus and the run was started within 5 minutes following the protocol advised by Qiagen (described before in Table 3).

For analysis of the data, the LightCycler 4 Software (Roche) was used. In a first step, the melting peaks were investigated using $T_M$ calling routine. Expression profiles were determined using the Qualitative detection protocol. We used two different housekeeping genes known from the literature: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a gene coding for an enzyme from glycolysis as well as Hypoxanthine-guanine phosphoribosyltransferase (HPRT), which acts mainly in purine recycling (Boda et al., 2009). For the determination of relative expression, the

Table 4: Composition of the RT-PCR master mix

| component | volume / $\mu$l |
|---|---|
| Nuclease-free water | 2 |
| Primer Mix (10 $\mu$M) | 1 |
| SYBR green Master Mix | 5 |
| **Total (per reaction)** | **8** |

comparative $C_T$ method was used, summarised in equation 3.

$$\text{relative expression} = 2^{-\Delta C_T} \quad \text{with} \quad \Delta C_T = C_T[\text{gene of interest}] - C_T[\text{housekeeer}] \tag{3}$$

All measurements were normalised to controls (controls being 100% or simply 1) or resilient animals (resilient animals being 100% or simply 1) to provide relative expression levels. Relative expression was calculated for normalisation with GAPDH and HPRT separately.

## 2.16 *In-situ* hybridisation

*In-situ* hybridisation (ISH) was performed to validate the results from the qRT-PCR via a different technique and gain information about the spatial distribution of selected transcripts. In this technique, a radioactively labelled RNA sequence complementary to the investigated RNA sequence is bound to the latter and allows for semi-quantitative measures of gene expression. Therefore, frozen brains were mounted in a cryostat microtome and 20 $\mu$m sections were cut in the coronal plane at a temperature of -16 °C. Samples were allowed to acclimate to the cryostat temperature for several minutes before cutting. Sections were thaw-mounted on superfrost slides, dried shortly and stored at -80 C.
In-situ hybridisation using $^{35}$S-UTP labelled ribonucleotide probes was performed as described previously (Schmidt et al., 2007). Briefly, sections were fixed in 4 % PFA and acetylated in 0.25 % acetic anhydride. Afterwards, slides were dehydrated in ascending concentrations of alcohol. On the dried slides, hybridization buffer containing between 1.5 and 2.0 x $10^6$ counts per minute of $^{35}$S-labelled riboprobe was applied with a volume of 100 $\mu$l per slide. Brain sections were coverslipped and incubated overnight at 55 °C. The next day, sections were rinsed and incubated with RNAse A. Finally, sections were desalted and dehydrated.
Radioactively labelled slides were apposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and were developed using an automated developing machine. Films were digitised and relative expression was measured by optical densitometry using the ImageJ software (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). For each animal, the mean of the bilateral structures of two sections was calculated if applicable, deducting the background from the value. Background signal was measured in a structure not expressing the gene of interest, which was the *stratum radiatum* in the case of the hippocampus. Values were normalised to controls similar to the qRT-PCR.

## 2.17 Protein isolation

Proteins were isolated from frozen hippocampal tissues. In a first step, a lysis buffer master mix was created by the addition of proteinase-inhibitor cocktail (1 $\mu$l per 1 ml lysis buffer). Then 200 $\mu$l of this mix were given to each sample and the samples were then homogenised first with a syringe with a 30-gauge needle, then a second time with a 27-gauge needle. Between samples needles and syringes were thoroughly cleaned. Homogenised samples were centrifuged for 30 min at 4 °C at 12000 rpm. Supernatant was transferred to a clean tube. Samples were then diluted 1:20 and the protein concentration was measured in duplicate using the DC Protein Assay kit from Bio-Rad (modified Lowry-assay) following the supplied protocol. Average values served as a basis to bring the samples to a final dilution of 40 $\frac{\mu g}{\mu l}$). Final samples were heated to 95 °C, placed on ice shortly and stored at -20 °C until further processing.

## 2.18 Western blot

Western blotting was performed to show the knockdown efficacy of the viral construct. Therefore, samples were loaded onto a NuPage 10 % Bis-Tris gel (Invitrogen). As a marker, the PageRuler Plus (Fermentas) was used. The gel was run at 90 V for 150 min in MES SDS running buffer. The finished Gel was trimmed and incubated for 10 min in transfer buffer together with the filter papers and membranes. The gel was then blotted for 40 min at 200 mA in a semidry blotter. For a quick check if protein transfer worked, the membrane was stained with ponceau S and afterwards rinsed with tap water. The membrane was then transferred to a small plastic box and was blocked for 30 min in 5 % bovine serum albumin (BSA) at RT. In the next step, the membrane was incubated overnight at 4 °C in a mixture of 1 % BSA with a 1:1000 dilution of the Arc antibody (ab23382; Abcam). The next day, 3 steps of washing (3 × 8 min in TBST) were done, followed by the second AB, DAKO polyclonal goat $\alpha$ rabbit Immunoglobulins / HRP (Abcam), was added with a dilution of 1:2000 in 1 % BSA and incubated for 2 h. Afterwards, 3 additional washing steps (3 × 8 min in TBST) were performed. All steps were performed on the shaker. The membrane was then incubated for 1 min in 1 ml ECL Amershan solution. The membrane was put in a film cassette and was exposed to a film for 5 sec. Final films were developed by hand.

Arc expression was normalised to tubulin, therefore the membrane was stripped by first rinsing it shortly with TBST and afterwards incubating 3 times (3 × 5min) with mild stripping buffer (Abcam). The membrane was then washed twice with TBST (2 × 5 min). Thereafter, the membrane was blocked with 5 % skimmed milk for 60 after which the first antibody was applied in 1 % skimmed milk (Abcam ab6160 tubulin goat polyclonal; 1:5000) and incubated overnight. The next day, 3 steps of washing (3 × 8 min in TBST) were done, followed by the second AB (Anti-rat IgG, HRP-linked Antibody #7077, Cell Signalling) was added with a dilution of 1:2000 in 1 % BSA and incubated for 2 h. Afterwards, 3 additional washing steps (3 × 8 min in TBST) were performed. All steps were performed on the shaker. The membrane was then incubated for 1 min in 1 ml ECL Amershan solution. The membrane was put in a film cassette and was exposed to a film for 30 sec. Final films were developed by hand. Films were digitised with a scanner and images were analysed using the Gels routine included in the ImageJ software (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda,

MD). The ratio of Arc to tubulin was calculated to normalise for differences in protein content.

### 2.19 Immunofluorescence

Immunofluorescence was performed on slices from perfused brains. All steps were performed at RT except when stated otherwise. Slices were carefully removed from slides in a petri dish filled with 0.1 M PB solution and transferred to a 12-well plate pre-loaded with 0.1 PB. Slices were then transferred to another 12-well plate filled with PB-mix (0.1 PB with 0.3 % Triton X-100) using a brush and washed while shaking for 10 min. This washing step was repeated twice (3 steps washing in total). A master mix was created fot the pre-incubation step using 1 ml of PB mix with 10 $\mu$l of donkey serum per sample. Of this solution, 1 ml was pipetted into each well, the sections were transferred and incubated for 1 h while shaking. For the next step, a master mix of 1 ml PB mix with 10 $\mu$l donkey serum and a final concentration of 1:700 of Arc antibody (ab23382; Abcam) was created. Slices were transferred into wells filled with 1 ml of the master mix and incubated over night at 4 °C on a shaker.
The next day, slices were washed 3 times with PB mix for 10 min each while shaking at RT. The master mix for the second AB was made of 1 ml PB mix, 10 $\mu$l donkey serum and 2 $\mu$l Alexa Fluor 488 donkey $\alpha$ rabbit (1:500). Slices were transferred to wells with 1 ml of the master mix and were incubated in the dark for 3 h on the shaker. From this step on, all following steps were performed in the dark or under no direct light. Slices were then washed 3 times in 0.1 PB (no Triton X-100) for 10 min each on the shaker. Finally, slices were transferred to a petri dish containing distilled water, mounted on uncoated slides and flattened with a fine brush. Slices were dried for 30 min, mounting medium containing DAPI staining (Vectashield hard set) was added and the slides were coverslipped. Finally, coverslips were fixed in place with small drops of natural balsam.

### 2.20 Knockdown virus of Arc

The virus for the knockdown experiment was generated and purified by GeneDetect (New Zealand) on the basis of an adeno-associated virus. The viral constructs either included a shRNA sequence against Arc (shARC) or a scrambled construct (SCR). Both types did include a GFP cassette.

### 2.21 Stereotactic surgery

Before the actual procedure, animals were quickly anaesthesised using isoflurane. Thereafter, animals were inserted and fixed in the stereotactic system under 4 % isoflurane anaesthesia. During the whole surgery, animals were placed on a warming bed to prevent extensive chilling of the animals. Animals were then injected with 200 $\mu$l of metacam (concentration: 62.5 $\frac{\mu g}{ml}$) for analgesia. Before the incision of the scalp, the head of the animals was freed of hair, treated with iodine, the eyes were covered with eye ointment and the status of anaesthesia was confirmed via a pinch between the toes. After the incision, the subcutaneous tissue was moved aside and the aneasthesia was reduced to roughly 1.5 % isoflurane (variable and depending on the respiration of the individual animal; animals were brought to roughly 30 to 35 respiratory cycles per minute).

The coordinates of bregma and lambda were determined under the binocular. The holes for bilateral injections were drilled with a 0.6 mm drillhead using the following coordinated from bregma: anterior/posterior + 1.9 mm; lateral ± 1.4 mm; ventral + 1.4 mm for experiment 3b (experiment 3a used the following coordinates: anterior/posterior + 1.9 mm; lateral ± 2 mm; ventral + 1.9 mm). Canulas for injection of the virus were pulled from glass canula blanks. The tip of the canula was cut off, the canula was filled with 1 $\mu$l of virus (shARC or SCR) and the canula was inserted into the brain. The injection was performed slowly (in the best case around 15 minutes) but varied slightly between animals. After the injection, 5 min were allowed to go past to prevent large spread of the virus. Finally, the scalp of the animals was sutured with 3 or 4 stitches, depending on the size of the incision. Animals were then transported back to their home cages for waking and were supplied with tap water containing low doses of metacam (concentration: 2.5 $\frac{\mu g}{ml}$) for postoperative analgesia for 1 week.

## 2.22 Statistical analyses

The statistical analyses were performed using the SPSS Statistics 17 package (Polar Engineering and Consulting). For comparisons between two values of a given variable (e.g. control vs stress), the two-tailed, independent samples Student's t-test was used. For variables with more than two values (e.g. control vs restraint stress vs food deprivation), One-way ANOVA was performed followed by Bonferroni *post-hoc* testing. For more complex datasets ($2 \times 2$ design; e.g. experiment 3b), univariate ANOVA was used. For datasets with multiple measurements of one variable at different timepoints (e.g. body weight over the course of the experiment), repeated-measures ANOVA was used. Correlations were done with a two-tailed, bivariate Pearsson's correlation analysis. The level of significance was set to $\alpha = 0.05$ for all tests, a trend was recognised at $\alpha = 0.10$. Values outside the 95 % confidence interval (CI) were defined as statistical outliers and excluded from the analyses. For the OR test and the sociability test in experiment 3b, 95 % CIs were calculated to investigate the difference to chance levels.
All figures were created using the SigmaPlot 10 software, with the help of Adobe Photoshop CS3 as well as Adobe Illustrator CS3.

# 3 Results

## 3.1 Experiment 1: Long-term effects of stress exposure on gene transcription in the hippocampus

### 3.1.1 Experiment 1a: Long-term effects following chronic social stress exposure

#### *3.1.1.1 Validity of the stress paradigm*

Parts of the data here (the results from the whole cohort) were already presented in the authors diploma thesis. They are reviewed here again to highlight the functionality of the stress paradigm. We found elevated levels of corticosterone 5 weeks after the stress exposure in stressed animals ($t_{45} = 2.824$, $p < 0.01$) as well as a decrease in thymus weight ($t_{42} = 3.264$, $p < 0.01$). Stressed animals also showed a trend for enlarged adrenals ($t_{43} = 1.685$, $p = 0.099$). In contrast, the stress animals that were used for the microarray study (a randomly selected subset of the animals described before), showed no significant difference in CORT levels or thymus weight, but significantly increased adrenals ($t_{21} = 2.877$, $p < 0.01$). These results are depicted in Figure 10.

#### *3.1.1.2 RNA quality*

We checked the qualtiy of the RNA in the Agilent BioAnaylyzer. The RIN for the samples of the control animals was 5.6 (Range: 4.3 - 6.2) for the CA1 and 5.3 (Range: 3.3 - 6.3) for the DG. In stressed animals the RNA quality was 4.6 (Range: 2.5 - 6.3) and 5.3. (Range: 2.3 - 6.4) for the CA1 and DG respectively. These data were also presented in the authors diploma thesis and are reviewed here, as they are important for the discussion of the results.

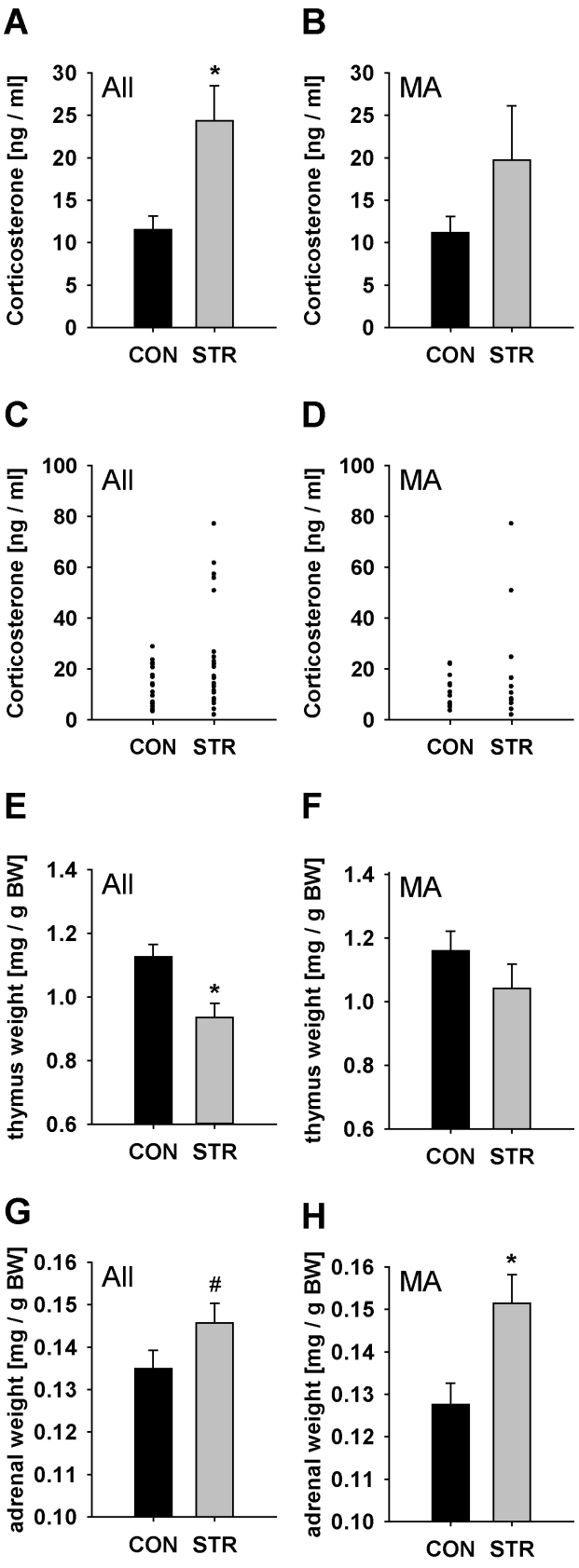


Figure 10: Overview of the effects of the chronic social stress exposure after 5 weeks of recovery and comparison between the whole cohort and the animals selected for the microarray study (discovery sample). Results from the whole cohort (All) are depicted on the left, while the results from the animals from the microarray (MA) are shown on the right. **(A)** Basal morning corticosterone levels of the whole cohort. Stressed animals showed significantly higher CORT levels. **(B)** Corticosterone levels in the selected animals did not differ between control and stress animals. **(C)** Scatter plot of the CORT levels from all animals. **(D)** Scatter plot for the CORT levels of the selected animals. **(E)** Relative thymus weight for the whole cohort. Stressed animals showed a significant involution of the thymus. **(F)** Animals selected for the microarray did not differ in relative thymus weight. **(G)** Relative adrenal weight for all animals. Stressed animals showed a trend for enlarged adrenal glands. **(H)** Stressed animals of the selected subgroup showed significantly enlarged adrenals when compared to their control counterparts. Data are given in mean + SEM; CON control; STR stress; All all animals from the whole cohort; MA only the subgroup of animals that were randomly selected for the microarray experiment; * significantly different to control animals $p < 0.05$; # different (trend) to control animals $p < 0.1$.

#### *3.1.1.3 Microarray analysis*

Analysis of the Illumina microarray revealed 40 regulated genes in the CA1 region, while no gene was significantly regulated in the DG region after correction for multiple testing. Significantly regulated genes from the CA1 region are depicted in Table 5. In a next step, the sequences spotted on the Illumina chips were double-checked. Unfortunately, the spotted sequences were of minor quality. Out of the 38 investigated sequences, 14 genes (36.8 %) were either not specific for the respective gene or yielded no BLAST results, while the sequences of 12 genes (31.6 %) hybridized with intronic sequences, leaving us with 12 genes (31.6 %) with a useful targeting sequence.

Table 5: Summary of significantly regulated genes in the hippocampal CA1 region 5 weeks after stress. Genes are ordered by adjusted p-value. Fold change is normalised to controls (a fold change of 2 means levels in stressed animals are double the levels from controls).

| number | symbol | fold change | adj. p-value | notes |
|---|---|---|---|---|
| 1 | Tcf4 | 2.09 | 0.011 | intronic sequence |
| 2 | Dnm1l | 1.98 | 0.014 | intronic sequence |
| 3 | Palm2 | 1.73 | 0.016 | ok |
| 4 | Bat2d | 2.10 | 0.027 | ok |
| 5 | A530025E09Rik | 1.86 | 0.029 | sequence not specific |
| 6 | LOC386330 | 1.95 | 0.029 | sequence not specific |
| 7 | LOC386101 | 2.39 | 0.029 | sequence not specific |
| 8 | A830055N07Rik | 2.04 | 0.029 | sequence not specific |
| 9 | Mtap1a | 2.12 | 0.030 | ok |
| 10 | Neto1 | 2.12 | 0.030 | checked by in-situ |
| 11 | Bat2d | 2.01 | 0.030 | ok |
| 12 | LOC382128 | 1.93 | 0.030 | sequence not specific |
| 13 | Fam178a | 1.84 | 0.030 | ok |
| 14 | Ankhd1 | 1.92 | 0.032 | sequence not specific |
| 15 | Ptprt | 1.82 | 0.032 | ok |
| 16 | Etfdh | 1.79 | 0.035 | intronic sequence |
| 17 | A130039H17Rik | 1.49 | 0.035 | sequence not in the gene |
| 18 | Snrp48 | 1.62 | 0.035 | intronic sequence |
| 19 | LOC386199 | 2.40 | 0.038 | sequence not specific |
| 20 | AK122498 | 1.92 | 0.038 | sequence not in the gene |
| 21 | Csnk1g1 | 2.03 | 0.038 | ok |
| 22 | Fancm | 1.81 | 0.038 | ok |
| 23 | Akap8 | 1.91 | 0.038 | intronic sequence |
| 24 | KIAA0513 | 1.81 | 0.039 | ok |
| 25 | Dlx6os2 | 1.56 | 0.040 | sequence not in the gene |
| 26 | Zzef1 | 1.50 | 0.040 | ok |
| 27 | Dopey1 | 1.73 | 0.040 | sequence not specific |
| 28 | Dopey1 | 1.78 | 0.040 | sequence not specific |
| 29 | Iffo1 | 1.60 | 0.040 | intronic sequence |
| 30 | Cacna2d1 | 1.75 | 0.040 | intronic sequence |
| 31 | Chd1 | 1.92 | 0.040 | ok |
| 32 | Trip12 | 1.80 | 0.040 | intronic sequence |
| 33 | D130017N08Rik | 2.03 | 0.040 | sequence not in the gene |
| 34 | Idh3b | 1.71 | 0.041 | intronic sequence |
| 35 | 2900060F21Rik | 1.55 | 0.041 | sequence not specific |
| 36 | Dnlac1 | 2.39 | 0.042 | ok |
| 37 | Rufy3 | 1.73 | 0.042 | intronic sequence |
| 38 | LOC386164 | 2.12 | 0.048 | sequence not specific |
| 39 | Gnaq | 1.94 | 0.049 | intronic sequence |
| 40 | Robo1 | 1.72 | 0.049 | intronic sequence |

### 3.1.2 Validation via qRT-PCR in the replication sample

#### *3.1.2.1 Validity of the stress paradigm*

The stress paradigm was performed in the same way as in the discovery sample with samples from experiment 2b. Here, we found significantly elevated levels of CORT 5 weeks after the stress exposure in stressed animals ($t_{94.565} = 3.622$, $p < 0.01$). We found no difference in thymus gland weight while the adrenal gland weight was higher in stressed animals, although not significantly (trend, $t_{113} = 1.907$, $p = 0.059$). In the subgroup selected for the qRT-PCR validation, we also found increased levels of basal morning CORT in stressed animals ($t_{14.271} = 5.838$, $p < 0.01$), whereas both adrenal weight and thymus weight were not significantly different to controls. The data is shown in Figure 11

#### *3.1.2.2 RNA quality*

No data about RNA quality is available from this experiment.

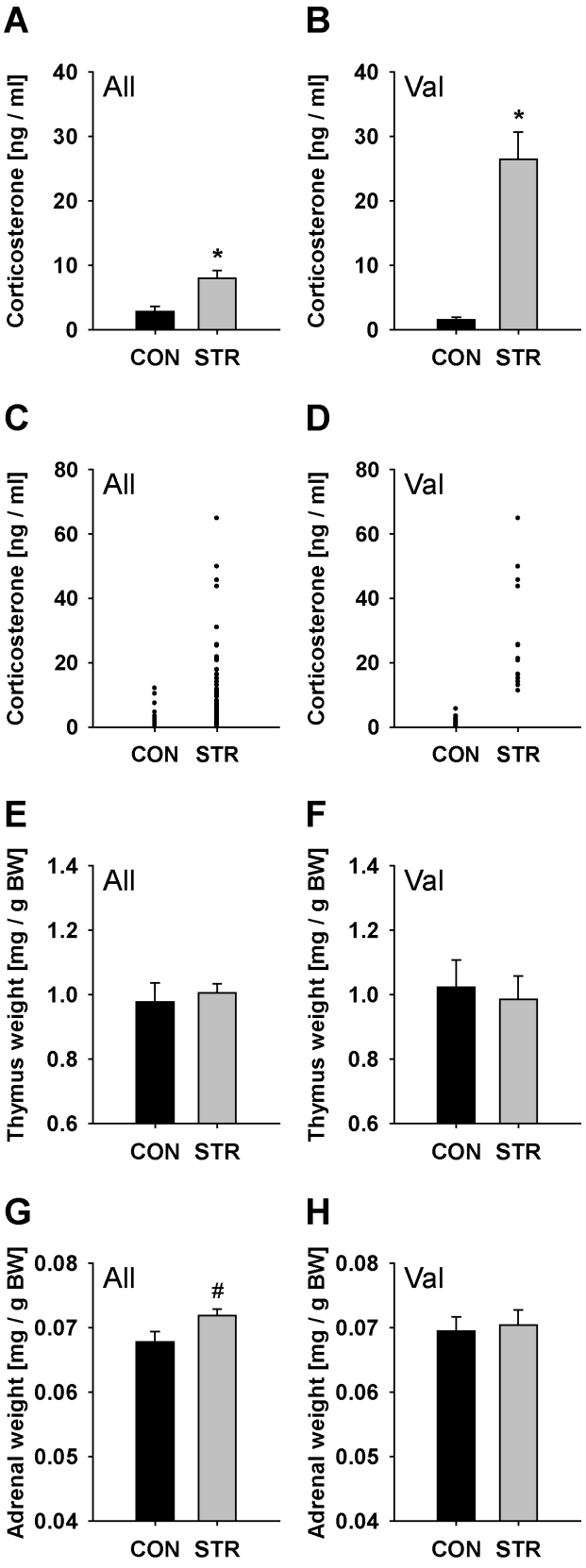


Figure 11: Overview of the effects of the chronic social stress exposure after 5 weeks of recovery and comparison between the whole cohort and the animals selected for the qRT-PCR validation (replication sample). Results from the whole cohort (All) are depicted on the left, while the results from the animals from the validation (Val) are shown on the right. **(A)** Basal morning corticosterone levels of the whole cohort. Stressed animals showed significantly higher CORT levels. **(B)** Corticosterone levels in the selected animals were also higher in the stress condition. **(C)** Scatter plot of the CORT levels from all animals. **(D)** Scatter plot for the CORT levels of the selected animals. **(E)** Relative thymus weight for the whole cohort. **(F)** Animals selected for the validation did not differ in relative thymus weight. **(G)** Relative adrenal weight for all animals. Stressed animals showed a trend for enlarged adrenal glands. **(H)** No difference was found for stressed animals of the selected subgroup. Data are given in mean + SEM; CON control; STR stress; All all animals from the whole cohort; Val only the subgroup of animals that were selected for the qRT-PCR experiment; * significantly different to control animals $p < 0.05$; # different (trend) to control animals $p < 0.1$.

#### *3.1.2.3 Quality control of the qRT-PCR*

Nevertheless, all 23 genes with a specific sequence were investigated via qRT-PCR, including all genes that were only represented with an intronic sequence. For the latter, intron-spanning primer pairs were designed and used for qRT-PCR. In a first step, we investigated HPRT as housekeeping gene in duplicate. Both runs showed no significant difference between control and stress animals, supporting its function as housekeeping gene (see Figures 12 A + B). As a second housekeeping gene, GAPDH was chosen and also revealed no difference between the groups (see Figure 12 C). We found a high degree of correlation ($r = 0.827$, $p < 0.001$, see Figure 12 D) between both HPRT runs, which gave us confidence in the high precision of the Roche LightCycler. In addition, the two different housekeeping genes showed a high correlation ($r = 0.647$, $p < 0.001$, see Figure 12 E).

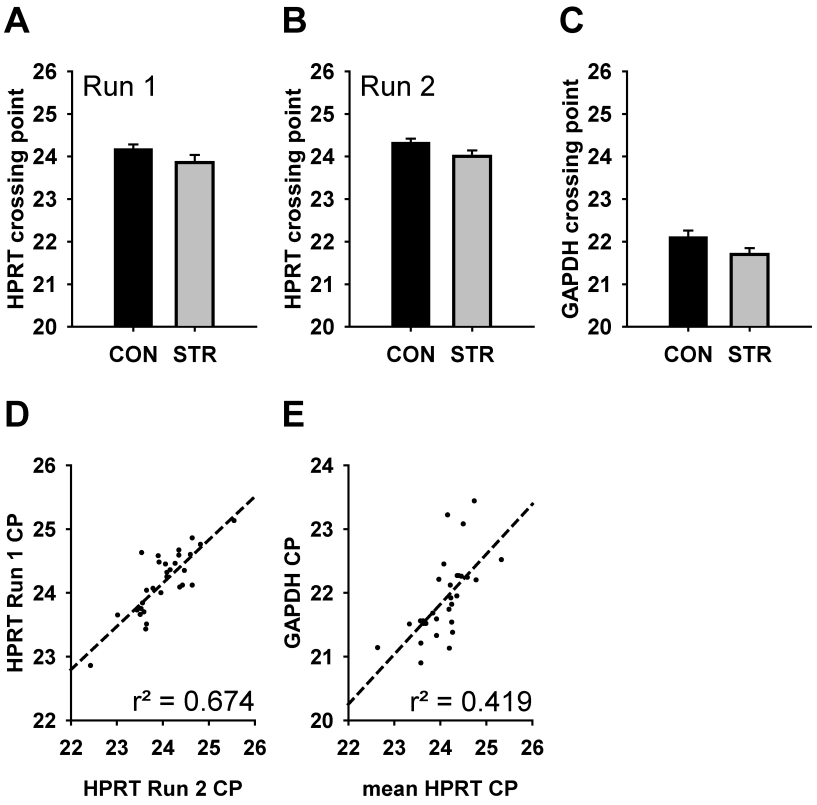


Figure 12: Comparison of the housekeeping genes HPRT and GAPDH. Absolute HPRT expression of **(A)** run 1 and **(B)** run 2 did not differ between control and stress animals. **(C)** The same was true for GAPDH. **(D)** We observed a strong correlation within HPRT runs and **(E)** also found good correlation between the different housekeeping genes. Data are given in mean + SEM.

Next, we looked at known stress-regulated genes in the qRT-PCR samples as a positive control. We found a downregulation of mRNA for both the GR ($t_{27} = 2.700$, $p < 0.05$) and the MR ($t_{27} = 2.258$, $p < 0.05$) in the previously stressed animals, as depicted in Figures 13 A + B, respectively.

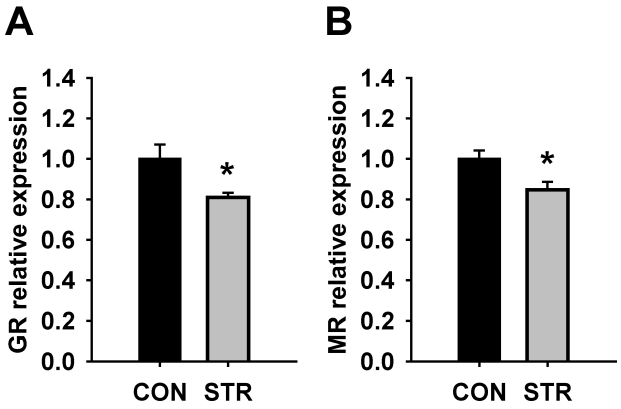


Figure 13: Relative expression of genes for positive control. **(A)** GR mRNA expression is significantly decreased in stressed animals. **(B)** The same holds true for the MR. Data are given in mean + SEM; * significantly different to control animals $p < 0.05$.

#### *3.1.2.4 Results from the qRT-PCR*

We investigated mRNA expression with normalisation to either HPRT, GAPDH or a combination of both (see Table 6). For the corresponding figure (Figure 14), the values from the combined approach were used. We found a significant upregulation of Mtap1 mRNA, although only in the combined approach ($t_{27} = 2.105$, $p < 0.05$). A significant downregulation was found for Akap8 ($t_{27} = 2.072$, $p < 0.05$), Iffo1 ($t_{28} = 3.079$, $p < 0.01$) and Rufy3 ($t_{17} = 2.537$, $p < 0.05$) while a trend was found for Fancm ($t_{28} = 1.764$, $p = 0.089$).

Finally, Table 7 compares the findings from the microarray with the results from the qRT-PCR. Here we saw that except in the case of Mtap1a, all regulated genes showed a different direction of regulation in the qRT-PCR compared to the microarray.

Table 6: Summary of the selected genes comparing the different housekeeper genes. The p-values below 0.1 are printed in bold. FC fold change; p-val p-value. Fold change is normalised to controls.

| | | **HPRT** | | **GAPDH** | | **combined** | | |
|---|---|---|---|---|---|---|---|---|
| **number** | **symbol** | **FC** | **p-val** | **FC** | **p-val** | **FC** | **p-val** | **notes** |
| 0 | GR | 0.86 | **0.012** | 0.76 | **0.077** | 0.81 | **0.017** | positive control |
| 0 | MR | 0.89 | **0.067** | 0.80 | **0.067** | 0.85 | **0.012** | positive control |
| 1 | Tcf4 | 1.02 | 0.793 | 0.91 | 0.504 | 0.97 | 0.652 | intronic sequence |
| 2 | Dnm1l | 0.92 | 0.268 | 0.80 | 0.242 | 0.86 | 0.212 | intronic sequence |
| 3 | Palm2 | 1.20 | 0.320 | 1.07 | 0.752 | 1.13 | 0.481 | ok |
| 4 | Bat2d | 0.91 | 0.212 | 0.79 | 0.191 | 0.85 | 0.148 | ok |
| 5 | A530025E09Rik | | | | | | | sequence not specific |
| 6 | LOC386330 | | | | | | | sequence not specific |
| 7 | LOC386101 | | | | | | | sequence not specific |
| 8 | A830055N07Rik | | | | | | | sequence not specific |
| 9 | Mtap1a | 1.17 | 0.107 | 1.14 | 0.148 | 1.16 | **0.045** | ok |
| 10 | Neto1 | | | | | | | checked by in-situ |
| 11 | Bat2d | 0.91 | 0.212 | 0.79 | 0.191 | 0.85 | 0.148 | ok |
| 12 | LOC382128 | | | | | | | sequence not specific |
| 13 | Fam178a | 0.95 | 0.660 | 0.90 | 0.541 | 0.92 | 0.542 | ok |
| 14 | Ankhd1 | | | | | | | sequence not specific |
| 15 | Ptprt | 1.01 | 0.800 | 0.90 | 0.406 | 0.96 | 0.518 | ok |
| 16 | Etfdh | 0.95 | 0.501 | 0.84 | 0.236 | 0.89 | 0.214 | intronic sequence |
| 17 | A130039H17Rik | | | | | | | sequence not in the gene |
| 18 | Snrp48 | 0.94 | 0.512 | 0.78 | 0.235 | 0.86 | 0.272 | intronic sequence |
| 19 | LOC386199 | | | | | | | sequence not specific |
| 20 | AK122498 | | | | | | | sequence not in the gene |
| 21 | Csnk1g1 | 0.97 | 0.782 | 0.79 | 0.305 | 0.88 | 0.386 | ok |
| 22 | Fancm | 0.88 | 0.190 | 0.75 | 0.114 | 0.81 | **0.089** | ok |
| 23 | Akap8 | 0.84 | **0.039** | 0.73 | **0.099** | 0.79 | **0.048** | intronic sequence |
| 24 | KIAA0513 | 0.95 | 0.532 | 0.85 | 0.168 | 0.90 | 0.133 | ok |
| 25 | Dlx6os2 | | | | | | | sequence not in the gene |
| 26 | Zzef1 | 1.02 | 0.791 | 0.93 | 0.538 | 0.97 | 0.750 | ok |
| 27 | Dopey1 | | | | | | | sequence not specific |
| 28 | Dopey1 | | | | | | | sequence not specific |
| 29 | Iffo1 | 0.78 | **0.014** | 0.71 | **0.020** | 0.74 | **0.005** | intronic sequence |
| 30 | Cacna2d1 | 1.02 | 0.776 | 0.90 | 0.545 | 0.96 | 0.722 | intronic sequence |
| 31 | Chd1 | 1.00 | 0.962 | 0.89 | 0.390 | 0.94 | 0.555 | ok |
| 32 | Trip12 | 1.06 | 0.485 | 0.94 | 0.739 | 1.00 | 0.987 | intronic sequence |
| 33 | D130017N08Rik | | | | | | | sequence not in the gene |
| 34 | Idh3b | 0.95 | 0.682 | 0.86 | 0.242 | 0.90 | 0.360 | intronic sequence |
| 35 | 2900060F21Rik | | | | | | | sequence not specific |
| 36 | Dnlac1 | 0.88 | **0.079** | 0.83 | 0.254 | 0.86 | 0.110 | ok |
| 37 | Rufy3 | 0.70 | **0.024** | 0.60 | **0.039** | 0.65 | **0.021** | intronic sequence |
| 38 | LOC386164 | | | | | | | sequence not specific |
| 39 | Gnaq | 1.01 | 0.883 | 0.87 | 0.389 | 0.94 | 0.530 | intronic sequence |
| 40 | Robo1 | 1.00 | 0.900 | 0.88 | 0.478 | 0.94 | 0.618 | intronic sequence |

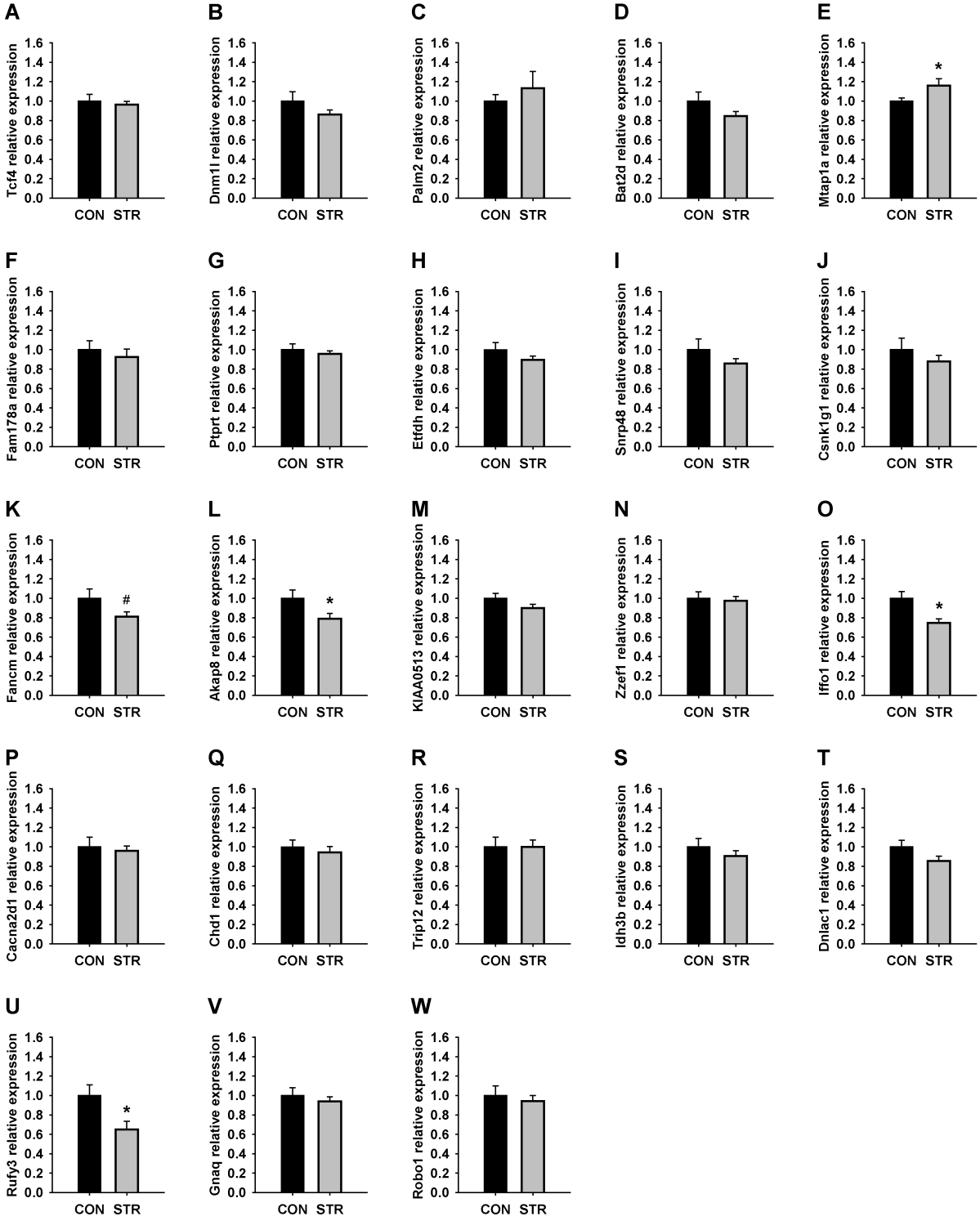


Figure 14: Relative expression of selected genes in whole hippocampus lysate. Expression for the different genes is shown in **(A)** - **(W)**. Data are given in mean + SEM; * significantly different to control animals $p < 0.05$; # different (trend) to control animals $p < 0.1$.

Table 7: Comparison between the microarray and the qRT-PCR. Genes that were significantly regulated in both the microarray and the RT-PCR are printed bold. FC fold change; p-val p-value. Fold change is normalised to controls

| | | **microarray** | | **RT-PCR** | | |
|---|---|---|---|---|---|---|
| **number** | **symbol** | **FC** | **p-val** | **FC** | **p-val** | **notes** |
| 0 | GR | 1.04 | 0.946 | 0.81 | 0.017 | positive control |
| 0 | MR | 1.31 | 0.066 | 0.85 | 0.012 | positive control |
| 1 | Tcf4 | 2.09 | 0.011 | 0.97 | 0.652 | intronic sequence |
| 2 | Dnm1l | 1.98 | 0.014 | 0.86 | 0.212 | intronic sequence |
| 3 | Palm2 | 1.73 | 0.016 | 1.13 | 0.481 | ok |
| 4 | Bat2d | 2.10 | 0.027 | 0.85 | 0.148 | ok |
| 5 | A530025E09Rik | 1.86 | 0.029 | | | sequence not specific |
| 6 | LOC386330 | 1.95 | 0.029 | | | sequence not specific |
| 7 | LOC386101 | 2.39 | 0.029 | | | sequence not specific |
| 8 | A830055N07Rik | 2.04 | 0.029 | | | sequence not specific |
| **9** | **Mtap1a** | **2.12** | **0.030** | **1.16** | **0.045** | **ok** |
| 10 | Neto1 | 2.12 | 0.030 | | | checked by in-situ |
| 11 | Bat2d | 2.01 | 0.030 | 0.85 | 0.148 | ok |
| 12 | LOC382128 | 1.93 | 0.030 | | | sequence not specific |
| 13 | Fam178a | 1.84 | 0.030 | 0.92 | 0.542 | ok |
| 14 | Ankhd1 | 1.92 | 0.032 | | | sequence not specific |
| 15 | Ptprt | 1.82 | 0.032 | 0.96 | 0.518 | ok |
| 16 | Etfdh | 1.79 | 0.035 | 0.89 | 0.214 | intronic sequence |
| 17 | A130039H17Rik | 1.49 | 0.035 | | | sequence not in the gene |
| 18 | Snrp48 | 1.62 | 0.035 | 0.86 | 0.272 | intronic sequence |
| 19 | LOC386199 | 2.40 | 0.038 | | | sequence not specific |
| 20 | AK122498 | 1.92 | 0.038 | | | sequence not in the gene |
| 21 | Csnk1g1 | 2.03 | 0.038 | 0.88 | 0.386 | ok |
| **22** | **Fancm** | **1.81** | **0.038** | **0.81** | **0.089** | **ok** |
| **23** | **Akap8** | **1.91** | **0.038** | **0.79** | **0.048** | **intronic sequence** |
| 24 | KIAA0513 | 1.81 | 0.039 | 0.90 | 0.133 | ok |
| 25 | Dlx6os2 | 1.56 | 0.040 | | | sequence not in the gene |
| 26 | Zzef1 | 1.50 | 0.040 | 0.97 | 0.750 | ok |
| 27 | Dopey1 | 1.73 | 0.040 | | | sequence not specific |
| 28 | Dopey1 | 1.78 | 0.040 | | | sequence not specific |
| **29** | **Iffo1** | **1.60** | **0.040** | **0.74** | **0.005** | **intronic sequence** |
| 30 | Cacna2d1 | 1.75 | 0.040 | 0.96 | 0.722 | intronic sequence |
| 31 | Chd1 | 1.92 | 0.040 | 0.94 | 0.555 | ok |
| 32 | Trip12 | 1.80 | 0.040 | 1.00 | 0.987 | intronic sequence |
| 33 | D130017N08Rik | 2.03 | 0.04 | | | sequence not in the gene |
| 34 | Idh3b | 1.71 | 0.041 | 0.90 | 0.360 | intronic sequence |
| 35 | 2900060F21Rik | 1.55 | 0.041 | | | sequence not specific |
| 36 | Dnlac1 | 2.39 | 0.042 | 0.86 | 0.110 | ok |
| **37** | **Rufy3** | **1.73** | **0.042** | **0.65** | **0.021** | **intronic sequence** |
| 38 | LOC386164 | 2.12 | 0.048 | | | sequence not specific |
| 39 | Gnaq | 1.94 | 0.049 | 0.94 | 0.530 | intronic sequence |
| 40 | Robo1 | 1.72 | 0.049 | 0.94 | 0.618 | intronic sequence |

### 3.1.3 Validation via *in-situ* hybridisation

After validation of the microarray via qRT-PCR, we used *in-situ* hybridisation for further confirmation and to learn more about the distribution of the selected transcripts. ISH was done on slides from the same animals used for the microarray, therefore the stress effects were already described in 3.1.1.1.

#### *3.1.3.1 Mtap1*

We were not able to construct a specific probe for Mtap1.

#### *3.1.3.2 Fancm*

As we showed a significant regulation of the Fancm gene in both the microarray as well as the qRT-PCR, we investigated the gene via *in-situ* hybridisation. However, in contrast to the other methods, we could not find a regulation of Fancm mRNA in the hippocampus (see Figure 15).

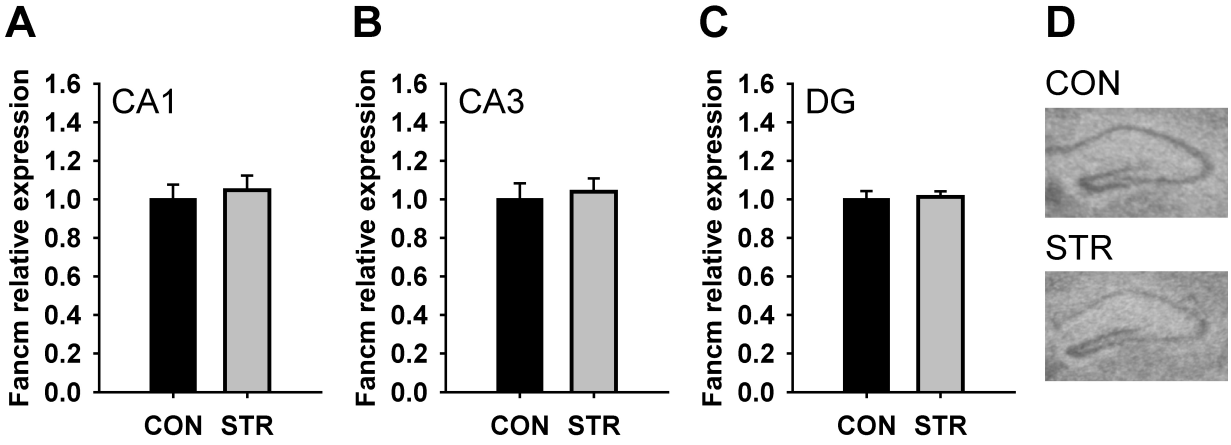


Figure 15: Relative expression of Fancm in the hippocampus 5 weeks after chronic social stress. **(A)** We found no differences in Fancm mRNA expression in the CA1 region. **(B)** The same was true for the CA3 region. **(C)** No difference was present in the DG region. **(D)** Representative images from the hippocampus. Data are given in mean + SEM.

#### *3.1.3.3 Akap8*

We were not able to design a functional ISH probe for Akap8.

#### *3.1.3.4 Iffo1*

One of the strongest candidates from the validation was Iffo1. We investigated Iffo1 mRNA expression in slides from the original microarray experiment, but did not find any effect in the hippocampus 5 weeks after stress exposure, as shown in Figure 16.

#### *3.1.3.5 Rufy3*

In the ISH study, we did not find any effects in the hippocampus in any of the investigated regions for the Rufy3 gene (see Figure 17).

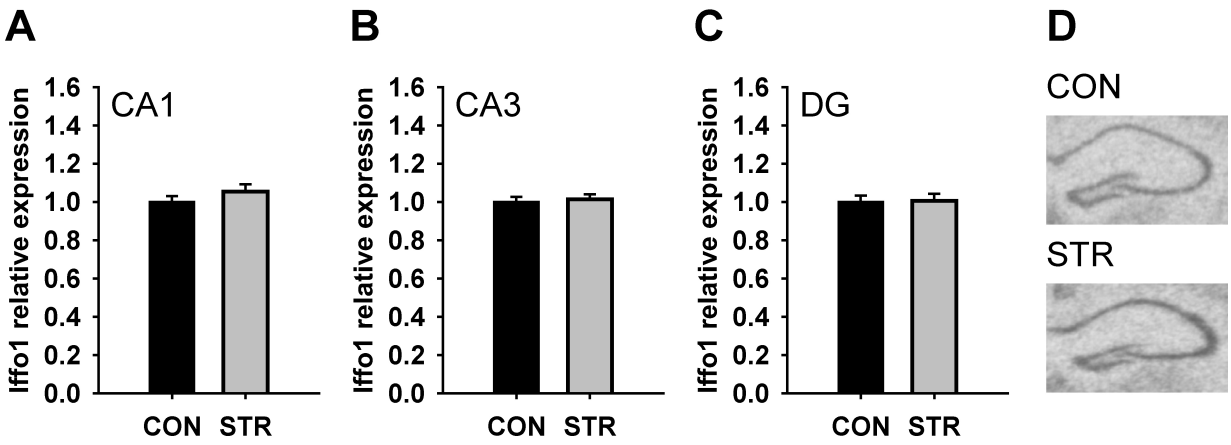


Figure 16: Relative expression of Iffo1 in the hippocampus 5 weeks after chronic social stress. **(A)** We found no differences in Iffo1 mRNA expression in the CA1 region. **(B)** The same was true for the CA3 region. **(C)** No difference was present in the DG region. **(D)** Representative images from the hippocampus. Data are given in mean + SEM.

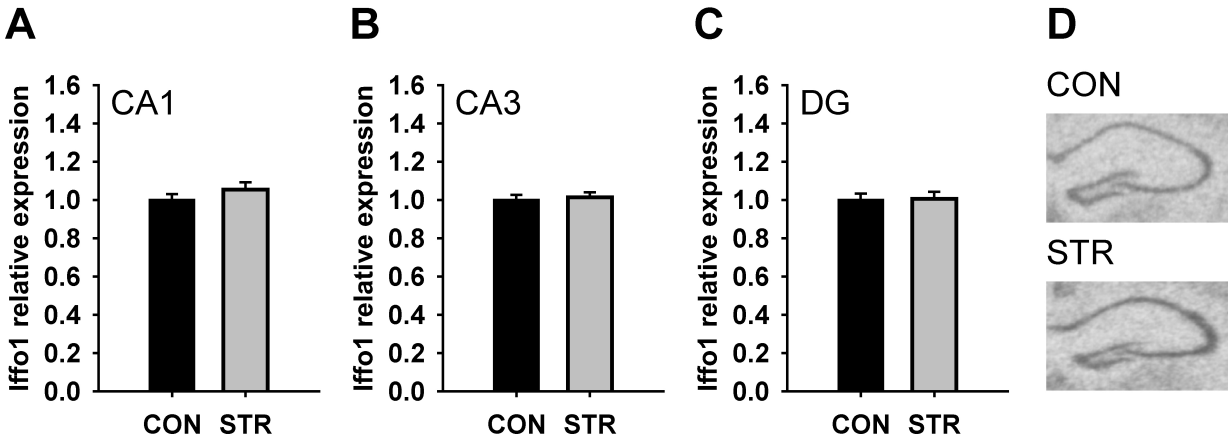


Figure 17: Relative expression of Rufy3 in the hippocampus 5 weeks after chronic social stress. **(A)** We found no differences in Iffo1 mRNA expression in the CA1 region. **(B)** The same was true for the CA3 region. **(C)** No difference was present in the DG region. **(D)** Representative images from the hippocampus. Data are given in mean + SEM.

### 3.1.4 Experiment 1b: The effect of acute stress exposure on selected candidate genes

#### *3.1.4.1 Iffo1*

Iffo1 was also investigated following acute stress, specifically after restrain and food deprivation paradigms. We found a significant downregulation of Iffo1 in all investigated regions (One-way ANOVA, CA1: $F_{2,18} = 13.953$, $p < 0.001$; CAC3: $F_{2,18} = 4.262$, $p < 0.05$; DG: $F_{2,18} = 9.427$, $p < 0.01$) as seen in Figure 18. Bonferroni *post-hoc* testing revealed that both the restrained group as well as the food deprivation group differed from controls, while no difference was present between the stress conditions.

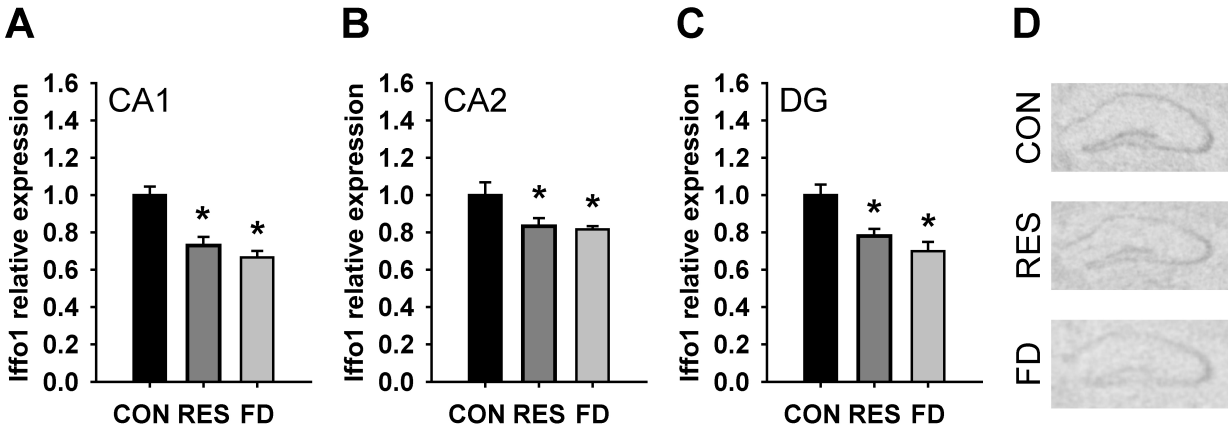


Figure 18: Relative expression of Iffo1 in the hippocampus after acute stress. **(A)** We found a significant downregulation of Iffo1 mRNA expression in the CA1 region after restraint as well as food deprivation. **(B)** The same was true for the CA3 region. **(C)** We also found decreased expression in the DG region. **(D)** Representative images from the hippocampus. Data are given in mean + SEM; * significantly different to control animals $p < 0.05$

### 3.1.5 Experiment 1c: Influence of the GR on selected genes via pharmacological activation

#### *3.1.5.1 Iffo1*

We also investigated the effects of dexamethasone treatment on Iffo1 expression. ANOVA revealed a significant effect of time in the CA1 and DG regions (CA1: $F_{3,48} = 4.513$, $p < 0.01$; DG: $F_{3,48} = 3.505$, $p < 0.05$) and an trend for treatment in the CA1 and CA3 regions (CA1: $F_{1,48} = 2.884$, $p = 0.097$; CA3: $F_{1,48} = 3.805$, $p = 0.058$). A Bonferroni *post-hoc* test showed that the 4 h timepoint was significantly different from the 1 h and 24 h timepoint. One-way ANOVA revealed that this time effect originated from the DEX-treated animals (CA1: $F_{3,23} = 5.213$, $p < 0.01$; DG: $F_{3,23} = 5.945$, $p < 0.01$) while no significant difference was found for vehicle-treated animals. Further *post-hoc* testing via t-tests showed decreased levels of Iffo1 expression in DEX-treated animals at 8 h in the CA3 region (trend, $t_{10} = 1.830$, $p = 0.097$).

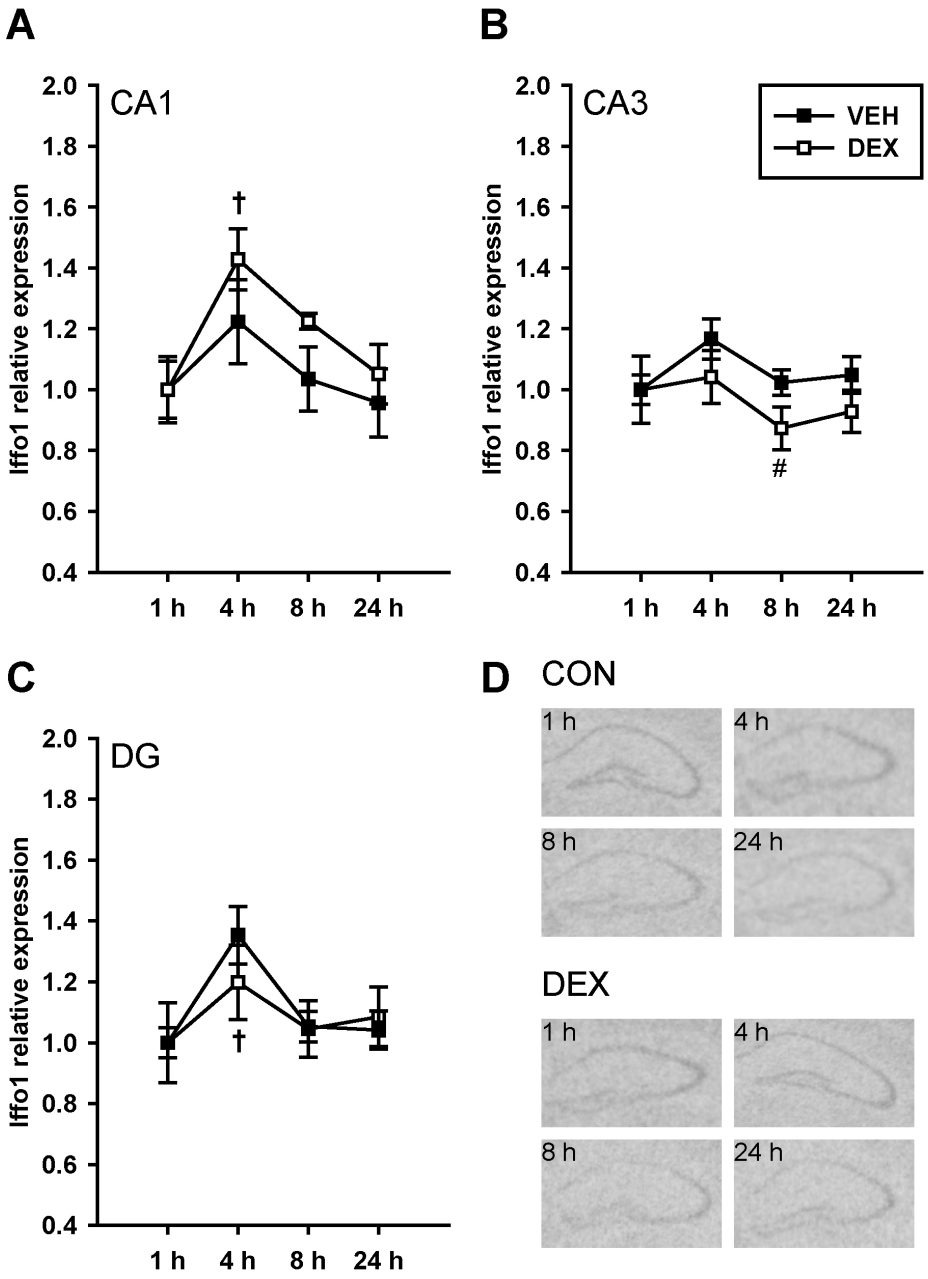


Figure 19: Relative expression of Iffo1 in the hippocampus after dexamethasone injection. **(A)** In the CA1 region, we found a significant increase in expression 4 h after injection in DEX-treated, but not in vehicle-treated animals. **(B)** Iffo1 expression did not fluctuate significantly over time, but at 8 h after the injection, DEX-treated animals showed lower expression of Iffo1 than their vehicle-treated counterparts. **(C)** In the DG region, we also found a significant increase in expression 4 h after injection in DEX-treated, but not in vehicle-treated animals. **(D)** Representative images from the hippocampus. Data are given in mean + SEM; # different to control animals $p < 0.1$; † significantly different to the 1 h and 24 h timepoints.

## 3.2 Experiment 2: Transcriptome differences between stress-vulnerable and stress-resilient animals

### 3.2.1 Vulnerability genes in the hippocampus

#### *3.2.1.1 Experiment 2a: Microarray of the discovery sample*

##### *3.2.1.1.1 Validity of the stress paradigm*

For the investigation of vulnerability genes in the brain, only animals from the stress condition were compared. For the animals selected for the microarray, we found significantly higher levels of CORT in the vulnerable group ($t_8 = 4.977$, $p < 0.01$) as seen in Figure 20

##### *3.2.1.1.2 RNA quality*

We checked the qualtiy of the RNA in the Agilent BioAnaylyzer. Again, RNA quality was only mediocre. For the samples of the vulnerable group, the RIN was 6.3 (Range: 5.6 - 7.1) for the CA1 region and 6.4 (Range: 5.7 - 7.0) for the DG region. Samples from the resilient group reached a RIN of 6.3 (Range: 5.4 - 7) in the CA1 region and 5.4 (Range: 2.5 - 7.3; one sample did not reach the RIN criteria at all).

##### *3.2.1.1.3 Microarray analysis*

Following our cut-off criteria (absolute differential score > 20), we found 2979 genes regulated in the CA1 region (1309 over-represented in vulnerable animals; 1670 over-represented in resilient animals) and 3263 genes regulated in the DG region (1734 over-represented in vulnerable animals; 1529 over-represented in resilient animals). Parts of the study are published in (Schmidt et al., 2010b). The whole dataset is accessible via GEO (accession number GSE11211; http://www.ncbi.nlm.nih.gov/gds?term=GSE11211) A number of genes were selected for further investigations based either on an interesting expression profile, previous studies or general importance in the field. For these genes, displayed in Table 8, a rigorous investigation of the spotted sequences was performed. Here, we found again that the array design showed a lack of diligence. While we found some genes with useful targeting sequences and responses (Ntrk3, Arc, Npy5r, Slc35b3, Gsk3b, Osbpl9), we also found many genes for which we only saw a response in

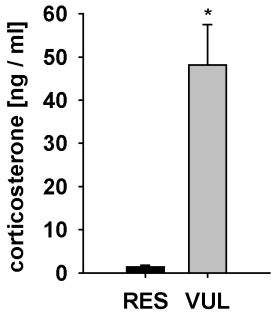


Figure 20: Corticosterone levels in the animals selected for the microarray. The vulnerable subgroup showed significantly higher levels of CORT than the resilient animals. * significantly different to resilient animals $p < 0.05$.

one sequence targeting an UTR sequence and no or a much weaker signal in sequences targeting within exon boundaries (Tm7sf3, Gria1, Gria2, Zfp292, Gabrg2, Homer1, CrhBP, Dag1, Slc6a15). The latter sequences probably detected unprocessed pre-mRNA.

Table 8: Summary of the spotted sequences from the selected hippocampal genes from the vulnerability microarray. Numbered gene names in the notes column refer to different transcript variants described in the ensembl mouse database (www.ensembl.org). FC fold change; diff_score differential score. Fold change is normalised to resilient animals (a fold change of 2 here means that vulnerable animals have double the amount of gene expression than resilient animals).

| | **CA1** | | **DG** | | | |
|---|---|---|---|---|---|---|
| **gene** | **FC** | **diff_score** | **FC** | **diff_score** | **target** | **notes** |
| Ntrk3 | 0.34 | -371 | 0.56 | -371 | scl000230.1_44-S | Ntrk3-002 |
| | 0.49 | -222 | 0.65 | -52 | scl00042.1_34-S | Ntrk3-002 |
| | 0.64 | -34 | 0.50 | -238 | scl018213.1_5-S | Ntrk3-001 |
| | 1.02 | 1 | 0.73 | -127 | scl00047.1_77-S | Ntrk3-001 |
| Tm7sf3 | 0.14 | -371 | 0.61 | -345 | scl28197.12_81-S | 3' UTR |
| | 0.94 | 0 | -6.17 | -2 | scl0001164.1_48-S | low detection |
| | 0.20 | 1 | 0.06 | -1 | scl0001183.1_67-S | low detection |
| Dusp6 | 0.54 | 1 | 4.68 | 3 | scl0003884.1_135-S | good target |
| | 1.35 | 135 | 1.78 | 80 | scl38496.5_152-S | good target |
| Gria1 | 0.54 | -204 | 1.01 | 1 | scl0014799.1_68-S | 5' UTR |
| | 0.92 | -16 | 0.90 | -24 | scl014799.15_1-S | good target |
| | 0.97 | -3 | 0.92 | -18 | scl41536.20_63-S | good target |
| Gria2 | 1.59 | 371 | 1.43 | 206 | scl0014800.2_192-S | 3' UTR |
| Arc | 2.41 | 371 | 1.17 | 23 | scl47086.3_589-S | good target |
| Zfp292 | 0.64 | -92 | 0.62 | -281 | scl030046.1_110-S | sequence potential in UTR |
| | 0.95 | -1 | 0.72 | -41 | scl0030046.1_16-S | good target |
| Gabrg2 | 0.89 | -7 | 1.20 | 27 | scl014406.1_194-S | good target |
| | 0.91 | 0 | 0.72 | -5 | scl0001338.1_15-S | Gabrg2 001,002,201 not 202,203 |
| | 1.11 | 0 | 7.09 | -2 | scl0014406.1_0-S | Gabrg2 001,002,201 not 202,203 |
| | 2.01 | 371 | 1.36 | 198 | scl0001414.1_316-S | 3' UTR |
| Npy5r | 1.43 | 6 | 0.21 | 145 | scl34746.2_217-S | good target |
| Homer1 | 0.69 | 1 | 1.11 | 0 | scl0003656.1_26-S | low detection |
| | 0.73 | 1 | 1.49 | -1 | scl0003685.1_136-S | low detection |
| | 1.64 | 224 | 1.37 | 31 | scl44526.12_201-S | 5' UTR homer 1b/c, d not a |
| | 2.28 | 280 | 1.28 | 18 | scl0003674.1_941-S | 5' UTR homer 1b/c, d not a |
| Slc35b3 | 0.40 | 1 | 0.63 | 1 | scl0003682.1_25-S | low detection |
| | 1.40 | 50 | 1.10 | 20 | scl44068.10_181-S | good target |
| CrhBP | 1.42 | 65 | 5.29 | 104 | scl012919.2_18-S | 3' UTR |
| Dag1 | 0.42 | -367 | 0.40 | -371 | scl013138.1_18-S | 3' UTR |
| | 0.53 | -344 | 0.49 | -371 | scl35355.5_339-S | 3' UTR |
| Slc6a15 | 0.52 | -102 | 1.00 | 0 | scl0103098.12_230-S | 3' UTR |
| | 1.44 | 1 | 2.40 | 5 | scl0003791.1_56-S | good target |
| Gsk3b | 0.64 | -87 | 0.53 | -175 | scl49158.12.6_21-S | good target |
| Osbpl9 | 1.25 | 40 | 1.18 | 26 | scl00100273.2_58-S | good target |

#### 3.2.1.2 Validation via qRT-PCR in the replication sample

##### 3.2.1.2.1 Validity of the stress paradigm

The replication sample (whole cohort) is the same as in experiment 2b (see 3.2.2.1.1), which will be described later, however a different set of animals were selected for the replication of the vulnerability genes. We found significantly elevated levels of CORT 5 weeks after the stress exposure in stressed animals ($t_{94.565} = 3.622$, $p < 0.01$). We found no difference in thymus gland weight while the adrenal gland weight was higher in stressed animals, although not significantly (trend, $t_{113} = 1.907$, $p = 0.059$). In the subgroup selected for the qRT-PCR validation, we found increased levels of basal morning CORT in vulnerable animals ($t_{11.117} = 5.811$, $p < 0.01$), whereas both adrenal weight and thymus weight were not significantly different to resilient animals (see Figure 21).

##### 3.2.1.2.2 RNA quality

No data about RNA quality is available from this experiment.

##### 3.2.1.2.3 Results from the qRT-PCR

The findings from this experiment are quite diverse. At the beginning, the first 7 genes were investigated in the RT-PCR. From these genes, we found a significant regulation in both Tm7sf3 and Arc (statistics given in Table 9). At this point 4 of the samples were lost (reducing the sample size from 12 per group to 10 per group). We continued our validation experiment and investigated Gabrg2, Npy5r and Homer1 out of which we found a significant regulation in Gabrg2 and Homer1. However, due to the magnitude of the effect, we decided to re-analyse all previous runs with only the 10 vs 10 samples (subset column). Interestingly, we now found strong effects in all genes but Gria2, demonstrating a clear selection bias within this experiment. In a next step, we reversely transcribed the samples again (2nd RT column) and continued to investigate Slc35b3, CrhBP, Dag1 and Slc6a15. Furthermore, we re-investigated Arc and Homer1. While we saw no significant effect in Homer1, the differences in Arc expression can be seen at least qualitatively. In this experiment, we also used 2 different housekeeping genes, GAPDH and HPRT, for which we found no different impact on the results, similar to 3.1.2.4. In a third step, we repeated the reverse transcription yet again, compared the samples with previous runs (Gria2, Homer1) and investigated Gsk3b and Osbpl9. Taken together, only Arc caught our eye as a candidate for further research, due to its good targeting sequence in the microarray and its strong regulation despite the huge variance in these experiments.

### 3.2.2 Experiment 2b: Vulnerability genes in peripheral lymphocytes

#### 3.2.2.1 Microarray of the discovery sample

##### 3.2.2.1.1 Validity of the stress paradigm

For the investigation of vulnerability genes in the blood, samples from the same animals selected in the replication cohort for the brain effects were used. Therefore, data about stress-related parameters can be found in 3.2.1.2.1.

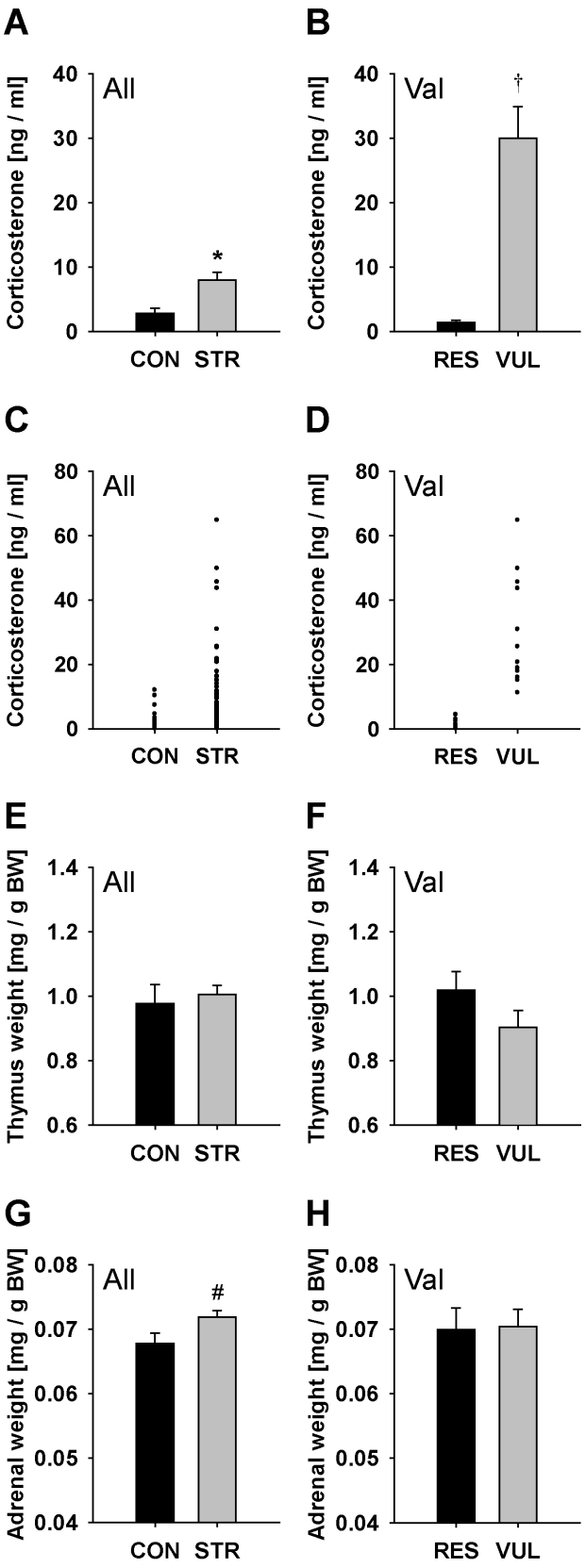


Figure 21: Overview of the effects of the chronic social stress exposure after 5 weeks of recovery and comparison between the whole cohort and the animals selected for the qRT-PCR validation (replication sample). Results from the whole cohort (All) are depicted on the left, while the results from the animals from the validation (Val) are shown on the right. Note that both the resilient and the vulnerable subgroups used for the validation are from within the stress group. **(A)** Basal morning corticosterone levels of the whole cohort. Stressed animals showed significantly higher CORT levels. **(B)** Corticosterone levels in the selected animals were higher in the vulnerable subgroup. **(C)** Scatter plot of the CORT levels from all animals. **(D)** Scatter plot for the CORT levels of the selected animals. **(E)** Relative thymus weight for the whole cohort. **(F)** Animals selected for the validation did not differ in relative thymus weight. **(G)** Relative adrenal weight for all animals. Stressed animals showed a trend for enlarged adrenal glands. **(H)** No difference was found for the selected subgroup. Data are given in mean + SEM; CON control; STR stress; RES resilient; VUL vulnerable; All all animals from the whole cohort; Val only the subgroup of animals that were selected for the qRT-PCR experiment; * significantly different to control animals $p < 0.05$; # different (trend) to control animals $p < 0.1$; † significantly different to resilient animals $p < 0.05$.

Table 9: Summary of the spotted sequences from the selected hippocampal genes from the vulnerability microarray. FC fold change; diff_score differential score.

| | **1st RT** | | | | **2nd RT** | | | | **3rd RT** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **all** | | **subset** | | **HPRT** | | **GAPDH** | | | |
| **gene** | **FC** | **p-val** | **FC** | **p-val** | **FC** | **p-val** | **FC** | **p-val** | **FC** | **p-val** |
| Ntrk3 | 1.16 | 0.326 | **1.37** | **0.021** | | | | | | |
| Tm7sf3 | **1.36** | **0.005** | **1.41** | **0.003** | | | | | | |
| Dusp6 | 1.20 | 0.459 | **1.68** | **0.008** | | | | | | |
| Gria1 | 1.05 | 0.812 | **1.34** | **0.020** | | | | | | |
| Gria2 | 1.06 | 0.758 | 1.25 | 0.154 | | | | | 1.11 | 0.448 |
| Arc | **2.95** | **0.001** | **3.37** | **0.001** | 1.41 | 0.061 | 1.39 | 0.053 | | |
| Zfp292 | 1.10 | 0.311 | **1.21** | **0.041** | | | | | | |
| Gabrg2 | | | **1.24** | **0.034** | | | | | | |
| Npy5r | | | 1.24 | 0.077 | | | | | | |
| Homer1 | | | **1.40** | **0.005** | 0.88 | 0.127 | 0.86 | 0.274 | 1.18 | 0.236 |
| Slc35b3 | | | | | 0.93 | 0.546 | 0.91 | 0.592 | | |
| CrhBP | | | | | 0.91 | 0.402 | 0.91 | 0.520 | | |
| Dag1 | | | | | 1.01 | 0.913 | 0.97 | 0.849 | | |
| Slc6a15 | | | | | 1.01 | 0.937 | 0.96 | 0.768 | | |
| Gsk3b | | | | | | | | | 0.96 | 0.777 |
| Osbpl9 | | | | | | | | | 0.91 | 0.476 |

#### *3.2.2.1.2 RNA quality*

We checked the quality of the RNA via the BioAnalyzer. We were able to isolate high-quality RNA, with a RIN of 8.2 (range 7.7 - 8.5) for the samples from resilient animals and 8.2 (range 7.6 - 8.6) for the samples from vulnerable animals.

#### *3.2.2.1.3 Results from the microarray*

The microarray analysis revealed no significantly regulated genes between stress vulnerable and stress resilient animals in lymphocytes. Nevertheless, we selected 10 genes based on an interesting regulatory pattern (see Table 10). The false discovery rate was predicted at 10 % for the first 2 genes, 20 % for the first 3 and 50 % for the remaining genes.

### *3.2.2.2 Validation via qRT-PCR in the discovery sample*

#### *3.2.2.2.1 Validity of the stress paradigm*

The validation was performed in samples from the same animals used for the microarray. Therefore, data about stress-related parameters can be found in 3.2.1.2.1.

#### *3.2.2.2.2 RNA quality*

RNA samples were split after quality assessment, so the quality is the same as in 3.2.2.1.2.

#### *3.2.2.2.3 Results from the qRT-PCR*

We were able to validate the microarray results for some of the genes. Hsp90b1, SLA, Casp1 and Clec4a3 showed a significant regulation similar to the microarray (for an overview and statistic details refer to Table 10), while Osbpl9, Klf6 and Nudt16 showed a trend in the right direction.

Table 10: Summary of the genes regulated in peripheral lymphocytes. FC fold change; adj. p-val adjusted p-value. Fold change is normalised to resilient animals.

| | **microarray** | | **qRT-PCR** | |
|---|---|---|---|---|
| **gene** | **FC** | **adj. p-val** | **FC** | **p-val** |
| Reep3 | 0.66 | 0.058 | 0.80 | 0.370 |
| Antrx2 | 0.64 | 0.058 | 0.85 | 0.598 |
| Clec4a3 | 0.70 | 0.169 | **0.48** | **0.004** |
| Hsp90b1 | 0.67 | 0.202 | **0.45** | **0.001** |
| Klf6 | 0.53 | 0.202 | 0.63 | 0.093 |
| Sla | 0.57 | 0.202 | **0.54** | **0.045** |
| Mtdh | 0.71 | 0.202 | 0.74 | 0.469 |
| Osbpl9 | 0.68 | 0.202 | 0.61 | 0.054 |
| Casp1 | 0.62 | 0.202 | **0.45** | **0.004** |
| Nudt16 | 0.65 | 0.202 | 0.30 | 0.090 |

### 3.2.3 Correlations between blood and brain

As we were in the fortunate position to have information about expression levels of selected genes in the hippocampus as well as in the blood of the same animals, we were able to compute the correlations between the domains.

#### *3.2.3.1 Combined correlations*

In a first step, we investigated the correlations of all the animals. An overview including the statistics is given in Figure 22. Our main findings here were the medium to strong correlation of the genes within one domain and some correlation with corticosterone levels 5 weeks after stress. We found almost no correlations between samples from the blood and samples from the brain.

#### *3.2.3.2 Correlations within the subgroups*

The correlation analysis for the subgroups revealed some very fascinating characteristics. It should be kept in mind here that also trends were included in the analysis. Together with the large number of single tests we performed, false positive results are expected. Therefore, the analysis is more focused on patterns and pattern changes than single correlation values. The correlation matrices are shown in Figure 23 for the resilient animals and in Figure 24 for the vulnerable animals. First of all, the correlation to the corticosterone levels 5 weeks after stress is absent. Furthermore, we now found a correlation between the corticosterone levels directly after stress and Osbpl9, Clec4a3, Arc, Dag1 and Slc6a15 expression. Intriguingly, this was only present in the vulnerable group. The next thing to note was the dissociation of single genes from their cluster. For example the Arc gene is correlated to most other genes from its domain in the combined analysis. However, in the resilient subgroup, we saw almost no correlations to other gene expression levels in the brain, while the correlations in the vulnerable animals is still strong. The same holds true for Tm7sf3 and Gabrg2 in the vulnerable subgroup. What's even more interesting is the fact that while these genes dissociated from the correlations within their domain, they strengthened their correlation with the other domain. We were now able to find some strong correlations between the domains, but these correlations are strongly sub-group dependent. For the resilient animals, Hsp90b1 in the blood strongly correlated with various genes in the brain

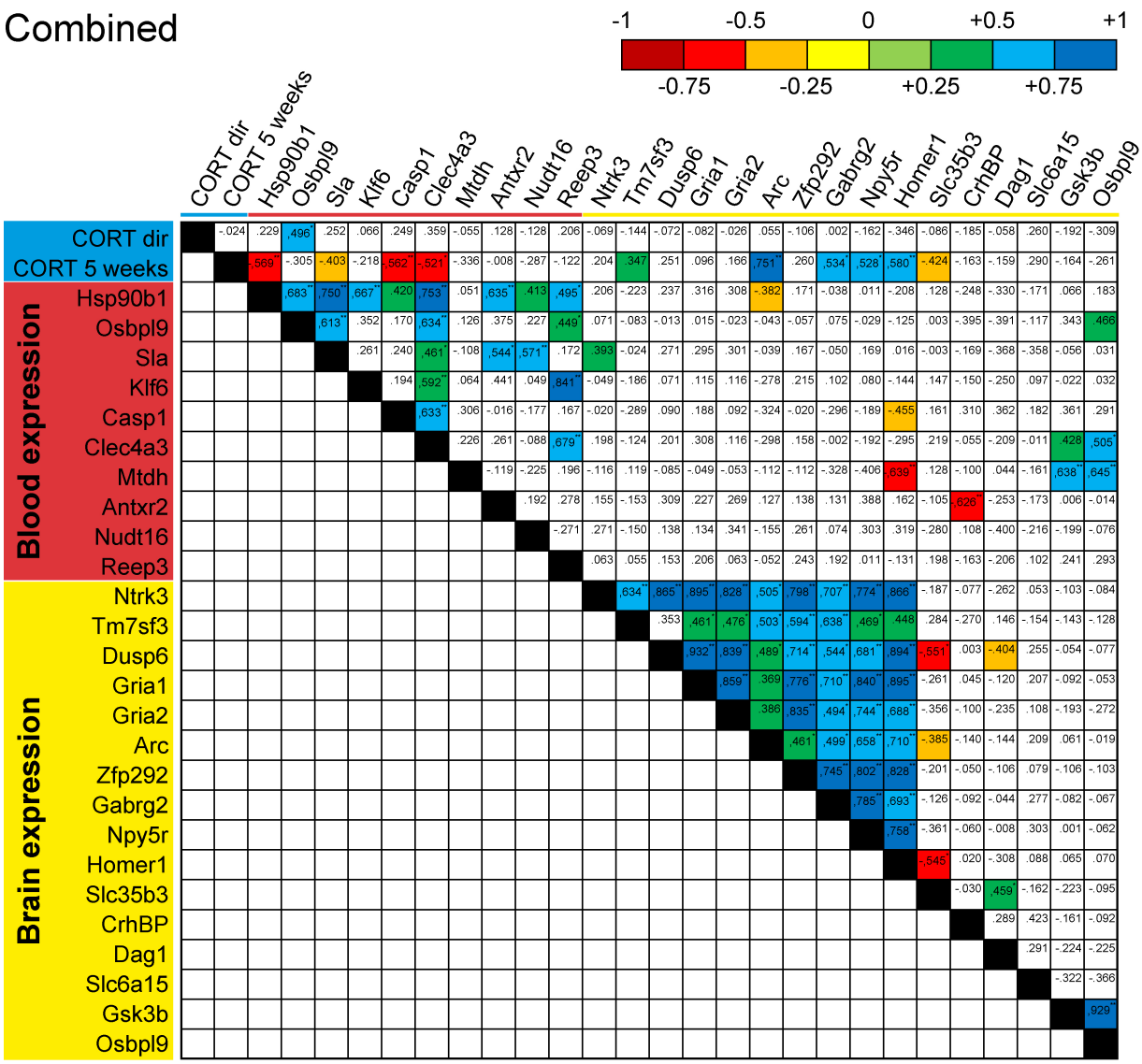


Figure 22: Correlation matrix within and between the selected genes. The matrix shows a strong correlation within the different domains. Correlations between the different domains are rare. Some genes correlate with the corticosterone levels 5 weeks after the stress. Pearson correlation (r) is colour-coded, see scale. P-values are omitted. Coloured fields indicate a trend ($p < 0.1$), if an asterix is added, levels reached significance (* $p < 0.05$; ** $p < 0.01$).

and Arc correlated with some gene expression levels in the blood. In the vulnerable animals, we found that Gabrg2 and especially Npy5r correlated with multiple gene expression levels in the blood. The last major difference we found was the emerge of the ‘‘negative correlation cluster’’ involving Hsp90b1, Osbpl9, Sla, Dag1 and Slc6a15.

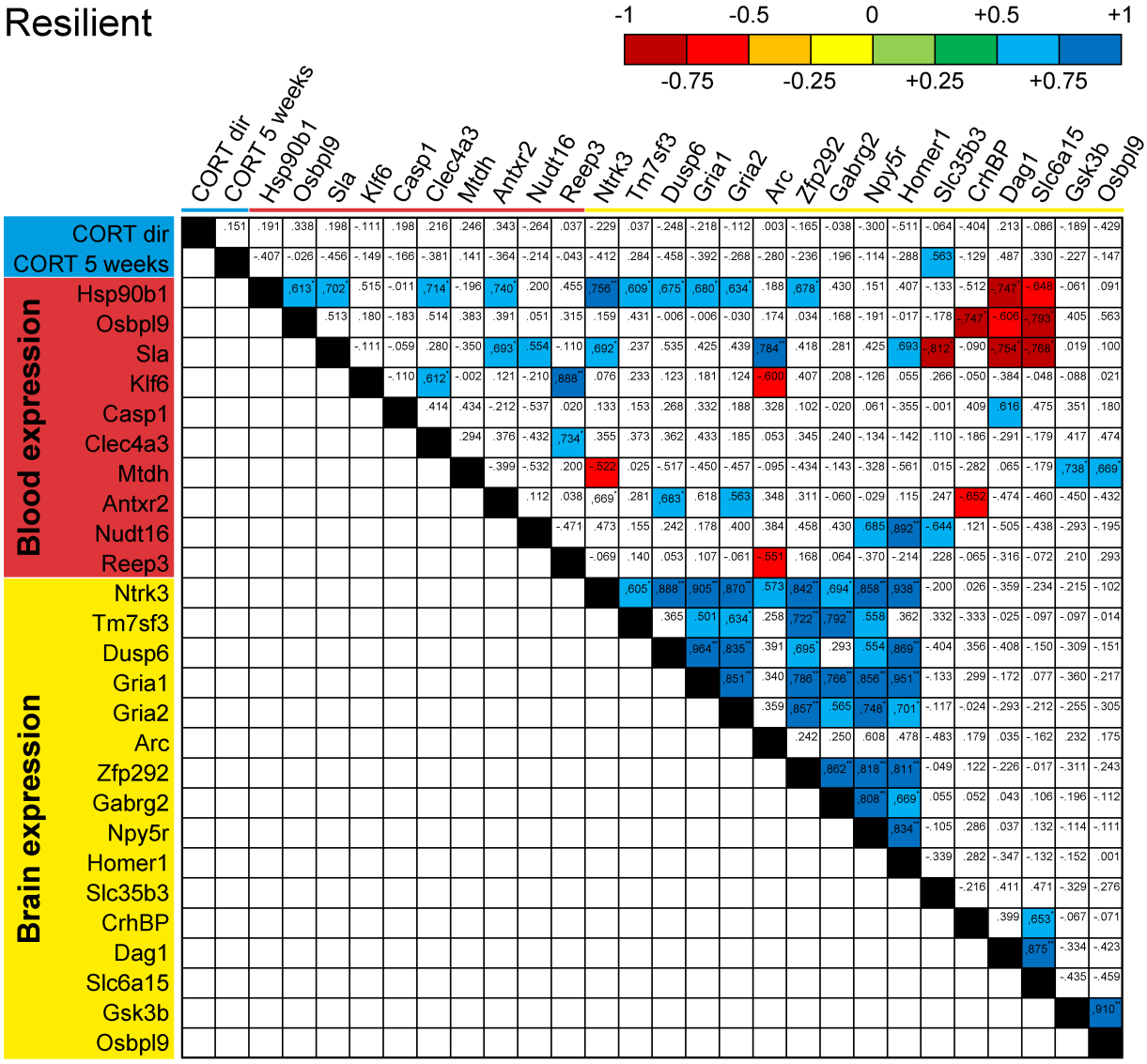


Figure 23: Correlation matrix within and between the selected genes in resilient animals only. Of note here are the lack of correlations to the CORT levels and the negative correlation cluster in the upper right corner. In addition, here we see the shift from correlations within the brain domain to correlations with blood expression levels in the Arc gene. We also see the strong correlation of Hsp90b1 with multiple brain gene expression patterns. Pearson correlation (r) is colour-coded, see scale. P-values are omitted. Coloured fields indicate a trend ($p < 0.1$), if an asterix is added, levels reached significance (* $p < 0.05$; ** $p < 0.01$).

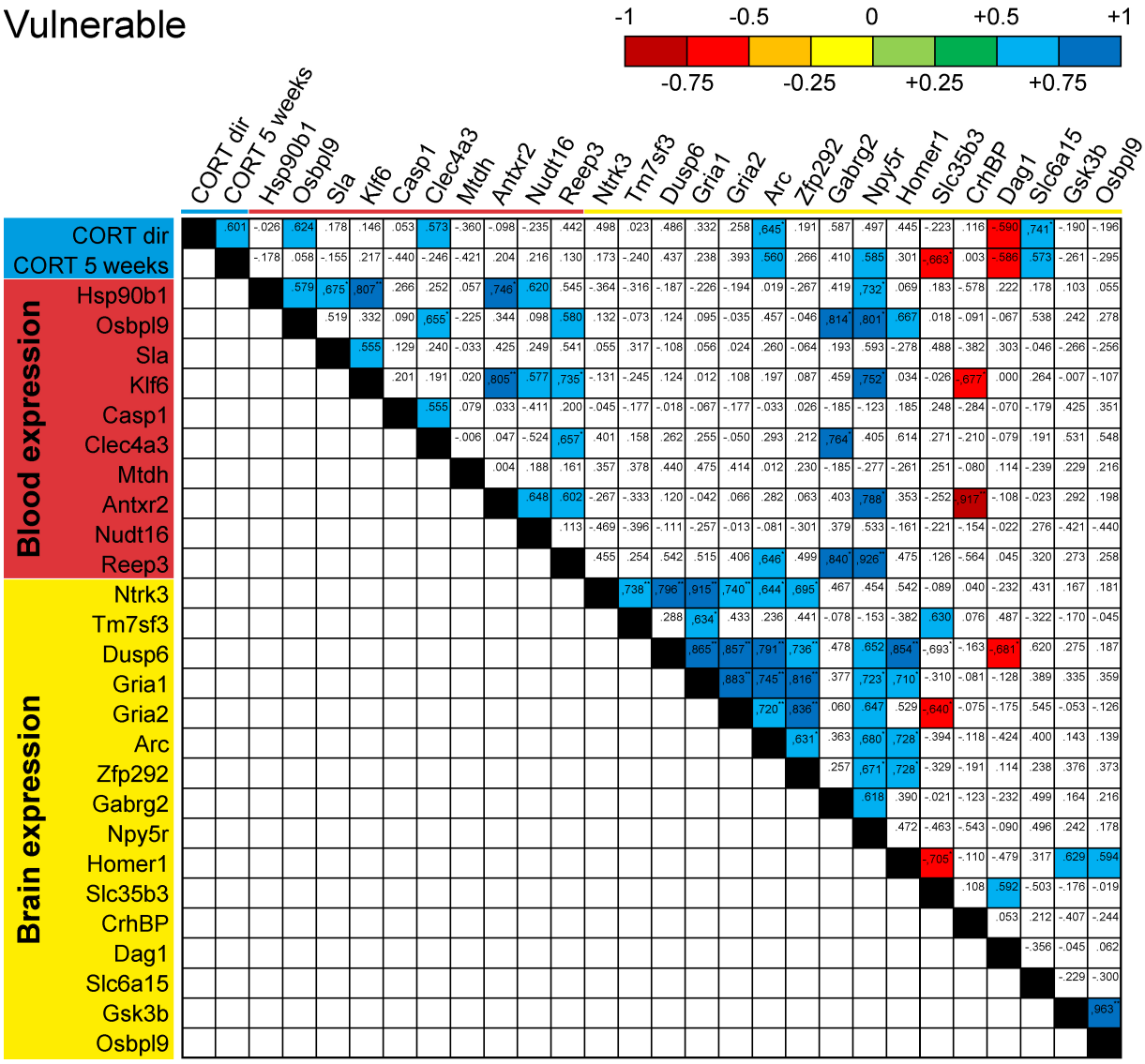


Figure 24: Correlation matrix within and between the selected genes in vulnerable animals only. Interestingly, we see some correlations with CORT levels taken directly after the stress exposure and gene expression levels. Furthermore, we see uncoupling of correlation with gene expression levels in the brain domain for Tm7sf3 and Gabrg2. While Gabrg2 now strongly correlates with blood gene expression levels, this is not the case for Tm7sf3. For Npy5r, the correlation to the brain genes remains intact while additional correlations to blood gene expression levels are found. Pearson correlation (r) is colour-coded, see scale. P-values are omitted. Coloured fields indicate a trend ($p < 0.1$), if an asterix is added, levels reached significance (* $p < 0.05$; ** $p < 0.01$).

## 3.3 Experiment 3: Causal influence of Arc on stress vulnerability

### 3.3.1 Experiment 3a: Confirmation of the Arc knockdown

In experiment 3a, we wanted to confirm the knockdown efficacy of the shARC construct. Therefore, we applied multiple molecular methods to target the problem.

#### *3.3.1.1 In-situ hybridisation*

The first step was the investigation of Arc mRNA. Here we found that the shARC virus seemed to have a strong knockdown effect, however the pattern closely matched the sites of potential cell damage seen in the histological staining (see Figure 25). Please note that no statistics were calculated due to the small sample size ($n = 2$).

#### *3.3.1.2 Western blot*

In addition, we investigated the knockdown efficacy via western blotting of the whole hippocampus. Here, we found a qualitative knockdown effect (see Figure 26). Please note that no statistics were calculated due to the small sample size ($n = 2$). Sample 1r does show an Arc expression not similar to the other 3 samples, but due to the small sample size, we do not know if this is true variation or a simple outlier. If this sample would be excluded, we would see a knockdown efficacy of roughly 40 %.

#### *3.3.1.3 Immunfluorescence*

We also performed immunofluorescent staining to investigate the knockdown effect. In contrast to the previous methods, this allowed us to selectively look at the cells actually expressing the viral construct, due to the GFP signal included in the viral construct (see Figure 27). We were not able to see a clear effect of viral knockdown on Arc protein expression.

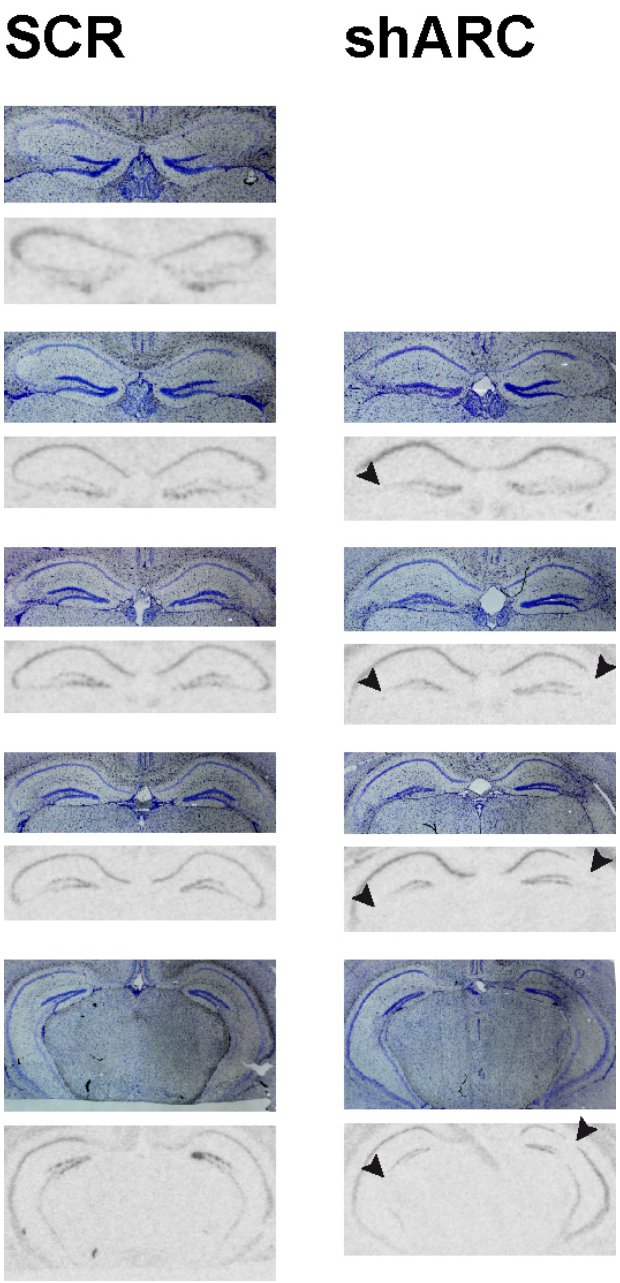


Figure 25: Overview of the viral knockdown on Arc mRNA. Histological (kresyl-violet staining) and in-situ results are paired. Slices from the scrambled group are shown on the left, slices from the Arc KD virus are shown on the right. Potential sites of knockdown are marked with black arrowheads.

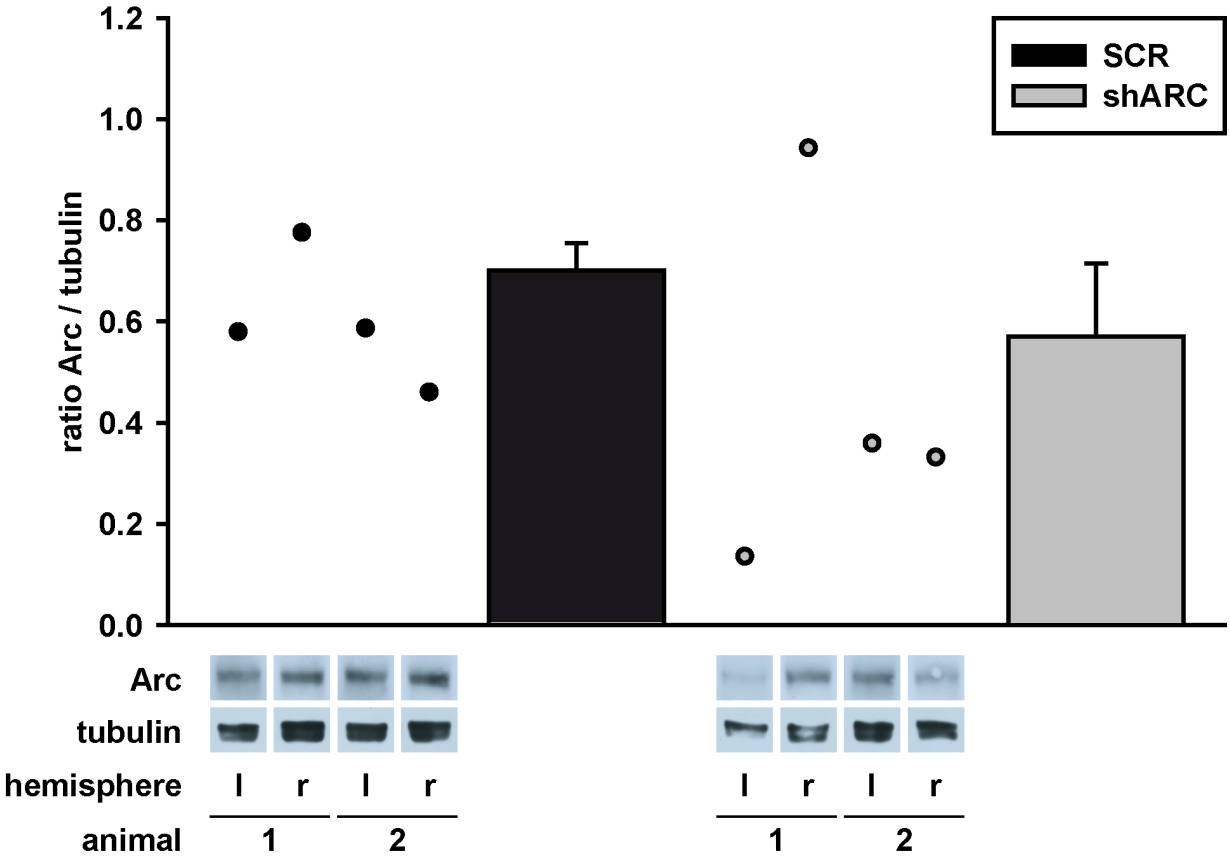


Figure 26: Knockdown efficacy of the shARC virus in the western blot. Here, we see a qualitative knockdown effect. No statistics were calculated due to the small sample size.

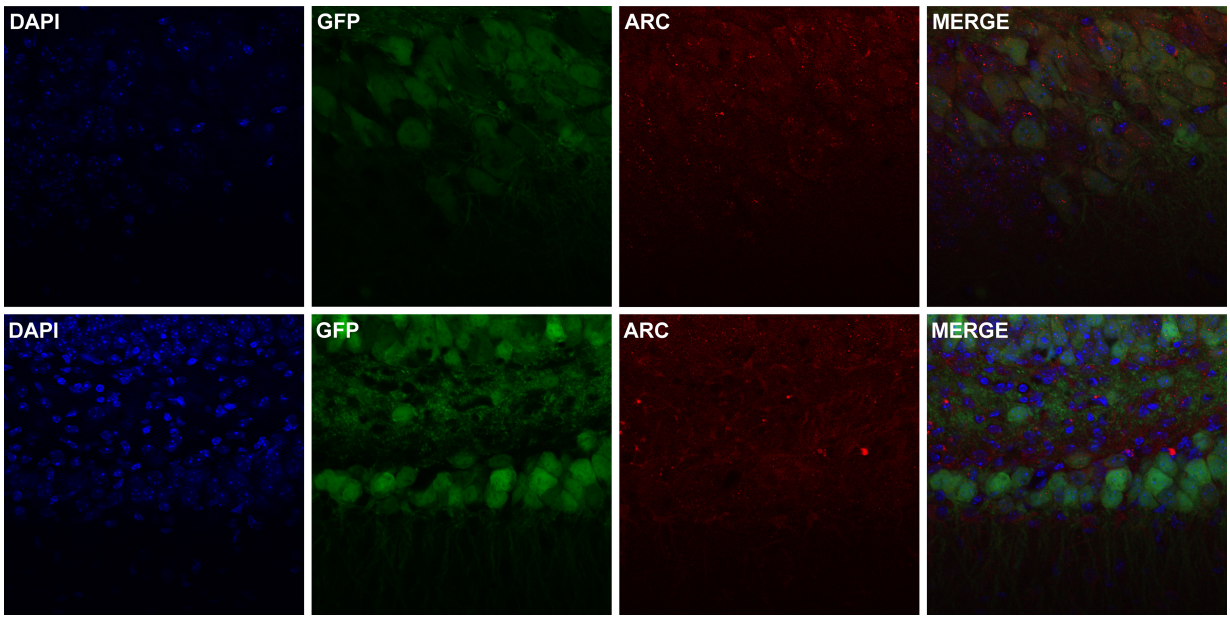


Figure 27: Immunofluorescent staining for testing the efficacy of the Arc knockdown.

### 3.3.2 Experiment 3b: Influence of Arc knockdown in the hippocampus on stress vulnerability

#### *3.3.2.1 Localisation*

Before the actual analysis of the data from the experiment, the hit rate and the localisation of the virus construct was assessed for every individual animal. Due to technical problems, the brain of animal 25 was lost during the perfusion process. In total, the CA1 region was hit bilaterally in 20 animals and unilaterally in 11 animals (24 bilaterally if the subiculum only is counted as CA1 hit), while the DG was hit bilaterally 33 times and unilaterally in 6 animals (see Figure 28 and Table 11 for details). Only animals hit bilaterally in either the CA1 region or the DG region were included in the following analyses ($n = 7$ animals were excluded in total).

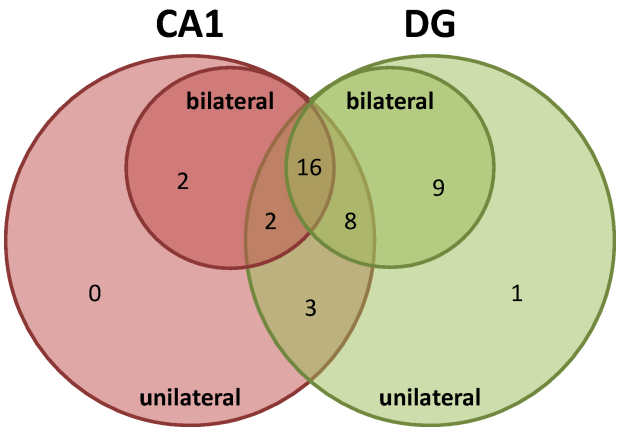


Figure 28: Venn-like diagramm depicting localisation of the shARC viral construct. Please note that animals which were not hit in any of the two regions were omitted from this diagram.

Table 11: Overview of the localisation of the shArc viral construct. ++ bilateral hit; + unilateral hit; - region not hit.

| animal | CA1 | DG |
|---|---|---|
| 1 | - | ++ |
| 3 | + | + |
| 4 | - | ++ |
| 5 | + | + |
| 6 | ++ | ++ |
| 7 | ++ | + |
| 8 | ++ | ++ |
| 9 | - | ++ |
| 10 | ++ | ++ |
| 11 | ++ | ++ |
| 12 | - | ++ |
| 13 | - | ++ |
| 14 | ++ | ++ |
| 15 | - | ++ |
| 16 | + | + |
| 17 | ++ | - |
| 18 | + | ++ |
| 19 | ++ | - |
| 20 | + | ++ |
| 21 | + | ++ |
| 22 | + | ++ |
| 23 | - | ++ |
| 24 | + | ++ |
| 25 | n.A. | n.A. |
| 26 | ++ | ++ |
| 27 | - | - |
| 28 | ++ | ++ |
| 29 | - | + |
| 30 | ++ | ++ |
| 31 | ++ | + |
| 32 | ++ | ++ |
| 33 | - | ++ |
| 34 | + | ++ |
| 35 | ++ | ++ |
| 36 | ++ | ++ |
| 37 | ++ | ++ |
| 38 | ++ | ++ |
| 39 | ++ | ++ |
| 40 | - | - |
| 41 | + | ++ |
| 42 | ++ | ++ |
| 43 | ++ | ++ |
| 44 | + | ++ |
| 45 | - | ++ |

#### *3.3.2.2 Body weight*

The body weight of the animals was monitored over the course of the experiment in different intervals, depending on the stage of the experiment (once per week during recovery before stress, twice per week during the social defeat as well as the following recovery and additionally directly before and directly after the SD). The data was analysed via repeated measures ANOVA in three different sets, once over all timepoints, only during the stress (subgroup of the first dataset) and only after the stress (recovery, also a subgroup of the first dataset). Sphericity was violated in all cases, therefore multivariate test statistics were used. For the whole experiment, we found a significant effect of time ($F_{19,15} = 14.786$, $p < 0.01$) as well as trend towards a time × condition interaction ($F_{19,15} = 2.005$, $p = 0.088$). For the stress phase, we found a significant effect of time ($F_{6,28} = 30.575$, $p < 0.01$), time × condition interaction ($F_{6,28} = 3.991$, $p < 0.01$) as well as a trend towards a time × virus interaction ($F_{6,28} = 2.052$, $p = 0.092$). Finally, the recovery phase was characterised by a time effect ($F_{9,25} = 16.911$, $p < 0.01$) and a time × condition interaction trend ($F_{9,25} = 1.943$, $p = 0.092$). Post hoc testing was only done between subjects (time effect omitted) and showed that 15 days after the end of the social defeat, the body weight of SD animals was higher compared to controls within the SCR-injected animals ($t_{15} = 1.777$, $p = 0.096$), as seen in Figure 29.

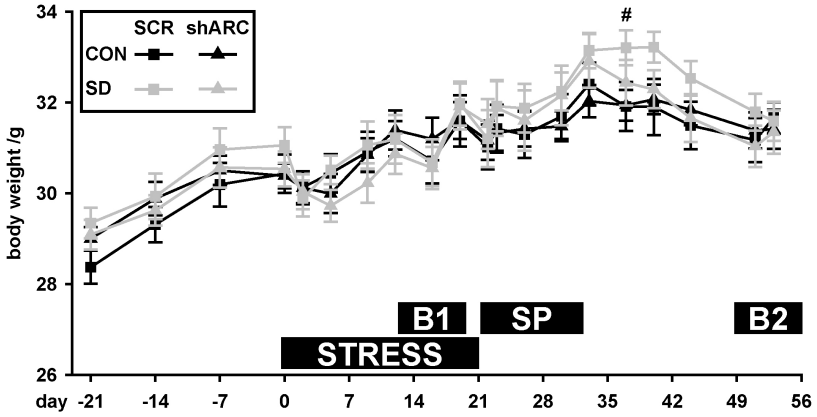


Figure 29: Absolute body weights of experiment 3b. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; STRESS = social defeat stress (3 weeks); B1 = behavioural testing phase during the stress; B2 = behavioural testing phase after recovery; SP = sucrose preference test; Data are given in mean ± SEM; # different (trend) to control animals within the same virus group $p < 0.1$.

#### *3.3.2.3 Fur state*

We also investigated the fur state of the animals during the stress as well as the recovery period. Much like the data for the body weight, the fur state was analysed over the whole experiment, only during stress and only during recovery. Sphericity was violated in all cases, therefore multivariate test statistics were used. For the whole experiment, we found a significant effect of time ($F_{19,15} = 21.926$, $p < 0.01$) as well as a time × condition interaction effect ($F_{19,15} = 21.640$, $p < 0.01$). If only the phase during the stress was analysed, we also found a significant effect of time ($F_{6,28} = 42.596$, $p < 0.01$) as well as a time × condition interaction effect ($F_{6,28} = 23.797$, $p < 0.01$). The same was true for the analysis of the recovery period (time: $F_{8,26} = 11.186$, $p < 0.01$; time × condition interaction: $F_{8,26} = 8.106$, $p < 0.01$). Within the SCR-injected animals, individuals that underwent SD showed higher fur state beginning from day 5 of the SD (day5: $t_{15} = 2.717$, $p < 0.05$; day9: $t_{15} = 4.903$, $p < 0.01$; day12: $t_{15} = 3.627$, $p < 0.01$; day16: $t_{15} = 3.723$, $p < 0.01$; day19: $t_{15} = 6.345$, $p < 0.01$; day22: $t_{15} = 3.088$, $p < 0.01$; day23: $t_{15} = 3.044$, $p < 0.01$; day26: $t_{15} = 1.840$, $p = 0.086$). This was also found within the shARC group (day5: $t_{18} = 1.818$, $p = 0.086$; day9: $t_{18} = 2.888$, $p < 0.01$; day12: $t_{18} = 3.964$, $p < 0.01$; day16: $t_{18} = 2.894$, $p < 0.01$; day19: $t_{18} = 3.627$, $p < 0.01$; day22: $t_{18} = 3.450$, $p < 0.01$; day23: $t_{18} = 2.260$, $p < 0.05$; day26: $t_{18} = 2.017$, $p = 0.059$). An overview is given in Figure 30.

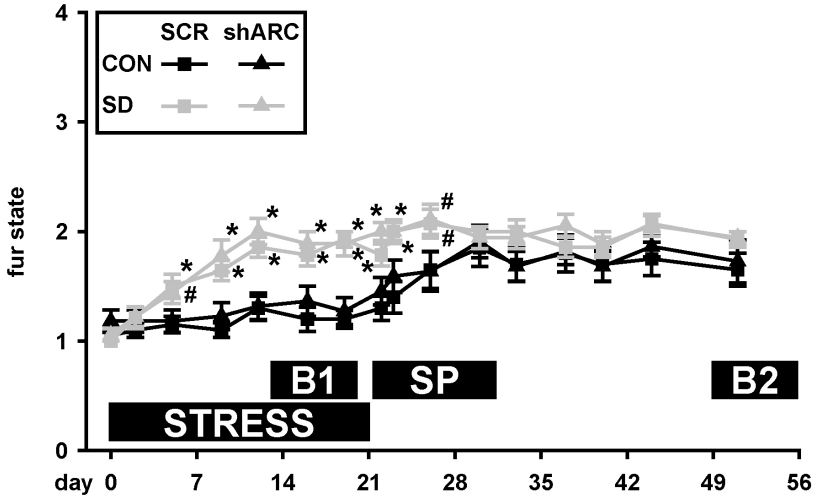


Figure 30: Fur state measured in experiment 3b. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; STRESS = social defeat stress (3 weeks); B1 = behavioural testing phase during the stress; B2 = behavioural testing phase after recovery; SP = sucrose preference test; Data are given in mean ± SEM; * significantly different to control animals within the same virus group $p < 0.05$; # different (trend) to control animals within the same virus group $p < 0.1$.

#### *3.3.2.4 Corticosterone*

Corticosterone is one of the major end-products of the HPA axis and can be used to measure stress system activity. Here, we assessed the responsivity of the HPA axis during the social defeat and after recovery (see Figure 31). We used repeated measures ANOVA and found that during the stress phase, we see an effect of time ($F_{2,31} = 185,412$, $p < 0.01$) as well as time × condition interaction ($F_{2,31} = 11.482$, $p < 0.01$). Post hoc testing showed that within the SCR-injected group, stressed animals show higher levels of corticosterone in the response as well as in the recovery (response: $t_{15} = 2.268$, $p < 0.05$; recovery: $t_{14} = 3.172$, $p < 0.01$). The same was true for animals injected with the shARC construct (response: $t_{18} = 2.661$, $p < 0.05$; recovery: $t_{18} = 3.550$, $p < 0.01$). In addition, we found that the corticosterone response was by tendency lower in stressed animals injected with shARC compared with SCR-injected animals ($t_{14} = 2.028$, $p = 0.062$). When investigating the corticosterone responsivity again after recovery, we only found a significant effect of time ($F_{2,32} = 86.998$, $p < 0.01$).

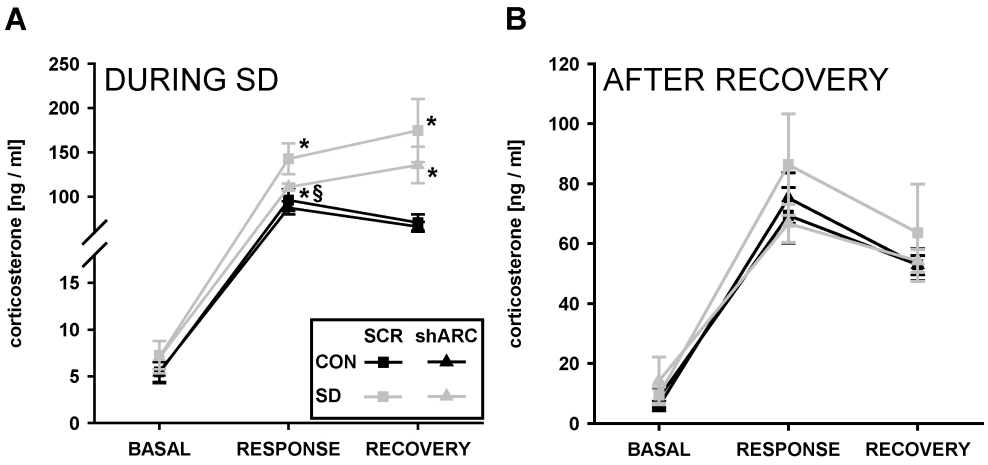


Figure 31: Corticosterone levels in the plasma from experiment 3b. **(A)** Corticosterone response tested during the social defeat. Please note the break and the resulting two different scales. **(B)** Corticosterone response tested after recovery. Please note here the different scale to subfigure A. BASAL = basal plasma levels taken in the morning; RESPONSE = plasma levels taken 30 minutes after onset of acute stress (acute stress lasted for 6 min); RECOVERY = plasma levels taken 90 minutes after onset of acute stress; CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean ± SEM ; * significantly different to control animals within the same virus group $p < 0.05$; § different (trend) to SCR-injected animals within the same condition $p < 0.1$.

#### *3.3.2.5 Adrenal glands*

The adrenal glands are the site of corticosterone production. We investigated the normalised weight of both adrenal glands (see Figure 32). ANOVA revealed a significant effect of condition ($F_{1,32} = 41.302$, $p < 0.01$), with no effect of virus or condition × virus interaction. Post hoc testing showed that animals which underwent social defeat had significantly heavier adrenal glands than control animals (within SCR-injected individuals: $t_{15} = 3.754$, $p < 0.01$; within shARC-injected individuals: $t_{17} = 5.867$, $p < 0.01$).

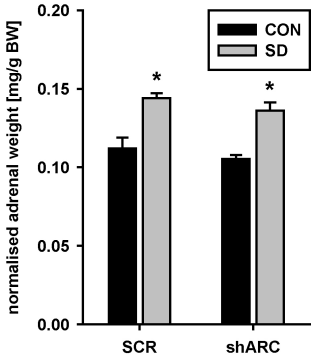


Figure 32: Normalised adrenal gland weight in experiment 3b. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; BW = body weight; Data are given in mean + SEM; * significantly different to control animals within the same virus group $p < 0.05$.

#### 3.3.2.6 Behaviour in the last week of social defeat

##### 3.3.2.6.1 Open field test

The open field test was used to assess anxiety-like behaviour and basic locomotion (see Figure 33). The test was performed in the morning and repeated in the afternoon. In the morning, ANOVA showed a significant condition effect for total locomotion ($F_{1,33} = 10.034$, $p < 0.01$), with no virus and no condition × virus interaction effect. Follow-up post-hoc testing showed that locomotion was significantly lower in stressed SCR-injected animals ($t_{15} = 2.607$, $p < 0.05$). When the total travelled distance was analysed in three segments of 300 s each, ANOVA revealed a condition effect for the second and third segment, but not for the first (Seg2: $F_{1,33} = 14.190$, $p < 0.01$; Seg3: $F_{1,33} = 26.661$, $p < 0.01$). Here again, the stressed SCR-injected animals showed lower levels of locomotion (Seg2: $t_{15} = 3.141$, $p < 0.01$; Seg3: $t_{15} = 4.305$, $p < 0.01$), but also the stressed shARC-injected animals, although only as a trend in the second segment (Seg2: $t_{15} = 2.026$, $p = 0.058$; Seg3: $t_{15} = 2.887$, $p < 0.05$). In the re-testing session in the afternoon, a significant condition effect was found for the total distance travelled over the whole test, as well as for all three segments (Total: $F_{1,33} = 25.942$, $p < 0.01$; Seg1: $F_{1,33} = 7.397$, $p < 0.05$; Seg2: $F_{1,33} = 31.474$, $p < 0.01$; Seg3: $F_{1,33} = 29.910$, $p < 0.01$). In addition, a trend for a virus effect was found in segment 2 (Seg2: $F_{1,33} = 3.288$, $p = 0.079$) and a significant condition × virus interaction effect was found for the total distance and segment 1, with a trend in segment 3 (Total: $F_{1,33} = 5.340$, $p < 0.05$; Seg1: $F_{1,33} = 6.394$, $p < 0.05$; Seg3: $F_{1,33} = 3.865$, $p = 0.058$). Post-hoc testing showed that the travelled distance was lower in stressed SCR-injected animals (Total: $t_{15} = 4.876$, $p < 0.01$; Seg1: $t_{15} = 3.630$, $p < 0.01$; Seg2: $t_{15} = 4.466$, $p < 0.01$; Seg3: $t_{15} = 4.622$, $p < 0.01$), as well as in shARC-injected animals in all but the first segment (Total: $t_{15} = 2.114$, $p < 0.05$; Seg2: $t_{15} = 3.301$, $p < 0.01$; Seg3: $t_{15} = 2.826$, $p < 0.05$). Furthermore, it was shown that shARC-injected animals show higher levels of locomotion than their SCR-injected counterparts, but only within the stress group and only as a trend in the different segments (Total: $t_{14} = 2.320$, $p < 0.01$; Seg1: $t_{14} = 1.942$, $p = 0.073$; Seg2: $t_{14} = 2.065$, $p = 0.058$; Seg3: $t_{14} = 1.946$, $p = 0.072$). ANOVA was also performed for the entries in the inner zone. In the morning session, a significant effect of condition was found in total as well as in segments 2 and 3 (Total: $F_{1,33} = 8.442$, $p < 0.01$; Seg2: $F_{1,33} = 6.609$, $p < 0.01$; Seg3: $F_{1,33} = 5.716$, $p < 0.05$). In addition, a trend towards a condition × virus interaction effect was present in segment 3 (Seg3: $F_{1,33} = 3.477$, $p = 0.071$). Post-hoc testing showed lower entries in the inner zone in stressed SCR-injected animals (Total: $t_{15} = 2.932$, $p < 0.05$; Seg2: $t_{15} = 1.941$, $p = 0.071$; Seg3: $t_{15} = 2.560$, $p < 0.05$). Animals injected with shARC showed higher number of entries in the stress, but not in the control condition (Total: $t_{14} = 2.960$, $p < 0.05$; Seg2: $t_{14} = 2.451$, $p < 0.05$; Seg3: $t_{15} = 1.766$, $p = 0.099$). In the afternoon, we found a significant effect of condition (Total: $F_{1,33} = 17.000$, $p < 0.01$; Seg2: $F_{1,33} = 11.273$, $p < 0.01$; Seg3: $F_{1,33} = 10.665$, $p < 0.01$) as well as condition × virus interaction (Total: $F_{1,33} = 10.866$, $p < 0.01$; Seg1: $F_{1,33} = 6.723$, $p < 0.05$; Seg2: $F_{1,33} = 3.584$, $p = 0.067$; Seg3: $F_{1,33} = 4.834$, $p < 0.05$). Stressed SCR-injected animals showed fewer entries (Total: $t_{15} = 3.662$, $p < 0.01$; Seg1: $t_{15} = 2.866$, $p < 0.05$; Seg2: $t_{15} = 3.177$, $p < 0.01$; Seg3: $t_{15} = 3.144$, $p < 0.01$) and compared to SCR-injected animals, shARC-injected animals had a higher number of entries, although only in the stress condition (Total: $t_{15} = 3.796$, $p < 0.01$; Seg1: $t_{15} = 2.242$, $p < 0.05$; Seg2: $t_{15} = 2.146$, $p < 0.01$; Seg3: $t_{15} = 3.055$, $p < 0.01$).

Finally, the time spent in the inner zone was analysed. In the morning seesion, ANOVA revealed a significant effect of condition (Total: $F_{1,33} = 6.483$, $p < 0.05$; Seg2: $F_{1,33} = 5.795$, $p < 0.05$; Seg3: $F_{1,33} = 4.373$, $p < 0.05$) as well as a trend towards a condition $\times$ virus interaction (Total: $F_{1,33} = 3.198$, $p = 0.083$). The following post-hoc tests showed that stressed animals injected with the SCR viral construct spent significantly less time in the inner zone (Total: $t_{15} = 2.254$, $p < 0.05$; Seg2: $t_{15} = 1.946$, $p = 0.068$; Seg3: $t_{15} = 2.187$, $p < 0.05$). In addition, there was an overall trend that shARC-injected animals spent more time in the inner zone, but only in the SD condition (Total: $t_{14} = 1.870$, $p = 0.083$). In the afternoon, the anaylsis showed a significant effect of condition (Total: $F_{1,33} = 12.046$, $p < 0.01$; Seg2: $F_{1,33} = 7.557$, $p < 0.01$; Seg3: $F_{1,33} = 3.964$, $p = 0.055$) as well as a condition $\times$ virus interaction effect (Total: $F_{1,33} = 12.046$, $p < 0.01$; Seg1: $F_{1,33} = 4.133$, $p = 0.050$; Seg2: $F_{1,33} = 3.515$, $p = 0.070$; Seg3: $F_{1,33} = 5.587$, $p < 0.05$). Post-hoc testing confirmed that stressed SCR-injected animals spent significantly less time in the inner zone (Total: $t_{15} = 3.135$, $p < 0.01$; Seg1: $t_{15} = 2.539$, $p < 0.05$; Seg2: $t_{15} = 2.548$, $p < 0.05$; Seg3: $t_{15} = 2.898$, $p < 0.05$) and that in the stress condition, animals injected with the shARC construct explored the inner zone for a longer period of time (Total: $t_{14} = 3.473$, $p < 0.01$; Seg1: $t_{14} = 2.057$, $p = 0.059$; Seg3: $t_{14} = 2.538$, $p < 0.05$). In addition, the first segment of the morning session was compared with the first segment of the afternoon session. Here, ANOVA showed a significant condition effect ($F_{1,33} = 5.013$, $p < 0.05$) for the travelled distance. Post-hoc testing revealed that only in SCR-injected animals, the individuals from the SD condition show lower levels of initial exploration ($t_{15} = 2.241$, $p < 0.05$, not shown as direct comparison in the figure).

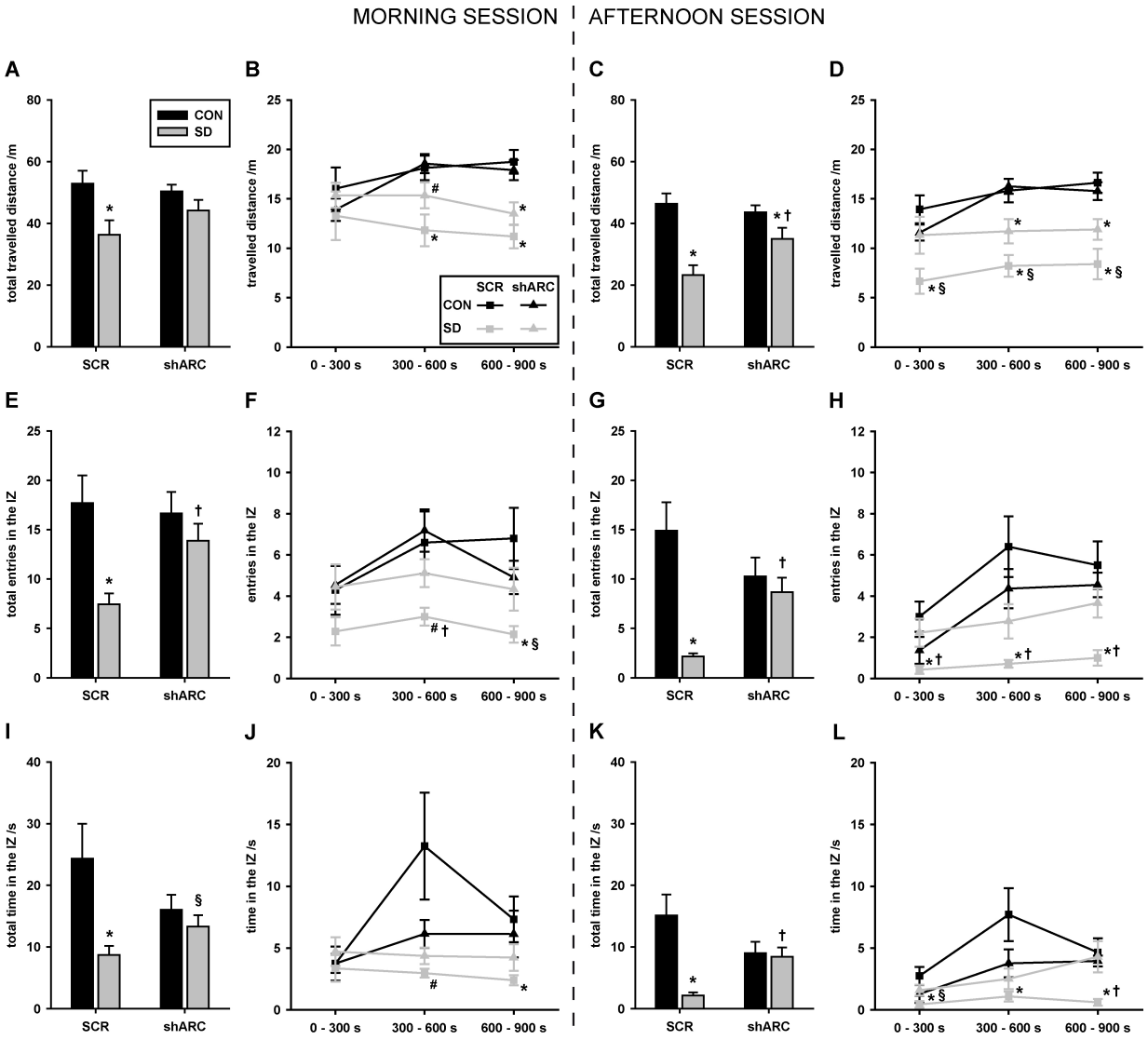


Figure 33: Results from the OF test performed in the last week of social defeat. **(A)** and **(B)** Distance travelled in total and split in segments, respectively, from the morning session. **(C)** and **(D)** Distance travelled in total and split in segments, respectively, from the afternoon session. **(E)** and **(F)** Entries in the inner zone in total and split in segments, respectively, from the morning session. **(G)** and **(H)** Entries in the inner zone in total and split in segments, respectively, from the afternoon session. **(I)** and **(J)** Time spent in the inner zone in total and split in segments, respectively, from the morning session. **(K)** and **(L)** Time spent in the inner zone in total and split in segments, respectively, from the afternoon session. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM (total) or mean ± SEM (segments); * significantly different to control animals within the same virus group $p < 0.05$; # different (trend) to control animals within the same virus group $p < 0.1$; † significantly different to SCR-injected animals within the same condition $p < 0.05$; § different (trend) to SCR-injected animals within the same condition $p < 0.1$.

#### *3.3.2.6.2 Object recognition test*

The spatial version of the object recognition test (identical object is switched to another location) was used to assess hippocampus-dependent learning and memory. In this test, 3 animals (numbers 34 and 36 from the SD shARC group and number 13 from the CON SCR group) had to be excluded as they showed no exploration at all during the test trial. Note that the investigated parameter here is a ratio of the time/entries near the displaced object to the total number of time/entries near both objects (displaced/non-displaced) to normalise for differences in activity. The 95 % confidence interval was computed for all groups to investigate general learning impairments. All of the groups were above 0.50 (50 %, chance level), as seen in Table 12. ANOVA showed a trend towards a virus effect for the time ratio ($F_{1,30} = 3.846$, $p = 0.059$) as well as a condition × virus interaction effect for both the entries and the time ratios (entries: $F_{1,30} = 5.147$, $p < 0.05$; time: $F_{1,30} = 3.247$, $p = 0.082$). Further post-hoc testing revealed a trend towards a lower object exploration ratio depending on the entries in SCR-injected stress animals ($t_{14} = 1.871$, $p = 0.082$). In addition, shARC-injected animals showed a higher exploration ratio than their SCR-injected counterparts within the SD condition only (entries: $t_{12} = 2.267$, $p < 0.05$; time: $t_{15} = 2.057$, $p = 0.062$), as depicted in Figure 34.

Table 12: Overview of the confidence intervals from the object recognition test during the social defeat.

| | | **95 % confidence interval** | | | |
|---|---|---|---|---|---|
| | | **entry ratio** | | **time ratio** | |
| **virus** | **condition** | **lower** | **upper** | **lower** | **upper** |
| **SCR** | **CON** | 0.6734 | 0.8400 | 0.6636 | 0.8428 |
| | **SD** | 0.5348 | 0.7621 | 0.5042 | 0.8052 |
| **shARC** | **CON** | 0.6315 | 0.7865 | 0.6983 | 0.8217 |
| | **SD** | 0.7015 | 0.8439 | 0.6973 | 0.9341 |

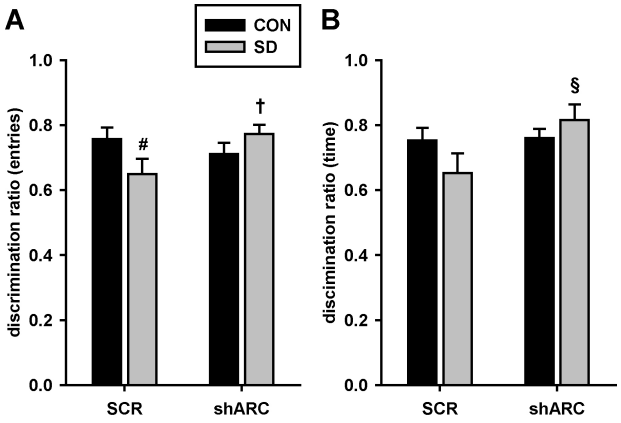


Figure 34: Results from the OR test performed in the last week of social defeat. **(A)** Exploration ratio calculated from the entries to the zone near the objects. **(B)** Exploration ratio calculated from the time spent in the zone near the objects. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM; # different (trend) to control animals within the same virus group $p < 0.1$; † significantly different to SCR-injected animals within the same condition $p < 0.05$; § different (trend) to SCR-injected animals within the same condition $p < 0.1$.

#### *3.3.2.6.3 Sociability test*

The sociability test was done to gain knowledge about the degree of motivation the animals show for social behaviour and interaction. The investigated parameter here is a ratio of interaction towards another mouse compared to both the other mouse and a dummy mouse. For the ratio based on the number of social interactions, ANOVA found a significant condition × virus interaction effect ($F_{1,33} = 4.512$, $p < 0.05$), while for the ratio based on the total time of interaction a condition as well as a virus effect were found (condition: $F_{1,33} = 4.668$, $p < 0.05$; virus: $F_{1,33} = 5.700$, $p < 0.05$). Post-hoc testing showed that SCR-injected stress animals showed a higher degree of interaction (number: $t_{15} = 2.118$, $p = 0.051$; time: $t_{15} = 2.566$, $p < 0.05$) and that within the stress condition, animals injected with the shARC construct show a lower degree of interaction than animals injected with SCR virus (number: $t_{14} = 2.213$, $p < 0.05$; time: $t_{14} = 2.310$, $p < 0.05$), as shown in Figure 35. Also for the sociability ratios, the confidence intervals were computed. The results show that only the animals from the stressed, SCR-injected group show a preference towards the social animal (see Table 13).

Table 13: Overview of the confidence intervals from the sociability test during the social defeat.

| | | **95 % confidence interval** | | | |
|---|---|---|---|---|---|
| | | **number ratio** | | **time ratio** | |
| **virus** | **condition** | **lower** | **upper** | **lower** | **upper** |
| **SCR** | **CON** | 0.418 | 0.660 | 0.363 | 0.723 |
| | **SD** | 0.579 | 0.821 | 0.689 | 0.934 |
| **shARC** | **CON** | 0.481 | 0.654 | 0.333 | 0.587 |
| | **SD** | 0.329 | 0.661 | 0.291 | 0.759 |

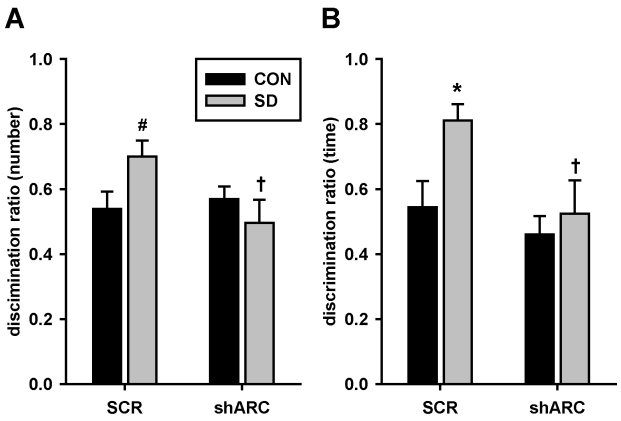


Figure 35: Results from the sociability test performed in the last week of social defeat. **(A)** Discrimination ratio calculated from the number of social interactions. **(B)** Discrimination ratio calculated from the total time of social interactions. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM; * significantly different to control animals within the same virus group $p < 0.05$; # different (trend) to control animals within the same virus group $p < 0.1$; † significantly different to SCR-injected animals within the same condition $p < 0.05$.

#### *3.3.2.6.4 Forced swim test*

The forced swim test was performed to investigate potential differences in coping styles. For three different behaviours - struggling, swimming and floating - two aspects were analysed: the number of behavioural bouts and the total time of the different behaviours. Animal 14 (from the CON shARC group) was injured during the tail-cutting procedure and omitted from the FST to prevent infection of the wound. ANOVA found no condition effect in any of the parameters, but a virus effect for the time struggling ($F_{1,32} = 6.277$, $p < 0.05$) and a trend towards a condition $\times$ virus effect also for the time struggling ($F_{1,32} = 3.236$, $p = 0.081$). Post-hoc testing showed a trend towards shorter struggling time in stressed animals injected with the shARC construct compared to control animals ($t_{17} = 1.987$, $p = 0.063$) and compared to SCR-injected animals ($t_{14} = 2.654$, $p < 0.05$), as seen in Figure 36.

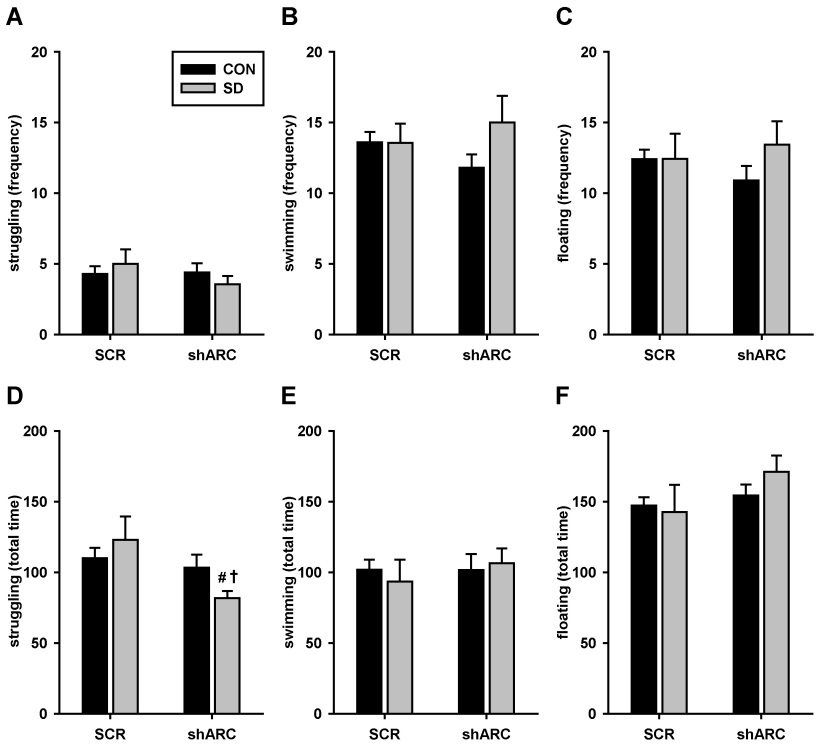


Figure 36: Results from the forced swim test performed in the last week of social defeat. **(A)** Frequency of the struggling behaviour. **(B)** Frequency of the swimming behaviour. **(C)** Frequency of the floating behaviour. **(D)** Total time the animal showed struggling behaviour. **(E)** Total time the animal showed swimming behaviour. **(F)** Total time the animal showed floating behaviour. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM; # different (trend) to control animals within the same virus group $p < 0.1$; † significantly different to SCR-injected animals within the same condition $p < 0.05$.

#### 3.3.2.7 Behaviour directly after the social defeat

##### 3.3.2.7.1 Sucrose preference test

The sucrose preference test is used to assess anhedonic behaviour. Repeated measures ANOVA revealed an effect of time ($F_{9,360} = 28.095$, $p < 0.01$) but no other within or between subject effect. Figure 37 shows the time course of the test.

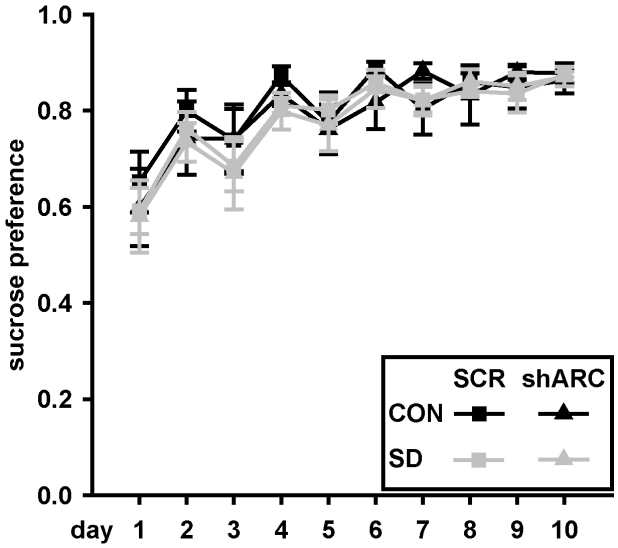


Figure 37: Results from the sucrose preference test performed directly after the social defeat. Sucrose preference describes the ratio sucrose solution is consumed compared to total fluid intake. The days do not describe a calendar day, but an interval of roughly 24 hours. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean ± SEM.

#### 3.3.2.8 Behaviour after recovery

##### 3.3.2.8.1 Open field test

The open field test was performed in the same way as during the stress procedure. In the morning session, no effect was found with ANOVA for the travelled distance (neither total nor split), entries in the inner zone (total) or time in the inner zone (neither total nor split), as seen in Figure 38. We found a trend towards a virus effect for the first segment of the inner zone entries ($F_{1,33} = 2.974$, $p = 0.094$). Further post-hoc testing of this variable did not yield any significant results. As before, the test was repeated in the afternoon. For the travelled distance, ANOVA showed a trend towards a condition effect in the last segment (Seg3: $F_{1,33} = 3.137$, $p = 0.086$). Post-hoc testing found a trend towards lower levels of locomotion (Seg3: $t_{15} = 1.923$, $p = 0.074$) in stresses SCR-injected animals compared to the relevant controls. For the entries in the inner zone, ANOVA revealed a trend towards a condition $\times$ virus interaction effect (Total: $F_{1,33} = 3.529$, $p = 0.069$; Seg2: $F_{1,33} = 2.970$, $p = 0.094$). Further investigations showed that SCR-injected stress animals explore less than their control counterparts (Total: $t_{15} = 2.062$, $p = 0.057$) and that within the control animals, individuals injected with shARC enter the inner zone less often than SCR-injected animals (Seg2: $t_{19} = 1.861$, $p = 0.078$). In addition, ANOVA showed a significant condition $\times$ virus interaction effect for the time spent in the inner zone (Total: $F_{1,33} = 6.171$, $p < 0.05$; Seg3: $F_{1,33} = 3.303$, $p = 0.078$). Post-hoc testing confirmed that SD animals injected with SCR viral construct spent less time in the inner zone than their control mates (Total: $t_{15} = 2.385$, $p < 0.05$). In addition, shARC-injected animals spent less time in the inner zone than SCR-injected animals, although only in the control condition (Total: $t_{19} = 2.144$, $p < 0.05$). Finally, the analysis comparing the morning and the afternoon session found a trend towards a condition $\times$ virus interaction effect ($F_{1,33} = 3.003$, $p = 0.092$) for the change in travelled distance, with no significant effects in the post-hoc test (data not shown as direct comparison in the figure).

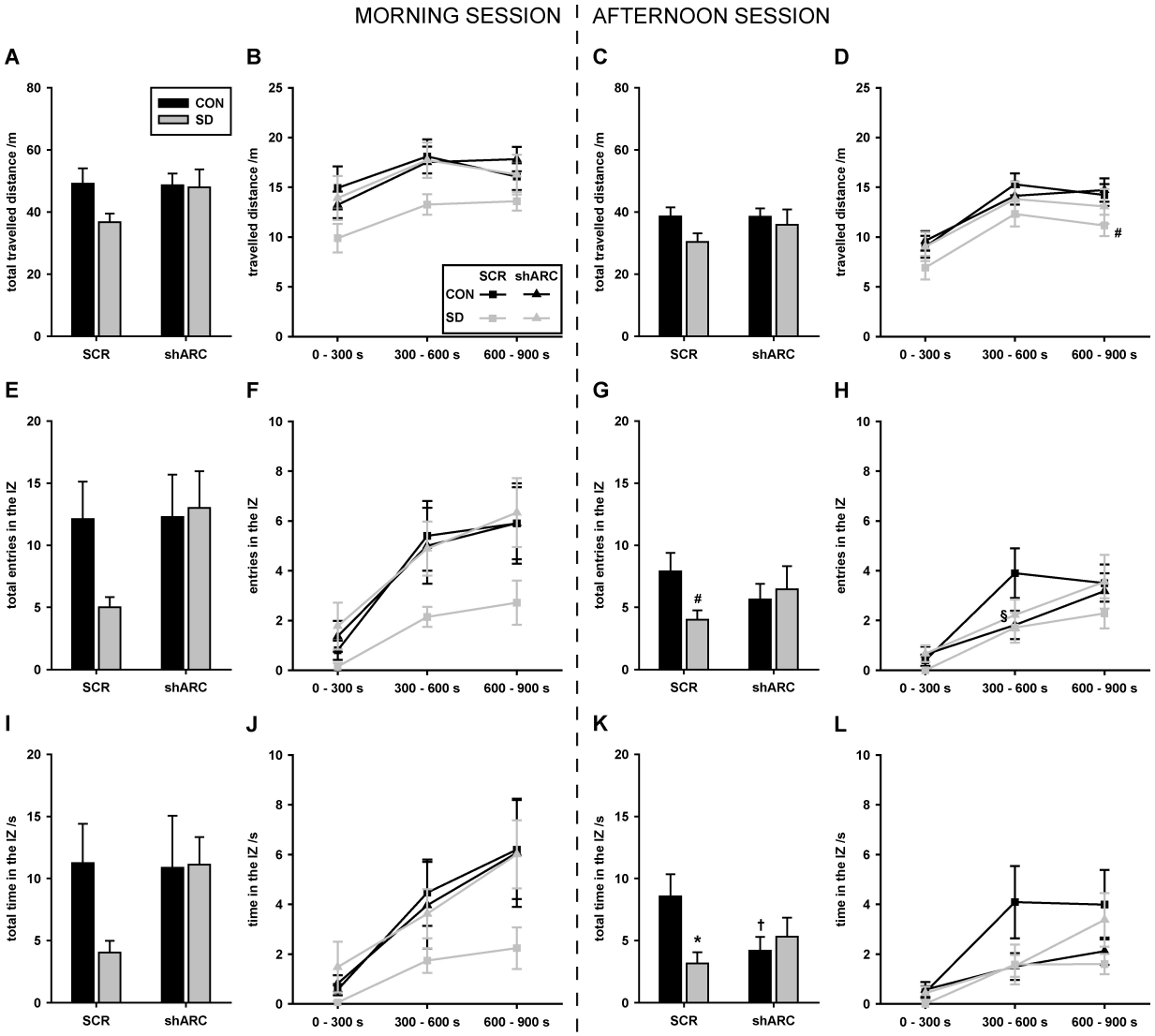


Figure 38: Results from the OF test performed after 3 weeks of recovery. **(A)** and **(B)** Distance travelled in total and split in segments, respectively, from the morning session. **(C)** and **(D)** Distance travelled in total and split in segments, respectively, from the afternoon session. **(E)** and **(F)** Entries in the inner zone in total and split in segments, respectively, from the morning session. **(G)** and **(H)** Entries in the inner zone in total and split in segments, respectively, from the afternoon session. **(I)** and **(J)** Time spent in the inner zone in total and split in segments, respectively, from the morning session. **(K)** and **(L)** Time spent in the inner zone in total and split in segments, respectively, from the afternoon session. For details on the statistics, please refer to the text. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM (total) or mean ± SEM (segments); * significantly different to control animals within the same virus group $p < 0.05$; # different (trend) to control animals within the same virus group $p < 0.1$; † significantly different to SCR-injected animals within the same condition $p < 0.05$; § different (trend) to SCR-injected animals within the same condition $p < 0.1$.

#### *3.3.2.8.2 Object recognition test*

The object recognition test was also repeated. The confidence intervals were calculated, showing that all the groups showed proper learning behaviour (see Table 14). ANOVA showed no significant differences between the groups, as depicted in Figure 39.

Table 14: Overview of the confidence intervals from the object recognition test after recovery.

| | | **95 % confidence interval** | | | |
|---|---|---|---|---|---|
| | | **entry ratio** | | **time ratio** | |
| **virus** | **condition** | **lower** | **upper** | **lower** | **upper** |
| **SCR** | **CON** | 0.5925 | 0.8515 | 0.6431 | 0.8769 |
| | **SD** | 0.5736 | 0.7810 | 0.5003 | 0.8386 |
| **shARC** | **CON** | 0.6223 | 0.8491 | 0.5777 | 0.8463 |
| | **SD** | 0.6515 | 0.8593 | 0.5877 | 0.8692 |

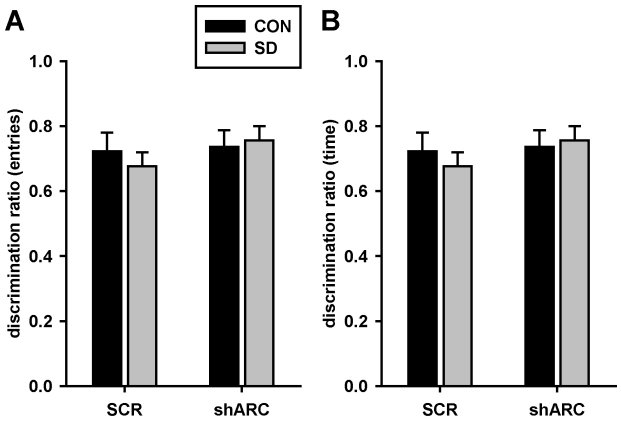


Figure 39: Results from the OR test performed after 3 weeks of recovery. **(A)** Discrimination ratio calculated from the entries to the zone near the objects. **(B)** Discrimination ratio calculated from the time spent in the zone near the objects. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM.

#### *3.3.2.8.3 Sociability test*

For the ratio based on the number of social interactions, ANOVA found no significant effects, but a trend was found for the ratio based on the total time of the interactions for both condition and virus (cond: $F_{1,33} = 3.762$, $p = 0.061$; virus: $F_{1,33} = 3.406$, $p < 0.074$). Post-hoc testing showed that SCR-injected stress animals showed a significantly higher degree of interaction ($t_{15} = 2.346$, $p < 0.05$), as described in Figure 40. Also for the sociability test, the confidence intervals were computed. The results show that only control animals injected with the SCR viral construct do not show a preference towards social interaction in both parameters (see Table 15).

Table 15: Overview of the confidence intervals from the sociability test after recovery.

| | | 95 % confidence interval | | | |
|---|---|---|---|---|---|
| | | number ratio | | time ratio | |
| **virus** | **condition** | **lower** | **upper** | **lower** | **upper** |
| **SCR** | **CON** | 0.4048 | 0.6814 | 0.3945 | 0.7786 |
| | **SD** | 0.5996 | 0.8064 | 0.7511 | 0.9214 |
| **shARC** | **CON** | 0.5228 | 0.6401 | 0.6117 | 0.8049 |
| | **SD** | 0.4469 | 0.7308 | 0.5460 | 0.8829 |

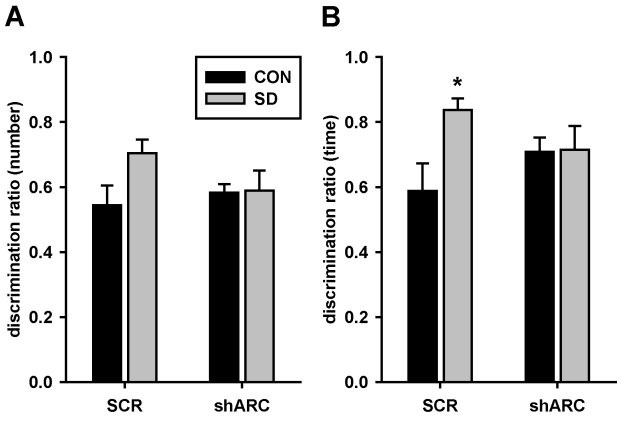


Figure 40: Results from the sociability test performed in the last week of social defeat. **(A)** Discrimination ratio calculated from the number of social interactions. **(B)** Discrimination ratio calculated from the total time of social interactions. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM; * significantly different to control animals within the same virus group $p < 0.05$.

#### *3.3.2.8.4 Forced swim test*

The forced swim test was performed after recovery to investigate potential differences in coping styles. For animals 27 and 43 (SD ARC and SD SCR, respectively) we encountered technical problems with the video recording, therefore these animals could not be analysed. ANOVA found a condition effect for the struggling frequency ($F_{1,31} = 6.185$, $p < 0.05$), a trend towards a virus effect for the struggling frequency ($F_{1,31} = 3.441$, $p = 0.073$) and a trend towards a condition $\times$ virus effect for the time struggling ($F_{1,31} = 2.881$, $p = 0.100$). Post-hoc testing showed a trend towards shorter struggling time and struggling frequency in SCR-injected stress animals compared to control animals (frequency: $t_{15} = 1.966$, $p = 0.068$; time: $t_{15} = 1.894$, $p = 0.078$). Within the control group, animals injected with the shARC viral construct show lower levels of struggling compared to SCR-injected animals (frequency: $t_{18} = 1.802$, $p = 0.088$; time: $t_{18} = 2.551$, $p < 0.05$), as seen in Figure 41.

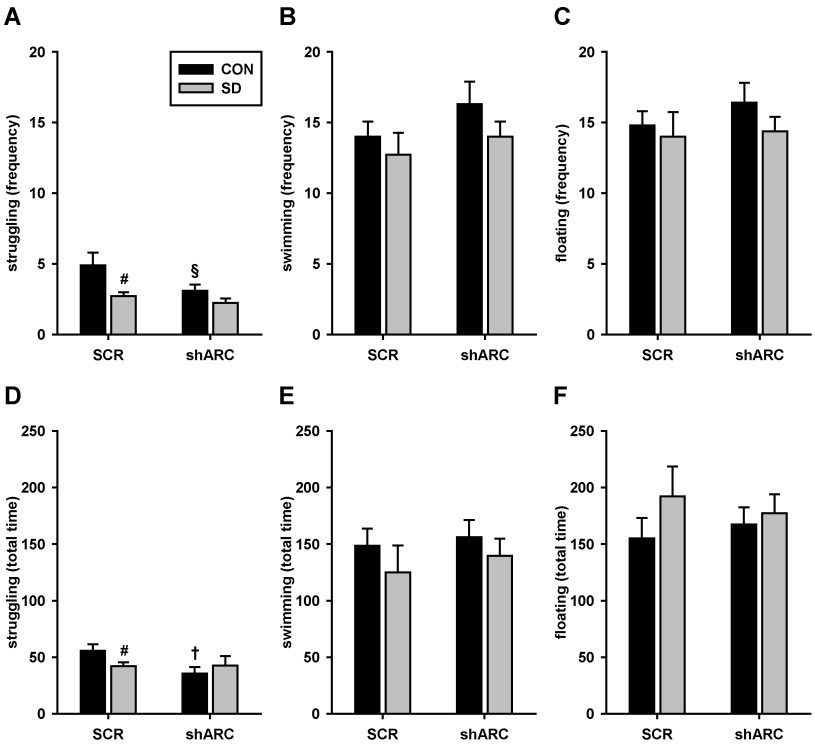


Figure 41: Results from the forced swim test performed in the last week of social defeat. **(A)** Frequency of the struggling behaviour. **(B)** Frequency of the swimming behaviour. **(C)** Frequency of the floating behaviour. **(D)** Total time the animal showed struggling behaviour. **(E)** Total time the animal showed swimming behaviour. **(F)** Total time the animal showed floating behaviour. CON = control; SD = social defeat; SCR = scrambled virus construct; shARC = short-hairpin ARC knockdown virus; Data are given in mean + SEM; # different (trend) to control animals within the same virus group $p < 0.1$; † significantly different to SCR-injected animals within the same condition $p < 0.05$; § different (trend) to SCR-injected animals within the same condition $p < 0.1$.

# 4 Discussion

## 4.1 Part 1: Long-term effects of stress exposure on gene transcription in the hippocampus

In the present study, we examined the effects of chronic social stress exposure in adolescent male mice on gene expression in the CA1 and DG subregions of the hippocampus. We were able to identify some stress-regulated transcripts via microarray in the discovery sample, however we were not able to replicate the results. This leads to 3 main possibilities: (1) We were not able to detect consistent differences due to technical difficulties; (2) Our hypothesis was false - no differences in gene expression arise in male mice 5 weeks and 12 months after exposure to chronic social stress during the adolescence; Or (3) our initial hypothesis is true - but only for a subset of animals.

### 4.1.1 Microarray - the pitfalls of whole genome expression studies

Technical variation is a problem in every empiric study and can only be controlled up to a certain level. The first problematic level of variation in our study was the quality of the RNA. While it looked OK by visual inspection of the electrophoreses curves of the BioAnalyzer, a systematic analysis via the corresponding software revealed only mediocre quality of the isolated RNA, a RIN roughly around 5. This was probably resulting from the LMPC method - while this method achieves very high regional specificity, other methods offer better RNA quality. Many researchers in the field now agree that the quality of the mRNA is probably the most important factor for the validity of the results and studies have shown that already at a RIN of 7.5, results can become unreliable (Medeiros et al., 2007; Copois et al., 2007). Unfortunately, we do not have any information about the RNA quality for the qRT-PCR validation in this experiment, however we know from other studies using the same protocol that the RNA quality is very good in that case (RIN around 9).

The next possible source of variation were the microarray chips themselves. As described in 3.1.1.3, only about 30 % of the sequences were of use to us. This is also reflected by the fact that from the first generation of chips (used in this study) to the second generation, about 31000 out of 40000 spotted sequences were changed (Eggle et al., 2009).

A common problem of whole genome analyses is the statistical background. Due to the huge number of single contrasts (roughly 46000 in our case), measures for correction of multiple testing must be applied. As classical methods like the Bonferroni correction might be too stringent for biological microarray studies, the FDR method has been used lately in various studies. While the results of classical methods normally yield a boolean result (true or false to a specific significance level), the FDR method ‘‘accepts’’ a number of false positive results but identifies a larger number of relevant genes. However, the theoretical value of 5 % was nowhere near our empiric value of up to 100 % false positives, depending on the method of validation.

Another factor might be the effect size of the differences. As we know from studies investigating the effect of stress exposure on single genes, the resulting changes are often not strong. For example the GR and MR in our model were found to be downregulated to about 80 % of the control value at the timepoint of the microarray. However, titration experiments have shown that

the detection capabilities of microarray technology show a steep decline for faint effects (Shippy et al., 2006)(P. Weber - personal communication). This might also be one of the reasons that microarray studies in samples with high effect sizes, for example tumor tissues in comparison to normal tissues, revealed more fruitful results up to now (Ratsch et al., 2011).
One of the reasons for the lack of validation might be the sample selection. As the qRT-PCR was done with tissue samples from a different cohort, this is a source of variation by itself. Nevertheless, especially for whole-genome studies, validation in a different cohort is absolutely necessary to decrease the risk of a cohort effect an yield meaningful results. Another point may be the difference in samples. While for the microarray LMPC material from the CA1 and DG regions were used, the qRT-PCR was performed on mRNA extracts from the whole hippocampus. Not only does the whole hippocampus lysate include cells from other hippocampal regions, but also the cells from the dorsal and the ventral parts were mixed. The LMPC for the microarray was done in slices exclusively from the dorsal part of the hippocampus, which might lead to different results as it has recently been shown that the dorsal and ventral part substantially differ in functionality and also gene expression (Fanselow and Dong, 2010).
The puzzling part remains the validation of the results via *in situ* hybridisation. The validation was done on slides from the same animals also used for the microarray and due to the nature of the ISH, regional specificity is given. Some authors have described differences in gene expression results depending on the location of the alignment sequence in the gene, but as the designated sequences for both the qRT-PCR and the *in situ* hybridisation were selected with the utmost care to either include the sequence from the microarray or lie in the direct vicinity, this cause seems unlikely.

### 4.1.2 Gene expression changes following chronic stress exposure

The notion that no differences in gene expression arose in our specific setting seems hardly probable. One of the main arguments against this hypothesis is the fact that we did find differences on a single gene level in studies with the exact same experimental setting. It has been found for example that the GR was downregulated in the CA1 region of the hippocampus in stressed mice 5 weeks after stress (Schmidt et al., 2007). Furthermore changes in gene expression in the hippocampus are commonly found after stress exposure, as described in the introduction. This downregulation has been confirmed via qRT-PCR in our validation sample, but was not detected via the microarray in the discovery sample, which also points to difficulties in the microarray. By now, whole-genome studies have been performed for various stress models and time points but yielding quite different results (see for expample (Bergström et al., 2007; Covington et al., 2011). While most studies find some sort of effects, the consensus between those studies is surprisingly small. So one may speculate that the stressed phenotype seen is not a homogenous phenotype, but that different paradigms do actually result in many different phenotypes with partly overlapping symptoms, which in turn are based on different genetic changes.

### 4.1.3 Subgroups within the stressed phenotypes

One reason for our results might be built upon biological variance. Recent studies in our group have shown that animals that underwent chronic stress exposure followed by 5 weeks of regeneration

can be divided into distinct subgroups. Some animals still showed elevated corticosterone levels 5 weeks after stress and were termed ‘‘stress vulnerable’’ while others recovered quickly and were termed ‘‘stress resilient’’ animals. Interestingly, it was shown that the vast majority of the stress effects, be it elevated CORT levels, enlarged adrenals, changes in gene expression or behavioural alterations, were present in the vulnerable animals only. In contrast, the resilient animals were not statistically different from controls in these parameters (Schmidt et al., 2010a). Extrapolated to our results, this might imply a selection bias. If our stress sample would include many vulnerable animals, the differences in gene expression would possibly be strong and in contrast when the ratio of resilient animals would be high, the differences might sink below the detection threshold. And indeed, when the whole cohort of animals for the experiment was analysed, CORT levels and other markers of stress were significantly higher in stressed animals than in controls, however the comparison of only the animals randomly selected for the microarray revealed no such difference between the groups. This is a strong hint that the ratio of stress resilient animals was high in the stress group, which potentially might attenuate differences in gene expression. In contrast, in the selection of animals for the qRT-PCR, animals with higher CORT levels were preferred. Nevertheless, no difference was found for adrenal gland weight and thymus weight. This might also explain some conflicting results between microarray and qRT-PCR.

### 4.1.4 Iffo1 - a candidate for future studies?

The only gene that showed at least some consistent effects was Iffo1. Virtually nothing is known about Iffo1, or intermediate filament family orphan 1. Our literature search found that only 2 publications so far describe the gene, both of them looking at protein networks, but not mentioning Iffo1 directly (Rual et al., 2005; Lim et al., 2006). It was found in the microarray in the discovery sample as well as in the qRT-PCR in the replication sample. However, while we found an upregulation by stress in the microarray, Iffo1 mRNA was downregulated in the qRT-PCR. No effect was found in ISH in the discovery sample. Due to the already discussed problems of the microarray and the difference in RNA quality, the rational decision must be to value the results from the qRT-PCR higher. This is supported by the fact that we also found downregulation of Iffo1 mRNA following acute stress. It might also be speculated that Iffo1 expression is mainly affected by stress differently in the dorsal and ventral part of the hippocampus. Both the microarray as well as the ISH of the long-term stress effects were performed mainly in the dorsal part. Here we found an upregulation and no effect respectively. The qRT-PCR, which included material of both regions, and the acute stress ISH (the slides here were mainly from the ventral part of the hippocampus) showed a downregulation of Iffo1 mRNA. Lastly, we investigated the timecourse of Iffo1 following GR activation. Here, we showed that Iffo1 expression can be induced via dexamethasone. However, this induction was mainly present in the CA1 and DG regions 4 h after injection. This was the same timepoint as in the restraint stress group, where we found a downregulation of Iffo1 mRNA. Again, this might be explained by differences in dorsal and ventral hippocampus, although the difference in distance between those two samples was relatively small. Interestingly, DEX treatment caused lower levels of Iffo1 in the CA3 region 8 h after injection. This also supports different roles of Iffo1 in different regions of the hippocampus and hints at compensatory mechanisms. From this regional specificity we can also deduct certain theories

about the microarray. As we saw that Iffo1 was upregulated following GR activation only in the CA1 and DG regions and we used exactly those two regions for the microarray, this might explain our finding of increased levels of Iffo1 in the microarray. This might be supported by the fact that the induction of Iffo1 was much stronger in the CA1 region than in the DG. Indeed, we only found Iffo1 regulated in the CA1, but not in the DG region. Furthermore, this might also explain the downregulation in the qRT-PCR. While for the microarray only the CA1 and DG regions were investigated, the samples for the qRT-PCR included the whole hippocampus - thereby also large amounts of cells of the CA3 region. This effect might be amplified by the fact that Iffo1 expression is highest in the CA3 region. Therefore, it is likely that the downregulation of Iffo1 that we saw in the qRT-PCR was solely originating from the CA3 region and masked a potential upregulation in the CA1 and DG region. However, this hypothesis fails to explain the downregulation of Iffo1 in the CA1 and DG after acute stress.
It might also be fruitful to speculate about the influence of the absolute CORT levels on Iffo1 expression. In the discovery sample, we saw an upregulation or no effect in Iffo1 expression depending in the technique. From the subgroup analysis we know that CORT levels were relatively low, even in the stressed animals. In contrast, the qRT-PCR and acute stress experiments found a downregulation. We know from these groups that the CORT levels were quite high (CORT levels from the restraint and food deprivation experiments were published in (Scharf et al., 2011)). This might suggest a biphasic response to CORT, with an increase at low levels and a decrease at high levels. However, the results from the DEX timecourse do not fit the picture and suggest a more complex interaction.
All in all, the data indicates that Iffo1 is differentially regulated by stress in the different subregions of the hippocampus. It seems plausible that in response to stress, Iffo1 gene expression is induced in the dorsal part of the hippocampus, presumably in the CA1 and DG regions, while the expression is decreased in the ventral part of the hippocampus, probably mainly in the CA3 region. Further studies are needed to investigate these hypotheses.

### 4.1.5 Conclusions

Taken together, we were not able to show consistent differences in gene expression in male mice that underwent aolescent chronic social stress 5 weeks and 12 months after the stress exposure. And while the quality of the microarrays was far from optimal, it was most probably in combination with the random selection of the animals that caused the effects to be to low to be detected. Therefore our initial hypothesis ''*Chronic social stress in adolescent male mice causes long-term differences in the transcriptome in the hippocampus*'' needs to be rejected and adapted to ''*Chronic social stress in adolescent male mice causes long-term differences in the transcriptome in the hippocampus*, but only in a subgroup of animals''. In the following part, we will try to further illucidate alterations in gene expression but pay a close regard to distinct subgroups in the stressed cohort.

## 4.2 Part 2: Transcriptome differences between stress-vulnerable and stress-resilient animals

In the second experiment of the thesis, we investigated genetic differences between stress-vulnerable and stress-resilient animals. Here, we were able to show that the expression of multiple genes is different between the subgroups in the hippocampal CA1 and DG fields. From the sequence analysis and the validation we saw that the Arc gene is one of the strongest candidates for further studies. In addition, we also looked at differences in gene expression in lymphocytes from peripheral blood. While no significant differences were found, we selected some genes based on an interesting expression profile for validation and were able to validate Clec4a3, Hsp90b1, Sla and Casp1. In the final stage of the experiment, we compared the expression profiles from the blood and from the brain. We found correlations between genes potentially involved in vulnerability in the brain with genes in the blood. Interestingly, the correlations were subgroup-specific, supporting the differences between stress-resilient and stress-vulnerable animals.

### 4.2.1 Differences in the hippocampus

After our initial findings comparing control with stress animals, we now focused on differences within the stressed phenotype. Therefore, we focused on animals that are strongly affected by the stress exposure and compared them with animals that do not seem to be affected by the stress experience. Corticosterone is an important effector in the stress system and was used as our classification criterion here. Although corticosterone alone is only a single parameter, studies from our group have shown that also other parameters, like central gene expression, behaviour or endocrine measurements vary when animals are selected based on their respective CORT levels (Schmidt et al., 2010a). As already discussed in the introduction, stress-vulnerable animals often exclusively show stress-induced effects while stress-resilient animals strongly resemble the controls. However, this is not the case for all parameters and in some cases the stress-vulnerable animals resemble the controls while stress-resilient animals differ from controls. So just by selecting via corticosterone levels, we probably compared true phenotypes that differ in stress vulnerability.
Interestingly, we often found differences in untranslated regions flanking the gene, but no differences for sequences that target the gene directly. These - on the first glance - conflicting results are very interesting. As in most cases the detection levels were quite similar for the UTR and the coding region, the difference is probably in the binding ‘‘availability’’ of the sequences. So in the case that we find differences in the UTR region, but not in the sequences targeting the coding region, like in the case of Gria1, this might indicate that: (i) the total number of mRNA molecules are the same (thereby the number of UTR sequences should also be the same) and (ii) that the sequences in the UTR are better accessible in one of the groups. One reason for this might be binding of either RNA-binding proteins (RBP) or micro-RNA molecules (miRNA) to the UTR sequence (Lee and Gorospe, 2011). MiRNA for example can bind to the 5’-UTR sequence and modulate the translation of the mRNA (rom et al., 2008). So it seems possible that the differences we see in the phenotype, which are probably mainly caused by differences on the protein level, are at least partly regulated not via differences in transcription *per se*, but via different regulation of the translation by small molecules modifying the mRNA. Altough not much is known about the influence of miRNA molecules on stress vulnerability, some studies have been

performed. For example a study by Uchida and colleagues showed that in rats, REST4 levels were increased in the mPFC following 180 min of maternal separation (Uchida et al., 2010). REST has been shown to regulate miRNAs in the brain and was implicated in synaptic plasticity (Conaco, 2006). Interestingly, the authors showed that OE of REST4 caused an increase in vulnerability, which was accompanied by differences in various miRNA molecules in the mPFC. Therefore, an influence of small RNA modulating molecules on stress vulnerability seems plausible. This form of regulation might also be responsible for some of the negative results in the validation. Almost all of the sequences for the qRT-PCR were designed to be intron-spanning, therefore only detecting the number of mRNA molecules.
It also might be possible that some exon probes were not specific, despite showing a good absolute detection signal. Actually, we found that for most of the genes with more than 1 spotted sequence, the results from the different probes were not overlapping.

### 4.2.2 Differences in the blood

We also investigated the differences in gene expression in peripheral blood. Here, we found no significant differences in the microarray. However, detecting relevant gene expression differences in data with large amounts of variables is a process that is still not fully understood and statistical descriptions are still being created and refined. Therefore, after applying a false discovery rate analysis yielding no results, we scanned the data by hand and selected genes that (i) showed a medium to high absolute detection, (ii) showed a reasonable clustering within the different groups and (iii) were represented by a solid sequence on the microarray. So despite the fact that the statistical analysis did not find any significant differences following correction for multiple testing, we were able to validate 4 out of the 10 selected genes in the qRT-PCR. This clearly highlights the need for better statistical techniques for analysis of data with a biological background.
It should also be mentioned that we did not find an overlap from the results from the vulnerability genes in the hippocampus and the vulnerability genes in peripheral blood. This is not too surprising as genes in the brain often have different functions than in the blood.
It is intriguing to speculate about the function of these differences in the blood, especially in the context of stress vulnerability. The most obvious influence might be bioavailability of CORT. Corticosterone is one of the major effectors in the stress system and is transported via the blood. However, only a small fraction of CORT is freely available in the blood and able to exert its hormone actions. The rest is bound to corticosterone binding protein (CBP) and thus prevented from playing its biological role, creating yet another mechanism to tightly control bodily functions. So it might be possible that the changes in the gene expression between the phenotypes might ultimately result in different bioavailability of CORT. Nevertheless it is still unclear how gene expression changes in lymphocytes might influence CBP function. Another hypothesis is the sensitivity to CORT. The gene expression changes we saw might reflect or result in different responses to corticosterone in those cells. This in turn can have a plethora of effects, for example differences in apoptosis and proliferation (D'Elia et al., 2010).

### 4.2.3 Correlations between brain and blood

One of the most fascinating aspects of the present experiment was the subgroup-specific correlations between the brain and blood gene expressions. Nevertheless, correlations should be interpreted with caution. It is easy to imply direct relations or even causality into these effects, however this is not correct. One should always keep in mind that correlations do not provide information about the direction of an effect (variable A influencing variable B results in the same correlation as variable B influencing variable A) or potential underlying factors (variable C influences both variables A and B). One example to always be aware of the data can be seen in the combined contrast matrix. Here, we saw correlations with the CORT levels 5 weeks after stress and some gene expression levels. But on second glance, nearly all the genes that correlate are the ones that show different expression patterns between stress-vulnerable and stress-resilient animals. This creates two distinct particle clouds in a 2D plot, resulting in a strong correlation. However, when the single clouds are analysed separately, this correlation does not hold true for most of the investigated genes, implying that no general correlation is present, only an artifact created by our selection of the animals.
We also saw some strong correlations between the genes within one domain (blood or brain). This is to be expected. When all the genes investigated here influence the phenotype, the single genes naturally create a specific gene expression pattern that should look relatively similar for the same phenotypes. One can also deduct some information about the quality and validity of the qRT-PCR samples from those correlations. As described in the results, we had to repeat the reverse transcription for the samples two times resulting in three different sets of samples due to technical difficulties. And as seen from the results section, we found different results for the same genes in different sets. We can also clearly see these differences, in the correlation analysis. The cluster of strongly correlating genes in the brain (Ntrk3 to Homer1) exactly describes the border to the set of genes investigated in the second and third reverse transcription. So what we see are strong correlations within one reverse transcription set, for the samples from the first and third reverse transcription. Interestingly, no such correlations were found for the samples from the second reverse transcription. To extrapolate these findings to the quality and the validity is difficult. Strong correlations could either mean valid data due to influence of the phenotype as described before, but might also suggest unspecificity of the results. Further studies are needed for a more specific statement.
Probably the most useful information we can deduct from the analysis of patterns and changes in patterns between the different subgroups. One of these changes is the uncoupling of correlations within domains in the subgroups. We saw this for Arc in the resilient animals and Tm7sf3 and Gabrg2 in the samples from the vulnerable animals. This might for example suggest an interaction effect with the phenotype. Assuming that the cluster of correlations really represents a pattern of expression underlying the phenotype, the uncoupling suggests a function of the gene in only one subgroup or different functions in the different subgroups. Let's use Arc as an example. Our data strongly suggests that vulnerable animals show a higher expression of Arc than resilient animals in the hippocampus. We also see a correlation of Arc with the other brain genes in vulnerable, but not in resilient animals. This might suggest a threshold model. So it might be possible that animals expressing high levels of Arc are stress-vulnerable, which is mirrored by high

or low expression of other vulnerability genes causing the correlations we see (this also implies an additive model of vulnerability). However, if the levels of Arc fall below a certain point (or better: range), the molecular machinery conveying high stress vulnerability loses functionality. In this case, it would not matter if other vulnerability genes would be strongly expressed - as they would not be able to induce their influence on stress vulnerability. This would also suggest a critical role of Arc in the process.
Furthermore, while some correlations are lost, new ones arise. This is especially interesting due to the fact that we now see correlations between the blood and the brain - however only when looking at subgroups. The most prominent example of gene expression in the blood is Hsp90b1. While almost no correlations can be found in the vulnerable animals, multiple gene expression levels in the hippocampus correlate with Hsp90b1 expression in the resilient animals. This again might suggest an interaction effect. It seems that Hsp90b1 is closely regulated together with vulnerability factors in the brain, although only in resilient animals. Hsp90b1 is involved in the co-complex of the GR and might therefore influence GR dynamics. It was also found to be significantly downregulated in the blood of vulnerable animals. So if Hsp90b1 levels fall below a certain critical range, stress resilience might be disturbed. This might explain why in vulnerable animals, no correlation is found.
The other way around, we found for example Npy5r expression to correlate with multiple blood gene expression levels. While the information from this direction is the same, its practical use is better. If these findings would translate into human research, it would enable researchers to predict Npy5r levels in the brain - just by investigating peripheral blood samples. A feat that is otherwise nearly impossible to achieve. For this case however, one would need information about the stress vulnerability of the patient.
An interesting feature is that we find correlations in the resilient subgroup. In this group, the variation between the samples is probably higher than in the vulnerable animals. While the vulnerable animals clearly represent the top 20 % of the CORT spectrum of the given population, selecting the animals from the resilient group is not so straightforward. Here, the majority of the animals are densely clustered regarding CORT levels. Furthermore, the lowest levels of CORT at the time of 5 weeks after stress often lie in ranges that are in the or below the lower detection range of the RIA. This problem is aggravated by the pulsatile release of corticosterone (Lightman et al., 2008). The possibility remains that a single measurement of corticosterone might just coincide with a nadir of secretion, confounding the results. Therefore, we would expect the rate of false positive detection to be higher in the resilient subgroup or we expect the population to be more diverse.
The last section also already stretches another point - the fact that we did not use a control group in this study. As we have demonstrated that vulnerable and resilient sub-groups exist in this and other studies, we face the problem of causality. From experiment 2 alone, we cannot decide if the changes we find are induced by the stress exposure or if they are innate. Surely, some of the genetic markup that influences the stress vulnerability of the individuals must be present before the stress exposure, hence creating the phenotypic differences we found. Therefore, it is clear that also the control group must include stress-resilient and stress-vulnerable animals - however we have at the moment no means of detecting this state other than stressing the animals. As a

correct control group, we would need separate control groups for the resilient and the vulnerable animals, but as we were not able to pair those groups, control groups were omitted.

### 4.2.4 Vulnerability or CORT response?

Due to our study design, we must face the question whether the effects we see reflect an innate state of vulnerability or just an response to CORT. Our vulnerable and resilient phenotypes are by definition also ''high CORT'' and 'low CORT'' conditions and therefore we cannot rule out the possibility of a simple CORT response without further experiments. However, we saw some aspects that hint at vulnerability instead of CORT response for some cases. The most prominent finding for this is the lack of correlations to the CORT levels at the time of sacrifice in most cases. While we saw some correlations in the combined analysis, those correlations mostly disappeared in the sub-group specific analyses. This seems plausible for the resilient group, as in this case the clustering of the CORT levels was very dense (ranging roughly from 0 to 5 ng/ml) hampering the detection of a correlation, but in the case of the vulnerable subgroup, the individual values were widely spread (a range of 50 ng/ml or higher), comparable even to the comparison of stress and control animals, which should allow for a safe calculation of the correlations. Nevertheless, we did not find a correlation to CORT levels 5 weeks after stress with most of the genes. In addition, we did not find a regulation of genes known to be regulated by chronic exposure to high CORT levels, like the GR or the MR, in our microarray. All of this supports the notion that we do see a phenotype of vulnerability and not just an response to CORT.

### 4.2.5 Arc as a candidate

The gene that sparked our interest most was Arc. In the microarray, we found a robust underrepresentation of the gene in the hippocampal CA1 region of vulnerable animals. Although we had a very high degree of variation in the validation, we found Arc to be regulated in the same direction in all the qRT-PCR runs. Also, the characteristics known from the literature and described in the introduction about Arc make it a very interesting candiate indeed.

### 4.2.6 Conclusions

In this experiment, we were able to show that stress-resilient and stress-vulnerable animals differ in their genetic profiles. We were also able to show that some correlations exist between gene expression in the brain and gene expression in the blood - these however are strictly dependent on the sub-group of the individual. The gene that caught our attention the most was Arc. Not only did we find a robust regulation in both the microarray and the validation, we also found the potential interaction effect in the correlation analysis. The hypothesis for this experiment was ''*Chronic social stress in adolescent male mice causes long-term differences in the transcriptome in the hippocampus, but only in a subgroup of animals*''. This can be confirmed based on the results from the experiment. Now, the question about the molecular effects of the genes we found

emerges. However, before we can delve deeper in the molecular mechanisms of these genes, we first need to address a more pressing question - the question of causality. For further studies, we need to clarify if the effects we saw were stress-induced changes in gene expression that do not influence the response to future stress exposure, which by itself is an interesting research field, but not the main topic of this study, or if the differences were already present before the stress and - at least partly - influenced the response to the stress and the recovery, resulting in the phenotypes we observed. Hence, the question of causality will be investigated in the next part of the study using Arc as a candidate gene.

## 4.3 Part 3: Causal influence of Arc on stress vulnerability

In the third and final part of this thesis, we investigated the Arc gene in more detail. We were especially interested in the potential causal relationship of Arc in stress vulnerability. As we knew from the previous studies that Arc mRNA expression is higher in vulnerable animals, our working hypothesis for this part was: *Knockdown of Arc expression in the hippocampus shifts the animals towards stress resilience and protects them from stress-induced changes.*

### 4.3.1 Investigation of causality - a new design for a new research question

To test this hypothesis, we used social defeat instead of the chronic social stress paradigm in which our original results were found. This was done for multiple reasons. In the previous studies, we repeatedly found that Arc expression was higher in vulnerable animals, however it was always measured after the actual stress exposure. From these studies, we cannot exclude the possibility that Arc expression was the same in both groups before the stress exposure and was consequently altered by the episode of stress, giving information about the influence of stress, but not about stress vulnerability. Therefore, the aim of this study was to modulate the Arc expression before the actual stress exposure. As already described in the introduction, the induction of Arc can be quite generic and also in the transport to the synapse, a multitude of factors are involved that are not exclusively targeting Arc. This renders it - to our best knowledge - virtually impossible to modify Arc expression pharmacologically without majorly affecting other genes or proteins. And while knockout animals are described in the literature Plath et al. (2006), none of the available animals show a knockout specifically in the hippocampus, our region of interest. Therefore, we decided to modulate Arc expression via viral-mediated shRNA knockdown. The latter has been successfully applied before and is excellent for *in vivo* knockdown of genes, due to its high protein and regional specificity (Sliva and Schnierle, 2010). However, the use of this technique excluded the chronic social stress paradigm, as it begins during the juvenile period of the animals at 4 weeks of age, resulting in two major flaws for this design: First of all, the animals need some time to recover from the surgery and it takes some time until viral expression - and thereby knockdown - reaches a certain threshold. We would have had to do surgery on the animals at latest at the age of 1 week, which would strongly influence the development of the animals, not even mentioning the vast technical difficulties. Secondly, it has been shown that Arc is critical in embryogenesis (Liu et al., 2000) and that Arc levels reach peak levels at pnd21 (Lyford et al., 1995), showing its influence in developmental processes. By modifying the Arc expression at such a young age, we would put further pressure on the developmental system, causing potential confounding factors

for the study. While the role of Arc during development for stress vulnerability later in life would be interesting, it lies beyond the scope of this thesis.

### 4.3.2 Arc knockdown partially prevents stress-induced changes in behaviour

In the last experiment, animals were phenoytped thoroughly using multiple behavioural tests. Here, we saw that the stress exposure caused a steep increase in anxiety-like behaviour in the open field test, which was present even after the recovery period (Dear reader: The data here are discussed on the basis that we saw a difference in the morning session of the OF test after recovery. From a pure statistical point of view, this is not correct. We did not find any effect in the ANOVA, preventing a post hoc analysis. However, based on the distribution of the data and in concordance with the previous test, I do believe this is a false negative result (type II error).). Increases in anxiety-like behaviour following social defeat or social disruption (comparable with social defeat: the test mouse is the resident and an aggressive intruder is introduced to the home cage) have been described in the literature (Kinsey et al., 2007; AJ and S, 1999). In addition, social defeat caused hypolocomotion, which is also in line with multiple previous studies (Carboni et al., 2006; Pistovcakova et al., 2005; AJ and S, 1999). Interestingly, knocking down Arc in the hippocampal formation prevented the increase in anxiety-like behaviour while for the hypolocomotion, the effects can be described as partial response or intermediate phenotype. This suggests that Arc has both a specific, as well as a general component in mediating stress vulnerability in the hippocampus. Another interesting aspect we found in the OF test was that stressed animals (injected with the SCR construct) showed the high anxiety-like phenotype in the afternoon already during the first 5 minutes of the test. Based on the findings from this experiment and from the experience with this setup in general, we normally see the phenotype in the second or third section of the test. This might suggest that these animals are unable to effectively shut down the stress system, activated during the morning session. While we did see that the feedback of corticosterone secretion is impaired in stressed animals, we only saw a condition effect (regardless of viral injection), while we saw a clear condition $\times$ virus effect in the OF. As it has been described that stress exposure can not only increase the amplitude in CORT secretion, but also the slope (meaning the curve of recovery is steeper in unstressed animals - CORT levels are elevated for a longer time in stressed animals)(Sarabdjitsingh et al., 2010; Maccari and Morley-Fletcher, 2007), it might be the case that this first segment in the afternoon serves as a readout for extended feedback of the stress system and that only at this later time point (animals were re-tested roughly 6 - 8 h after the first session), the interaction effect becomes visible. Another possibility might be that while CORT levels in the morning did not differ significantly, the OF test in the afternoon might be the result of differences in afternoon (peak) CORT levels. During the stress exposure, we also found a slight decrease in spatial memory, which was prevented by shARC injection. While it has been described that Arc is vital for learning and memory (Bramham et al., 2010), we here see a rescue of memory function via Arc knockdown. However, as our inter-trial interval in this case was 30 minutes - categorizing the test in the domain of short-term memory - our data nicely fits the still disputed observation that Arc is involved in the long-term formation of memory circuits, but not in short-term memory (Plath et al., 2006). This in turn also shows that Arc has both a function in memory as well as in

stress vulnerability and that these functions seem to be - at least partly - dissociated. Regarding social behaviour, we intriguingly found an increase in social interaction in the stress condition, both when tested during the stress as well as after recovery, whereas the literature strongly suggest a decrease in this behaviour (Covington et al., 2011; Komatsu et al., 2011; Wilkinson et al., 2011; Hollis et al., 2010). One possible explanation might be our control group. While stressed animals were housed in - mainly sensory - contact with another conspecific during the stress, controls were single housed and indeed a recent paper questions this kind of control group for social defeat experiments. So it might be that control animals are either socially deprived or that for stress animals, quick evaluation of the social status (resulting in fighting or immediate submission during the physical encounters) is promoted. This however does not explain that with the exact same setup, we consistently found a decrease in sociability following social defeat (unpublished observations). In concordance with the literature, we did not find robust changes in depression-like behaviour in the FST (Slattery et al., 2011), although other studies showed an effect (Becker et al., 2007). We also saw no effect of the stress exposure on sucrose preference, which is also found in multiple studies (Slattery et al., 2011; Hollis et al., 2010; Razzoli et al., 2009). Taken together we saw a preventive effect of the Arc knockdown in all stress-induced behavioural changes. This strongly suggests that Arc has a central role in the manifestation of stress-induced maladaptions.

### 4.3.3 Arc knockdown does not influence basic physiological parameters

In addition to the behavioural changes, we also investigated different physiological parameters. While in rats, stress-induced changes in body weight seem to be straightforward and are nicely described (Tamashiro et al., 2007), the literature for mice is more diverse. Here, we found a generic condition effect of stress exposure on body weight. However, this was only attributed to a single measurement of body weight when analysed separately. Nevertheless, this one timepoint seems very interesting. From the data we see that body weight reaches a peak roughly at the end of the sucrose preference test and declines again afterwards - in all animals but the stressed SCR-injected group. While this single point of data does not allow for a detailed description of the molecular workings, it does encourage speculation due to its peculiar timing. So it might be possible that although the ratio of sucrose solution consumption was the same in all groups, the sucrose consumption served as some sort of coping mechanism, especially for stressed individuals (Dallman et al., 2005). A study in rats has shown that consumption of sucrose solution can decrease responses from the stress system and interestingly this inhibition was weaker in rats that consumed saccharin solution, suggesting that the caloric component plays an important role (Ulrich-Lai et al., 2007). When the sucrose preference test was discontinued the stressed animals might have compensated the loss of the coping factor by a higher intake of food, which might explain why they kept their peak body weights for longer than the other animals. A recent study suggested that Arc expression is regulating cellular responses to cocaine (Caffino et al., 2011) and while addiction and stress coping should not be directly compared, it nevertheless shows the function of Arc to modulate the reward system, which might be the underlying factor in the present case.

Furthermore, we investigated the fur state of the animals. As suggested from previous experiments

and the literature, stress exposure caused a decrease in fur quality (Wagner et al., 2011; Hartmann et al., 2012). Here, we saw no effect of the Arc knockdown. Following the stress exposure, fur quality started to deteriorate in control animals. This might have been an effect of the testing period. Behavioural tests put strain on the homeostasis of the animals and strongly aversive tests like the here-used FST might be enough to cause this change. Additionally, the animals were tested in a separate testing room in our animal facility and after testing relocated to their previous housing room, which also might have served as a slight aversive experience. Possibly a combination of multiple small effects, which by themselves would not induce such a response, caused the phenotype we saw under those conditions. It should be noted that we did not observe abnormal behaviour in control animals in the following behavioural tests.
We also investigated the weight of the adrenal glands of the animals. An increase in size of the adrenal gland via hyperplasia as well as hypertrophy is a well documented effect of chronic stress exposure (Ulrich-Lai et al., 2006; Hartmann et al., 2012; Wagner et al., 2011), which is another indicator of the validity of the stress exposure in this experiment. We found no protective effect of the Arc knockdown on adrenal gland weight. Interestingly, we found no differences in basal levels of corticosterone. Elevated levels of CORT are a common finding following chronic stress exposure (see for example: (Joëls and Baram, 2009)), but measuring basal levels of CORT with only a single measurement might not yield optimal results, due to the pulsatility of the HPA axis, potentially confounding the results (please note that basal CORT levels had to be taken some days after the response test to not influence the other behavioural readouts, so the actual timepoint of measurement was after cessation of the chronic stress procedure; this might have been enough time for CORT levels to normalise). However, we saw a significantly stronger response to an acute stressor in the stressed animals, concomitant with impaired feedback, as already shortly mentioned in the discussion of the OF data. Although shARC-injected stressed animals also differ from their respective controls, they again can be described as intermediate phenotype (see also hypolocomotion in the OF). In contrast to many behavioural findings, the changes in HPA-axis kinetics were not detectable any more after the recovery period. This shows once again the complexity of the stress response but also generates additional questions. For example, the fact that we saw (after recovery) differences in adrenal gland weight, but not in basal or stimulated CORT levels, hints at another layer of modulation - maybe responsivity of the adrenal gland - not tackled in this experiment. Here, we saw that Arc expression in the hippocampus is independent (or at least only dependent to a weaker degree) from most of the physiological effects of stress exposure and the interaction of Arc with the plethora of stress-induced alterations is still poorly understood.

### 4.3.4 Possible molecular mechanisms of Arc in stress vulnerability

As a thorough investigation of the function of Arc in the molecular machinery modulating stress vulnerability could possibly fill multiple theses, it is beyond the scope of this pioneer work. Nevertheless, some potential pathways will be discussed and set in context with known findings. A good start here would be the influence of Arc in LTP and synaptic plasticity. Arc acts downstream of multiple cascades described in detail in the introduction, including activation of the major effector ERK via the NMDAR or BDNF (Bramham et al., 2010). While these cascades

are used in the formation of memory, they are also in use when conveying activation of the stress system. As the hippocampus is an important regulator of the HPA-axis and it has been shown that the hippocampus is altered by exposure to chronic stress (reviewed for example in (de Kloet et al., 2005)), it can be assumed that some kind of molecular memory exists (which is often referred to as allostatic load (Danese and McEwen, 2011)) in the hippocampus which influences future stress responses. So the hypothesis would be that stress causes activation of AMPAR/BDNF cascades with the downstream effector ERK in the hippocampus, which is then - with the critical help of Arc - consolidated, in turn (mal-)adapting the stress system for future activation. Individuals with lower levels of Arc would thereby exhibit roughly similar levels of an acute stress response, while the adaption to the stressful environment would be disrupted. Large parts of these cascades have already been implicated in stress vulnerability or at least some evidence is available that they are involved. However it may just be the case that most of these systems converge on Arc, making it a putative central molecule in stress vulnerability. For example, animals vulnerable to chronic stress have a higher AMPAR binding, which ultimately will lead to stronger activation of Arc (Schmidt et al., 2010b). Interesting data also comes from the work with CRHR1 KO animals. These animals have been shown to be resilient to stress in multiple parameters (Wang et al., 2011b) and recent data have shown that by this KO, the increase in AMPAR mEPSCs amplitudes caused by shock and possibly responsible for the consolidation of fear memories could be prevented (Thoeringer et al., 2012), again suggesting that another model of stress resilience is associated with lower AMPAR activation. In this context, the recent finding that the CRHR1-associated anxiogenic effects derive exclusively from the glutamatergic population of neurons might tighten the link between maldaptive behaviour (stronger anxiety-like response in the OF test) and glutamatergic activation (Refojo et al., 2011). The CRHR1 is also upstream of BDNF. The Nestler lab has shown in multiple studies that in vulnerable animals, activating influences of this pathway are overrepresented, including BDNF itself, AKT and ERK among others. It should be noted that another recent study showed that when rats with either BDNF overexpression or BDNF knockdown were subjected to chronic mild stress, BDNF OE countered stress-induced changes in the sucrose preference test as well as the FST (Taliaz et al., 2011). However, the BDNF OE itself caused profound effects on these tests even without the stress exposure, hinting that the BDNF overexpression per se and not the interaction with the environmental stimulus caused the latter effects. KD of BDNF did not prevent changes in the sucrose preference test, but was able to prevent hypolocomotion in the OF test in a condition $\times$ stress interaction manner. In addition, it was found that BDNF was downregulated in vulnerable, but not in resilient animals. Unfortunately, the OE experiment was performed in adult animals, while the KD experiment (and the categorisation into vulnerable and resilient animals) was performed in young rats, making direct comparison somewhat unprecise. While the authors discuss it differently, a large proportion of the data of the study supports the direction found in this study (associating lower activation of the pathway with stress resilience). It is believed that Arc supports LTP partly due to strengthening of synapses via stabilisation of F-actin. As other factors acting on F-actin stabilisation have been identified recently linking stress and behaviour, for example DRR1 (Schmidt et al., 2011a), it might be the case that modulation of the actin system plays an important role in stress and individual vulnerability. However, the potential

link between stress vulnerability and the actin system still needs to be investigated directly in separate studies.
Another interesting feature of Arc is its involvement in neurogenesis. Arc has - in contrast to other IEGs - been found very early in the life of dentate granule cells, one of the few selected populations of cells in the mammalian central nervous system that show neurogenesis during the whole lifetime. Intriguingly, Arc expression seems to get stronger in cells that ultimately survive and differentiate into neurons, leading the authors to the speculation that it might be possible that the early expression of Arc might support cell survival or determines cell fate (Kuipers et al., 2009). While this has not been directly proven, it might give Arc a direct role in synaptic plasticity and neurogenesis with the possibility that lower levels of Arc might decrease the rate of cell survival. Synaptic plasticity in turn has been found to be important in the development of stress-induced changes and reduction of neurogenesis in the DG following chronic stress exposure is a well replicated finding (for review see (Lucassen et al., 2010)). For example, inhibition of Cdk5, which is implicated in synaptic plasticity, in the DG reduced stress-induced depression-like behaviour (Zhu et al., 2011). Therefore, neurogenesis in the DG might be another vector how Arc might influence stress vulnerability.
Noteworthy here is also the robustness of the results. Despite our switch in paradigm (CSS to SD) and our switch in mouse strain, even from the outbred CD1 to the inbred C57BL/6 strain, we still were able to see the effects here predicted from the previous design. This strongly suggests that what we describe here is a general and maybe even strongly conserved system.

### 4.3.5 The possible evolutionary justification of Arc

At first glance, the existence of a gene like Arc, which primes an individual susceptible against environmental stressors when expressed in high doses, is counterintuitive. However, stress itself is not malevolent per se. Evolution has shown that it is mostly the species which can adapt best to a change in environment are the ones to perform best. So what we consider to be a maladaption might be beneficial, depending on the circumstances. For this problem, the recently formulated mismatch hypothesis might provide some answers (Nederhof and Schmidt, 2011). In short, this hypothesis states that adaption is mainly non-optimal when the environment changes after the adaption process and does not match the prospected environment any more. In the case of Arc for example, this might mean that when animals with high levels of Arc are exposed to a highly stressful environment nicely adapt to this environment. When then tested under low-stress conditions - like in experiment 2 in this thesis, 5 weeks after the stress under standard conditions - the animals are still adapted to high stress conditions and perform worse than their counterparts. However, it seems possible that if the animals would have been tested under high-stress conditions, these animals might have excelled. Vice versa, animals with low levels of Arc might not be able to adequately adapt to a stressful world, and stay primed to non-stress conditions. When then tested under those conditions they show good results while they might perform worse under high-stress conditions. So the evolutionary benefit of Arc expression might be critically dependent on the consistency of the individual environments.

### 4.3.6 Conclusions

In this experiment we used chronic social defeat to elicit physiological and behavioural changes in C57Bl/6 mice. In addition, we were able to prevent most of the behavioural alterations, but not physiological alterations, via knockdown of Arc in the hippocampal formation. Therefore, we can partly accept our initial hypothesis *Knockdown of Arc expression in the hippocampus shifts the animals towards stress resilience and protects them from stress-induced changes* for the behavioural, but not for the physiological changes. This once again shows that the stress response is a complex process and by no means is exclusively regulated by a single pathway.

## 4.4 Synopsis

The results from this thesis nicely demonstate the complexity of the stress response and especially the complexity of the regulation of the latter. In this thesis, we used a step-by-step strategy to achieve the aims and the discussion so far led us from the more general-oriented experiments to a highly-specific, region-selective manipulation of a single gene. This last section should now illustrate shortly the way back through complexity levels to integrate the findings in a more generalised context and show the importance of such a step.
Such an approach was already started in the section on experiment 3 by discussing the involvement of Arc in multiple pathways implicated in stress vulnerability and stress resilience. Although Arc seems to play a prominent role in these pathways (which is for example also suggested from the correlations in experiment 2), it is probably not the only effector protein for the downstream alterations. This is backed up for example by the finding that modulation of Arc does not influence physiological parameters in the same extent that we saw in the behaviour. A model with multiple inputs, but also multiple outputs, seems more plausible and naturalistic for the molecular basis of stress vulnerability. But this in turn requires us to not only look at specialised studies but also explore other intersections. And while already some knowledge is available for some factors that also came up in the vulnerability microarray, like Gria1 and Gria2 (as discussed before), the involvement of other factors is more unclear. One interesting example would be Homer1, which was also regulated in the vulnerability microarray. It has for example been shown that Arc and Homer1 (Homer1a is also an IEG) seem to be active in the same cells and can both be activated by BDNF (Bottai et al., 2002). The next step would then be to investigate the involvement of Arc in a general stress context and the still unclear molecular transition from stress to stress vulnerability. We found Arc here in animals that were selected based on their corticosterone profiles. However, the fact that we were able to translate these findings to another strain of mice and another stress model, in combination with the literature about different possibilities to induce Arc and the nature of these activator molecules (meaning they are molecules commonly found regulated after various different forms of stress exposure and are molecules involved in relatively general pathways - see introduction) strongly suggests an important general role of Arc. Nevertheless, this still needs further studies.
All in all, this thesis provides both a good conceptual framework for studies of stress vulnerability as well as the description of Arc in this specific context. In addition, we were able to show that Arc is causally as well as critically involved in stress vulnerability and might be an important factor not only for our specific setup, but also in a more general context.

## 4.5 Future directions

While this thesis explains various findings, it also generated multiple interesting new research questions. The following experiments all refer to Arc for future experiments, as it seems the most promising candidate for future studies based on the known findings from the literature (this should not devaluate findings like Iffo1 for example - they might be similar in terms of potential, but Arc seem the less risky candiate due to known findings). In addition, Arc is for me the latest finding at the end of a series of experiments building on each other during this thesis and thereby the natural candidate to continue working with (please note that this is pure personal preference). We determined here that Arc expression is necessary for most of the stress-induced changes, however it would be very interesting to investigate if Arc is sufficient for multiple models of stress vulnerability. Specifically, it would be interesting to see if it is possible in a model based upon a suggested upstream molecule of Arc (for example lower AMPAR levels or binding causing stress vulnerability) to rescue the stress-induced changes (restoring the maladaptive response) purely via Arc OE in these vulnerable animals. In addition, it would be very interesting to test the potential mismatch aspect of Arc expression by (in the best case) subjecting animals both to good and stressful environments and testing them under low- and high stress conditions with both Arc knockdown and OE. However, this would result in a $2 \times 2 \times 2 \times 3$ (or 4 depending on the needed viral control groups), which represents a much to dense design in addition to the obvious technical and statistical difficulties. Nevertheless, as we already showed that animals with Arc KD are protected from the effects of stressful experience, it might be rewarding to test how animals with the same KD fare under stimulating conditions. Stimulating conditions like enriched environment have been shown to improve certain behavioural readouts in animals and if the KD would also impair positive development, this would strengthen the mismatch/plasticity aspect and would be a strong incentive for more detailed studies in this field.

# 5 Summary

Stress is part of everyday life. And while acute and short periods of stress can help to overcome challenges, exposure to chronic - and especially uncontrollable - stress can lead to maladaption of the organism, which can ultimately increase the risk of disease. However, vulnerability to stress and vulnerability to risk increases are strongly dependent on the individual. The molecular underpinnings of this vulnerability and resilience are still largely unknown. Therefore, the present thesis aims at identifying novel molecules involved in modulating individual stress vulnerability in the brain of male mice. In a first step, we investigated long-term gene expression changes in the hippocampus of male mice that underwent chronic social stress. Adolescent male CD1 mice were subjected to 7 weeks of chronic social stress and were investigated after 5 weeks of recovery via an unbiased whole-genome approach utilising microarray technology. Here, we did not find strong differences caused by the stress exposure, possibly because not all animals were affected by the stress exposure. Nevertheless, we identified Iffo1 as a gene that seems to be affected by at least acute stress and might also be involved in the long-term effect of chronic stress exposure. In the next step, we classified the animals from the same paradigm into stress-vulnerable and stress-resilient individuals based on their corticosterone levels after recovery. Animals which still showed elevated levels of corticosterone 5 weeks after stress were defined as vulnerable, while animals in which levels returned to baseline comparable to controls were termed stress resilient. With an additional whole-genome experiment, we were able to show distinct patterns of gene expression between the groups, including genes like Arc, Gria1 and Gria2. In addition, we also investigated differences in peripheral lymphocytes, which showed regulation in genes like Hsp90b1 or SLA. When we compared the expression profiles between brain and peripheral blood, we showed that stress-vulnerable and stress-resilient animals show different patterns of correlations. In the final part of the thesis, we decreased the expression of Arc, one of the genes we found overrepresented in vulnerable individuals, in the hippocampal formation of male mice via AAV-mediated shRNA knockdown. As we perfomed the modulation of Arc before the stress exposure, we were able to investigate the causal influence of Arc expression on stress exposure. Animals that were subjected to 3 weeks of chronic social defeat, showed an increase in anxiety-related behaviour, impairement in spatial memory, an increase in social behaviour and did not differ in depression-like behaviour. Concomitant with the behavioural alterations, stressed animals showed alterations in multiple physiological parameters, like increased adrenal glands or corticosterone response. Intruigingly, we were able to prevent most of the behavioural, but not the physiological, changes with the Arc knockdown. This strongly suggests that Arc is at least partly causally involved in the molecular machinery that underlies stress vulnerability. As Arc is a downstream molecule in multiple pathways already connected to stress vulnerability or stress in general, it might be that Arc actually is one of the major molecular factors that translate the effects of these pathways.

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# List of Figures

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## List of Abbrevations

11$\beta$-HSD 2 11$\beta$-hydroxysteroid dehydrogenase 2

5-HT1A 5-hydroxytryptamin (serotonin) receptor 1a

AAV adeno-associated virus

AB antibody

ACh acetylcholine

ACTH corticotropin (Adrenocorticotropic hormone)

AMPA 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid

AMPAR AMPA receptor

ANOVA analysis of variance

Arc/Arg3.1 activity-regulated cytoskeleton-associated protein

AS-ODN antisense oligodeoxynucleotide

avBNST anteroventral part of the bed nucleus of the stria terminalis

AVP arginine vasopressin

BCP 1-Bromo-3-chloropropane

BDNF brain-derived neurotrophic factor

BLA basolateral amygdala

BNST bed nucleus of the stria terminalis

bp base pair

BSA bovine serum albumin

BW body weight

CB1 cannabinoid receptor 1

CBP corticosterone binding protein

cDNA complimentary DNA

CeA central amygala

CI confidence interval

CMS chronic mild stress

CON control

CORT corticosterone

CRE cAMP response element

CRF/CRH corticotropin-releasing factor/hormone

cRNA complimentary RNA

CSS chronic social stress

DEPC Diethylpyrocarbonate

DEX dexamethasone

DHPG dihydroxyphenylglycine

DMH dorsomedial hypothalamus

DNA desoxyribonucleic acid

dNTP desoxynucleoside triphosphate

dsDNA double-stranded DNA

DSM-IV Diagnostic and Statistical Manual of Mental Disorders, $4^{\text{th}}$ edition

DTE dendritic targeting elements

EDTA ethylenediaminetetraacetic acid

EJC exon-junction complex

ELS early life stress

EPM elevated plus maze

ERK extracellular signalregulated kinase

FD food deprivation

FDR false discovery rate

Fkbp5 Fk506 binding protein

FMRP fragile-X mental retardation protein

FST forced swim test

GABA $\gamma$-aminobutyric acid

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GluR1 glutamate receptor 1

GluR2 glutamate receptor 2

GR glucocorticoid receptor

GRE glucocorticoid response elements

HPA hypothalamic-pituitary-adrenal

HPRT Hypoxanthine-guanine phosphoribosyltransferase

HRP horesradish peroxidase

IEG immediate-early genes

ISH in situ hybridisation

ITI inter-trial interval

KD knockdown

KO knockout

LMPC laser-microdissection and pressure-catapulting

LTD long-term depression

LTP long-term potentiation

mAChR muscarinic acetylcholine receptor

MeA medial amygdala

MEF2 myocite-enhance factor-2

mGluR metabolic glutamate receptor

miRNA micro-RNA molecules

mPFC medial prefrontal cortex

mPFCd dorsal part of the mPFC

mPFCv ventral part of the medial prefrontal cortex

mPOA medial preoptic area

MR mineralocorticoid receptor

mRNA messenger ribonucleic acid

mRNP messenger ribonucleoprotein particles

MS maternal separation

MWM morris water maze

NAc nucleus accumbens

NMD nonsense-mediated mRNA decay

NMDA N-Methyl-D-Aspartate

NMDAR NMDA receptor

NTS nucleus of the solitary tract (*nucleus tractus solitarius*)

OF open field (test)

OR object recognition (test)

ORF open reading frame

PB phosphate buffer

PFA paraformaldehyde

PKA protein kinase A

PKC protein kinase C

PND post-natal day

PNS prenatal stress

POMC pro-opiomelanocortin

pPVN peri-PVN region

PVN hypothalamic paraventricular nucleus

qRT-PCR quantitave reverse-transcription polymerase chain reaction

RBP RNA-binding proteins

RES (stress-)resilient

RES restraint stress

RIA radioimmunoassay

RIN RNA integrity number

rpm revolution per minute

RT room temperature

s.c. subcutaneous

SARE synaptic activity response elemen

SCN suprachiasmatic nucleus

SD social defeat

SEM standard error of the mean

shARC short-hairpin RNA against Arc

shRNA short-hairpin RNA

SHRP stress-hyporesponsive period

SNP single-nucleotide polymorphism

SNS sympathetic nervous system

SRE serum response element

SRF serum response factor

STR stress

TBST Tris-buffered saline with tween 20

TST tail suspension test

UBE3A Ubiquitin-protein ligase E3A

UTR untranslated region

UV dentage gyrus

UV ultraviolet

VEH vehicle

VGLUT1 vesicular glutamate transporter 1

VGLUT2 vesicular glutamate transporter 2

vLS ventral lateral septum

VMH ventromedial hypothalamic nucleus

vSUB ventral subiculum

VUL (stress-)vulnerable

WT wildtype

ZRE zeste-like factor response element

# Curriculum Vitae

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## Publication record

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## Assertation (Erklärung)

Hiermit versichere ich eidesstattlich, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.

Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, den 31.01.2012