Metabotropic Glutamate Receptors – Regulation of Acute and Chronic Stress-Related Behavior and Physiology

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

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Für meine Familie

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Introduction

The Introduction section includes chapters taken and adapted from Peterlik et al. (2016a):

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1. Stress and its physiological systems

Hans Selye, the founder of the modern stress concept, stated “everybody knows what stress is and nobody knows what it is” (Selye, 1973). In spite of its frequent use, the word “stress” is at best an ambiguous term. In fact, it has been used to describe both what creates the stress and the response of the body to it. In order to circumvent this ambiguity, two different terms have been introduced: “stressor” and “stress response”. A stressor is defined as anything that disrupts physiological balance independent of whether it is an actual or anticipated disruption of homeostasis or an anticipated threat to well-being (Bartolomucci, 2007; Chrousos, 2009). On the other hand, the stress response is an adaptive behavioral and physiological reaction aiming to re-establish homeostasis even in the most demanding of circumstances (Chrousos, 2009; Dhabhar, 2002; McEwen, 2004), thereby involving an efficient and highly conserved set of interconnected physiological systems (Ulrich-Lai and Herman, 2009). In mammalian species, the two major stress system mediating behavioral and physiological responses are the autonomic nervous system (ANS), especially its sympathetic branch (SNS), and the hypothalamic-pituitary-adrenocortical (HPA) axis. The activation of the SNS provides the most immediate response to stressor exposure within seconds via exclusively neuronal pathways. These pathways originate in the thoracolumbal regions of the spinal cord and in turn project to end organs and to chromaffin cells in the adrenal medulla (Figure 1a). In the latter case, they trigger the release of adrenaline/noradrenaline into the blood (Mason, 1968; Ulrich-Lai and Herman, 2009). In contrast to the fast acting SNS, activation of the HPA axis, which is driven by hormones, takes longer to develop. Stimulation of the HPA axis is triggered by the secretion of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus into the portal circulation of the pituitary. By binding to respective receptors expressed in the anterior pituitary, CRH promotes there the synthesis and secretion of adrenocorticotropic hormone (ACTH) into the peripheral blood, which, in turn, stimulates adrenal cortical cells to produce and secrete glucocorticoids (GC) into the blood stream (Figure 1b). Termination of the stress response is achieved by negative feedback inhibition via GC acting at glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) at different brain levels, e.g. the hippocampus and hypothalamus (Harris et al., 2013; Keller-Wood, 2015; Lupien et al., 2009).

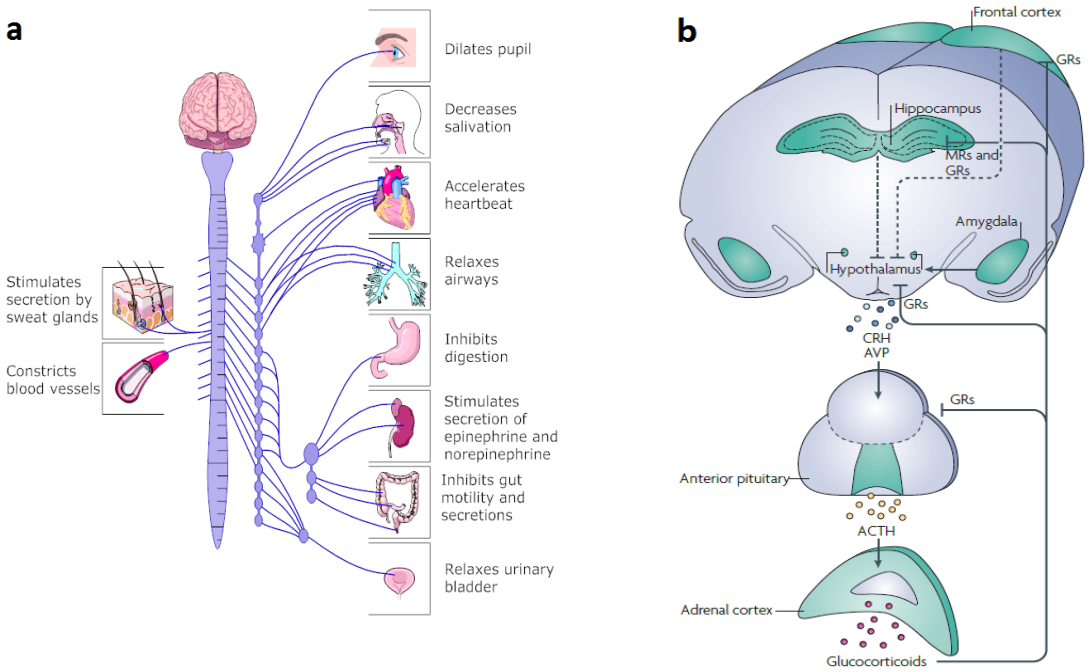


Figure 1. Schematic illustration of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenocortical (HPA) axis. The SNS (left-hand side; schematic overview adapted from http://wc1.smartdraw.com/cmsstorage/exampleimages/bf159507-dfa1-47d1-910e-f6903c30c35c.png) and the HPA axis (right-hand side; adapted from Lupien et al. (2009)) represent the primary mammalian systems responsible for maintaining or reinstating homeostasis during stress. (a) Stressor exposure results in activation of preganglionic sympathetic neurons in the intermediolateral cell column of the thoracolumbal region of the spinal cord. These preganglionic neurons project to pre- or paravertebral ganglia that in turn project to end organs and to chromaffin cells of the adrenal medulla. This sympathetic activation represents the classic "fight or flight" response that generally increases circulating levels of adrenaline (primarily from the adrenal medulla) and noradrenaline (primarily from sympathetic nerves), which in turn increases heart rate and force of contraction, peripheral vasoconstriction, and energy mobilization. In addition, the parasympathetic tone can also be modulated during stress. In the parasympathetic system (not shown), activation of craniosacral preganglionic nuclei activates postganglionic nuclei located in or near the end organs that they innervate; parasympathetic actions are generally opposite to those of the sympathetic system. (b) For the HPA axis, stressor exposure activates hypophysiotrophic neurons in the paraventricular nucleus (PVN) of the hypothalamus that secrete releasing hormones, such as corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), into the portal circulation of the median eminence. These releasing hormones act in the anterior pituitary to promote the secretion of adrenocorticotropic hormone (ACTH), which in turn acts on the inner adrenal cortex (zona fasciculata) to initiate the synthesis and release of glucocorticoids (GC). Following activation of the system, and once the perceived stressor has subsided, feedback loops are triggered at various levels of the system in order to shut down the HPA axis and to return to a homeostatic point. Adapted from Ulrich-Lai and Herman (2009).

1.1 Acute, repeated and chronic stress

Although the term "stress" commonly bears a negative meaning, an acute stress response is one of the most important mechanisms of an organism to adapt appropriately to challenges and threats in the environment. An acute stress response results in immediate behavioral and physiological changes including enhanced attention and arousal, increased energy mobilization and increased cardiovascular and respiratory rates, whereas digestive and reproductive functions are inhibited (Charmandari et al., 2005). The fundamental process through which the organism actively adjusts to stressful challenges is referred to as "allostasis" and represents an essential component of maintaining homeostasis (McEwen, 1998). When not overstrained, these changes are adaptive and beneficial as they increase an individual's chance of survival. In case of excessive and prolonged activation during repeated or chronic stressor exposure, these adaptive systems may become overstimulated, a condition termed "allostatic load" (McEwen and Stellar, 1993; McEwen and Wingfield, 2003; McEwen, 1998). Accordingly, inappropriate, severe or prolonged stress is associated with changes in the brain that impair its ability to regulate appropriately physiological and behavioral responses (Arnsten, 2009; McEwen, 2007). Thus, important criteria to distinguish stress are its duration and intensity. While acute stress is defined to last for minutes to hours, chronic stress persists for days to month (Dhabhar, 2000). On the other hand, stress intensity can be measured by the magnitude of heart rate and blood pressure and by stress hormone levels (GC, catecholamines) in blood. Acute stressor exposure results in an increase of blood GC levels after 15-30 min and a decline to basal levels 60-120 min later (de Kloet et al., 2005; Keeney et al., 2006). In contrast, chronic stressor exposure may result in persistently elevated levels of plasma corticosterone (CORT). For instance, sustained increased basal plasma CORT levels were reported following chronic subordination for 14 days (Albeck et al., 1997) or repeated restraint stress for 7 days with 1.5 h/day (Zelena et al., 1999) compared to unstressed controls. Such prolonged elevations of plasma CORT levels are considered the most toxic because they are most likely to result in long-term or permanent changes in emotional, physiological and behavioral responses that influence susceptibility to disease (Chrousos, 2009; de Kloet et al., 2005; Karatsoreos et al., 2010). Interestingly, although HPA axis hyperactivity (hypercorticism) has been generally linked to prolonged stressor exposure, there is accumulating evidence for even opposite alterations (Heim et al., 2000). There are chronic or repeated stress models leading even to reduced or unaffected plasma GC levels. For instance, chronic isolation

stress resulted in reduced basal plasma CORT (Djordjevic et al., 2012), whereas repeated exposure to noise did not affect basal plasma CORT levels (Armario et al., 1986). Concurrently, chronic or repeated exposure to the same (homotypic) stressor is often associated with an adaptation of the stress response (Bartolomucci, 2007; Wood et al., 2010), a mechanism that enables the organism to habituate to innocuous, not life threatening challenges. However, despite adaptation to these familiar stressors, it is important for the individual to respond adequately to novel, possibly dangerous threats. So far, possible mechanisms underlying habituation to familiar and sensitization to subsequent novel stressors include functional changes at the level of the adrenal gland (Uschold-Schmidt et al., 2012) or altered negative feedback regulation of the HPA axis (Aguilera, 1994).

Of note, the consequences of chronic or repeated stressor exposure can vary based on individual differences. For example, while some individuals perceive a challenging situation as stressful, others that are resilient can actively cope with the same situation. Evidence supports the hypothesis that genetic predisposition (DeRijk and de Kloet, 2005; Gillespie et al., 2009) as well as adverse (early) life experiences (Eiland and McEwen, 2012; Gola et al., 2012; Veenema et al., 2008) strongly increase the individual's vulnerability to stress-related pathological conditions.

Until now, numerous studies have focused on physiological and behavioral consequences of an acute stress response that are generally well understood. Instead, although chronic stress-induced physiological and behavioral alterations are likely to play a major role in the etiology of various diseases, the detailed underlying mechanisms are less well understood due to, at least in part, the lack of appropriate animal models (Langgartner et al., 2015).

1.2 Psychosocial stress

Hans Selye's theory of the "general adaptation syndrome" involved the description of an organism's reaction to stressors of exclusively physical nature including intoxication or cold (Selye, 1998). Even nowadays many physical stressors are frequently used in laboratory such as restraint or forced swim stress. However, the major limitation these stress paradigms have is that they do not mimic the animal's natural situation, *i.e.* when exposed to psychological and social stressors (Bartolomucci et al., 2005). More importantly, physical stressors do not reflect the typical situations humans have to face in today's highly demanding society. Nowadays it is widely acknowledged that, for instance,

work-related stress represents a common feature of modern life at high costs of an individual's health and performance. Most of the challenges and threats humans face today are of psychological nature, such as changing world of work, demand for flexible contracts accompanied with increased job insecurity as well as poor work-life balance. Concurrently, psychological stress occurs when an individual perceives that these environmental demands strain or exceed the own adaptive capacity to deal with (Cohen et al., 2007). Of note, most of these challenges include social interactions between individuals like competition for resources or social rank and workplace bullying. Accordingly, social stress represents one of the most potent but also naturalistic type of challenges (Koolhaas et al., 1997; Tamashiro et al., 2005). Psychosocial stress, the combination of psychological and social aspects of stress, is accepted as a major risk factor for the development of a wide variety of somatic and affective disorders in humans (Bennett et al., 1998; Heim and Nemeroff, 2001; Lupien et al., 2009; Sgoifo and Meerlo, 2002). In view of this, animal models utilizing a chronic psychosocial stress component represent the most promising approach on a preclinical basis to unravel the mechanism underlying chronic stress-promoted pathologies as they more accurately reflect the human situation. The chronic subordinate colony housing (CSC) model has emerged as one such appropriate model, as it combines chronic, psychological and social aspects of stress, and promotes the development of both somatic and affective pathologies (Langgartner et al., 2015; Peterlik et al., 2016a; Reber et al., 2007; detailed description see below).

1.3 Established rodent models for the evaluation of stress

Understanding the biological basis of the stress response is essential for a better comprehension of the etiology of stress-related disorders. Animal models have turned out to be instrumental in this respect and, like in humans, animals use coping strategies when exposed to stress. They can express both active coping mechanisms manifested by aggressive behaviors as well as exploratory activity or passive coping manifested by freezing, immobility and submission (Franklin et al., 2012). All these behaviors can be reliably measured in different animal models. In the following, a number of animal models are discussed in which the animal's stress response is reflected either upon exposure to acute or to a chronic stressor. Paradigms that employ acute stressor exposure include stress-induced hyperthermia (SIH), the forced swim test (FST), the tail suspension test (TST), elevated plus maze (EPM) and learned helplessness (LH), to name a few. On the

other hand, chronic mild stress/chronic unpredictable stress (CMS/CUS), chronic social defeat stress (CSDS) and chronic subordinate colony housing (CSC) represent chronic stress models, all of which employ relatively long-term exposure to inescapable or uncontrollable stress events.

1.3.1 Assessment of acute stress in rodents

The SIH Test

In general, SIH is known to be a physiological phenomenon when a mammalian organism is confronted with an either physical or psychological stressor (Adriaan Bouwknecht et al., 2007; Olivier et al., 2003; Vinkers et al., 2013; Zethof et al., 1994). Notably, the SIH test in rodents is also referred to a physiological animal model for anxiety (Adriaan Bouwknecht et al., 2007). Typical for the test is that, modified from the version originally described by Borsini et al. (1989), the basal temperature is measured rectally (T1) followed by a second rectal measurement 15 minutes later (T2). During these 15 minutes, the temperature usually rises due to the physical stress the animal is exposed to (handling, rectal measurement). Conveniently, potential anxiolytic-like effects of drugs are measured by a decrease in the SIH response (Adriaan Bouwknecht et al., 2007). Those measurements of the body temperature are not dependent on the animal's motoric activity, which makes SIH different from other mild stress models/anxiety tests that depend on locomotor performance of the animal, for instance the EPM or open field test.

The FST and TST

The FST and TST are the two most widely used preclinical screening tests that allow rapid detection of substances with potential antidepressant-like activity (good predictive validity). In general, both tests are based on the same principle, which is the measurement of the duration of immobility while rodents are exposed to an acute, short-term (minutes) inescapable situation. In the FST, first described by Porsolt et al. (1977a,b), a mouse or a rat is placed in a water-filled cylinder, in which the animal is unable to escape from. Following an initial period of escape-oriented movements, the animal will eventually display an immobile posture, a passive behavior characterized by the absence of movements except those necessary to keep the head above the water level. By contrast, in the TST, immobility is scored while mice are suspended by their tails and, as water is not required, this test is not confounded by challenges of thermoregulation. In both tests, the

immobility is typically interpreted as an expression of behavioral despair (Cryan et al., 2005a, 2005b; Lucki, 2001), which can be reversed by the acute administration of compounds with antidepressant potential. So, testing of new substances in these stress models allows a simple and rapid screening of potential antidepressant activity by the measurement of their acute effect on immobility. However, this poses a problem for the model, as antidepressants used in depressed humans in the clinics generally require many weeks of administration to elevate mood. Nevertheless, both the FST and TST are currently popular models mostly due to their low cost, their ease of use and their reliability across laboratories (Borsini and Meli, 1988; Holmes, 2003).

The EPM test

The EPM represents one of the most widely used anxiety models, in which anxiety-related behavior is typically measured by indices of open-arm avoidance and locomotor activity by the frequency of closed-arm entries (Lister, 1987; Rodgers et al., 1999). In principle, this test exploits the balance between the preference of rodents for avoiding open exposure to potential predators *vs.* exploration for possible rewards. When placed in the center portion of the plus-maze and allowed to explore each of the arms freely, animals with higher anxiety levels will show reduced open-arm activity and vice versa. This tendency can be, for instance, suppressed by anxiolytics and potentiated by anxiogenic agents (Belzung and Griebel, 2001). As short-term exposure of animals to heights and bright open spaces demonstrates an acutely stressful situation, the EPM can also be used and interpreted as a test for mild acute stressor exposure (Salomé et al., 2006).

The LH model

The LH model can be basically viewed as analogous to the abovementioned tests, with the difference that it involves a series of stressors over a few hours or even days (Nestler and Hyman, 2010). Following an uncontrollable stressor such as exposure to inescapable electric foot shocks, animals eventually will either display increased escape latency or completely fail to escape from a subsequent situation in which escape is possible (Seligman and Beagley, 1975; Seligman et al., 1975; Willner, 1984). Importantly, escape deficits can be reversed by a variety of antidepressants (Henn and Vollmayr, 2005). Following one or more sessions of inescapable shock, animals have been shown to develop persistent changes that are reminiscent of depression, including weight loss, alterations in

sleep pattern, changes in HPA axis activity and loss of spines in hippocampal regions (Cryan and Mombereau, 2004; Nestler et al., 2002a, 2002b). The attractiveness of LH is that the model is based on the consideration that cognitive functions (e.g. learning) are linked to other behavioral outcomes (e.g. neurovegetative modalities), and thus, this model helps to provide a reasonably integrated and broad picture of depressive symptomatology analogous to the human situation. However, the major drawback of the model is that most of the depression-like symptomatology does not persist beyond 2-3 days following cessation of the uncontrollable shock. Moreover, another limitation is – in contrast to the FST and TST – the difficulty to replicate between laboratories, particularly in mice.

Until today, these acute stress models clearly represent the first line of behavioral tests used to rapidly screen putative antidepressant and anxiolytic compounds and to phenotype transgenic animals. Even though direct links to emotional disorders in man are obviously weak due to utilizing only acute stressors and testing only acute antidepressant/anxiolytic responses, these acute stress models have helped enormously to reveal important molecular players within the CNS emotion circuitry (Krishnan and Nestler, 2011, 2010, 2008; Krishnan et al., 2008).

1.3.2 Assessment of chronic stress in rodents

While acute stress paradigms are used broadly for their ease, automation potential, and rapid phenotyping abilities, they offer singular readouts that often cannot be unambiguously interpreted. For instance, increased immobility in the FST is typically interpreted as an expression of despair. However, it can also be understood as a successful and adaptive behavioral response that functions to conserve energy (West, 1990). Today's chronic stress models are distinguished by their remarkable ability to simultaneously produce a set of behavioral alterations with strong face validity for depression and anxiety disorders (behavioral manifestations that should be similar to the symptoms observed in affected humans). Based on the clinical evidence that chronic stress significantly increases pathogenesis of affective diseases, these stress models are potentially of high value to better understand the underlying physiological mechanisms (Krishnan and Nestler, 2008; Nestler and Hyman, 2010). Basically, they are composed of repeated and/or permanent applications of uncontrollable and unpredictable stressors that are associated with quantifiable molecular, behavioral and physiological changes.

The CMS model

In the CMS model, also referred to as chronic unpredictable stress (CUS) paradigm (Willner, 1997; Willner et al., 1992), rodents are exposed to a variety of relatively mild, mostly physical, stressors such as restraint, isolation housing, disruption of light-dark cycles, intermittently for relatively prolonged time periods (e.g. several weeks). Typically, a variety of stressors is used within the CMS schedule in order to prevent or delay habituation, which can occur rapidly when a single stressor is presented repeatedly (Willner, 1997; Willner et al., 1992). In addition to a reduction in sucrose preference (Muscat and Willner, 1992), CMS has also been shown to result in a number of other changes that are difficult to objectively quantify, such as grooming deficits and changes in aggressive and sexual behavior. Interestingly, many of these changes can be reversed by chronic antidepressants applied either during the stress procedure or as a post-stress treatment (Guidotti et al., 2013; Strekalova et al., 2006). However, as the CMS model only employs physical stressors and often lacks cross-laboratory reliability, other approaches to develop chronic psychosocial stress-based models, more reminiscent of human depression, have emerged in recent years.

The CSDS model

The stress models described above are based exclusively on physical stressors, and thus, are lacking the relevance of mimicking most important situations that human beings encounter in everyday life – *i.e.* social interactions (Brinkborg et al., 2011; DeVries et al., 2007; Kouzis and Eaton, 1998; Vega et al., 2004). As opposed to CMS, the CSDS model clearly includes an important social stress component, and thus displays remarkable strength as it relies on innate social behavior. The model is based on the principle that two animals interact socially and physically such that one achieves dominant status and the other becomes subordinate. Much of the preclinical aggression research has been conducted so far in territorial male resident rats or mice confronting an intruder conspecific. As a consequence of territoriality, the resident will attack unfamiliar males intruding in its home cage. However, there are many versions of CSDS for mice and rats. For example, a typical procedure in mice lasts for 21 days where the experimental animal is exposed repeatedly to 10 intermittent bouts (5-10 min, once daily) of social defeat. Here, the experimental mice are forced to intrude into cage space occupied by a larger mouse of a more aggressive strain, leading to subordination of the experimental mice. In addition to

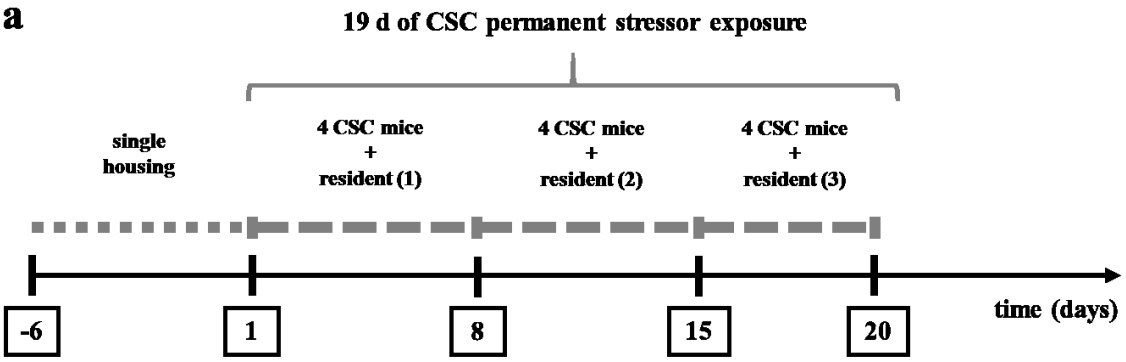
the short-time physical stress during direct contact with the dominant male, the experimental mice are exposed to additional psychological stress in form of prolonged "not physical" contact by housing them for 24 h in the same cage as the residents, but with a transparent partition allowing only sensory interaction (Wagner et al., 2014, 2011). Other laboratories expose the experimental mice also daily to 10 min of physical interaction with a resident, followed by 24 h of sensory contact, but only for 10 consecutive days (Berton et al., 2006; Razzoli et al., 2011; Tsankova et al., 2006). For rats there are protocols where the experimental animals are placed in a resident's home cage for 5 min physical interaction followed by 10 min of sensory threat for 4 consecutive days (Duclot and Kabbaj, 2013; Hollis et al., 2010), or where the intruders are placed in the cage of the resident for intermittent physical interaction until submission followed by 30 min of sensory threat for 1 to 3 consecutive days (Razzoli et al., 2009, 2007, 2006). Following repeated exposure to a dominant encounter, independent of the protocol used, animals reliably show decreased sucrose preference, indicative of an anhedonia-like state, and show reduced social interaction/sociability as well as alterations in HPA axis and autonomic function (Avgustinovich et al., 2005; Hollis and Kabbaj, 2014; Krishnan et al., 2007). Importantly, many of these changes can be reversed by chronic, but not acute, antidepressant drug administration, illustrating pharmacological validity of this stress model (Balsevich et al., 2014; Der-Avakian et al., 2014; Venzala et al., 2012).

The CSC model

The CSC paradigm was established by Reber et al. (2007) and represents a chronic psychosocial stress model with similarities to the CSDS model, but with the difference of applying psychosocial stress not only intermittently but permanently over a period of 19 days (24 h per day; Figure 2a). This chronic stress model is a very reliable animal model in combining chronic, psychological and social aspects of stress. In doing so, and as compared to the other stress models of above, it more comprehensively mimics the type of health compromising stressors that humans are exposed to in daily life. Typically, four male mice are housed together with a larger male resident in its homecage for 19 consecutive days. This results in immediate subordination of the four intruder CSC mice, and a hierarchy within each colony is formed, in which the resident clearly obtains the dominant position. To avoid habituation to the dominant mouse, the four CSC mice are transferred into the homecage of a novel larger male resident mouse on days 8 and 15.

Single-housed animals that remain undisturbed serve as unstressed controls (SHC; Reber and Neumann, 2008). Importantly, studies by the group of Reber S.O. clearly demonstrate that CSC exposure leads to the development of affective and somatic changes and also results in reduced GC signaling (Langgartner et al., 2015; Figure 2b), and thus provides a powerful experimental tool to study the mechanisms underlying several relevant stress-induced conditions. In detail, is has been shown that exposure to CSC alters several parameters indicative of chronic stress, including reduced body weight gain, decreased thymus weight and increased pituitary and adrenal weights (Reber et al., 2007; Uschold-Schmidt et al., 2012). The latter finding is accompanied by a reduced responsiveness of adrenal explants to an ACTH challenge *in vitro*. Importantly, adrenal ACTH sensitivity seems to be not only diminished under *in vitro* conditions, as CSC mice show unaffected basal morning plasma CORT despite elevated plasma ACTH levels in comparison with SHC mice. Moreover, CSC mice show basal evening hypocorticism, suggested by decreased basal evening plasma CORT levels compared to SHC mice (Reber et al., 2007; Uschold-Schmidt et al., 2013, 2012). The decline in GC signaling is further amplified by a reduced GC sensitivity seen in lipopolysaccharide-stimulated splenocytes (Reber et al., 2007) and plate-bound anti-CD3-stimulated T helper (Th) 2 cells from peripheral lymph nodes (Schmidt et al., 2010) of CSC compared to SHC mice. These are interesting findings, as an insufficient GC signaling can be observed in numerous affective and somatic disorders in man following chronic psychosocial stressor exposure (Caplan et al., 1979; Heim et al., 2000; Yehuda, 1997a, 1997b). In addition, CSC-stressed mice develop spontaneous colonic inflammation, indicated by an increased secretion of proinflammatory cytokines from mesenteric lymph node cells *in vitro* and an increased histological damage score of colonic tissue (Peters et al., 2012; Reber et al., 2007; Veenema et al., 2008). Moreover, CSC exposure was also shown to increase the risk for the development of inflammation-induced colorectal cancer (CRC), indicated by the development of macroscopic suspect lesions, as well as a trend towards an increased incidence of low- and/or high-grade colonic dysplasia (Peters et al., 2012). Interestingly, in humans, inflammatory bowel disease (IBD) has been shown to be a consequence of chronic life stress and colorectal cancer poses one of the most serious complications in these patients (Cairns et al., 2010; Eaden, 2004, 2003; Eaden et al., 2000; van Hogezand et al., 2002). Furthermore, IBD was also shown to be comorbid in patients suffering from depression (Bitton et al., 2003; Duffy et al., 1991; Levenstein et al., 2000). These findings further indicate that the CSC paradigm is an appropriate model of chronic psychosocial stress with

high construct validity (*i.e.* high disease relevance of methods by which the animal model is constructed; Nestler and Hyman, 2010). With respect to their behavior, CSC mice show reduced open-arm activity on the EPM and reduced center-activity in an open field after 19 days of CSC, further illustrating that chronic stressor exposure increases anxiety-related behavior, a phenomenon that co-occurs with depression in humans (Kennedy, 2008). Moreover, CSC mice spend a similar time investigating an empty cage and a cage with an unknown conspecific during the social preference/avoidance test (SPAT) on day 20 of CSC, suggesting a lack of social preference (Slattery et al., 2012). In addition, CSC mice also show an increased ethanol (EtOH) preference and total intake, which was already shown following 14 days of CSC exposure (Peters et al., 2013). Interestingly, in humans, chronic psychosocial stress represents a strong risk factor for the development of substance abuse such as alcoholism, which is often co-morbid with anxiety disorders (Boger et al., 2014; Cooper et al., 2014; Morley et al., 2014). Taken together, the CSC paradigm represents an animal model that utilizes a chronic psychosocial stress component and results in decreased GC signaling and concomitant affective and somatic pathologies, in particular the stress-induced anxiogenic phenotype and the systemic proinflammatory phenotype (Figure 2b). Thus, the CSC model is likely to have more translational value than other stress models in animals, e.g. when compared to the CMS or CSDS model, which lack either social or truly chronic components. Most interestingly, CSC exposure is associated with hypocorticism, a phenomenon that is not apparent after CMS or CSDS, but occurs in humans suffering from mood disorders. In contrast, CMS and CSDS are associated with greatly elevated plasma CORT levels (hypercorticism). Interestingly, in addition to mice, the CSC paradigm was also established in rats (Nyuyki et al., 2012). However, to date the effects of CSC exposure in rats are not that well characterized as compared to mice. Overall, the CSC paradigm is a promising animal model that makes it also possible to gain further insight into how stress-induced pathophysiological changes (e.g. HPA axis alterations) eventually lead to affective and somatic disorders in man.



b

	CSC vs. SHC	CSC causes …
anxiety-related behavior (EPM, LDB, EPF, OF, SPAT)	↑	affective changes
EtOH preference and total intake	↑	
social preference	↓	
IFN-γ production of mes LN cells *in vitro*	↑	somatic changes
colonic histological damage score (inflammation)	↑	
risk for colorectal cancer	↑	
pituitary weight	↑	decreased GC signaling
adrenal weight	↑	
basal morning plasma ACTH	↑ hypocorticism	
basal morning plasma CORT	⇔	
basal evening plasma CORT	↓	
adrenal ACTH sensitivity *in vitro*	↓	
GC sensitivity of LPS-stimulated splenocytes *in vitro*	↓ GC resistance	
GC sensitivity of anti-CD3-stimulated Th2 LN cells *in vitro*	↓	

Figure 2. Schematic illustration of the experimental design of the chronic subordinate colony housing (CSC) paradigm in mice and a summary of the main effects of CSC on behavioral, immunological and physiological parameters. (a) In the CSC paradigm, male mice weighing 19-21 g are housed singly for one week before they are assigned to the single-housed control (SHC) or the CSC group in a weight-matched manner. In order to induce chronic psychosocial stress, CSC mice are housed together with a larger dominant male for 19 consecutive days. In detail, four experimental CSC mice are put into the homecage of resident (1) on day 1 of CSC, resulting in immediate subordination of the four intruder CSC mice. The latter are then housed together with this dominant resident (1) for 7 consecutive days. On day 8, and again on day 15 of CSC, the four experimental CSC mice are transferred into the homecages of resident (2) (day 8) and resident (3) (day 15), respectively, in order to avoid habituation. On day 19, CSC and SHC mice are usually tested for their innate or physiological anxiety and on day 20 immunological and physiological parameter are assessed. (b) Compared to SHC mice, CSC mice show affective and somatic changes and develop decreased glucocorticoid (GC) signaling. Thus, the CSC paradigm represents a promising animal model to mimic

diseases in which decreased GC signaling is a core feature and to unravel the underlying mechanisms of stress-related pathology in humans. Abbreviations: EPM, elevated plus-maze; LDB, light-dark box; EPF, elevated platform; OF, open field; SPAT, social preference/avoidance test; mes LN cells, mesenterial lymph node cells; ACTH, adrenocorticotropic hormone; CORT, corticosterone; LPS, lipopolysaccharide; Th2, T helper 2; Adapted from Peterlik et al. (2016a).

Taken together, the rodent models explained above can be performed in mice as well as in rats. Which species to use probably depends on the particular research question to be answered. However, in case of the CSC model, mice are the first choice to gain more knowledge about the role of the brain glutamatergic neurotransmitter system in the development of the resulting affective and somatic changes, as the effects of chronic psychosocial stressor exposure using this model are much better characterized and robust in mice. Other advantages of mouse models are lower costs and less space necessary. Moreover, mouse models also offer the possibility to use transgenic animals, e.g. knockout mice, in order to analyze the underlying mechanisms.

2. The glutamate system is implicated in stress-related physiology and disorders

Major depression, anxiety, and drug abuse disorders represent the most prevalent stress-related psychiatric conditions and are an enormous health concern worldwide (Aas et al., 2012; Cryan and Holmes, 2005; Schneiderman et al., 2005). The etiology of these pathologies is complex, with chronic psychosocial stressors being the most acknowledged risk factors (Caspi et al., 2003; Cryan and Holmes, 2005; Cryan and Slattery, 2007; de Kloet et al., 2005; Schneiderman et al., 2005; Virtanen and Kivimäki, 2012; Virtanen et al., 2013). These factors include continuing adverse conditions, such as social decline along with poverty, or life events that possess a high degree of chronic threat (e.g. medical disabilities), long lasting negative emotions and experience of personal loss (de Kloet et al., 2005; Hammen, 2005; Virtanen and Kivimäki, 2012; Virtanen et al., 2013). The majority of these types of factors has been demonstrated to increase both anxiety- and depression-related behavior, but also alcohol and drug abuse (Blanchard et al., 2005, 2006; Kotov et al., 2010; Schmidt et al., 2007). Indeed, and not surprisingly, there's a high comorbidity between anxiety and depression/mood disorders, with approximately half of

the patients suffering from major depression also meeting criteria for comorbid anxiety (Kennedy, 2008).

As disorders of mood and emotion may show a common excessive or inappropriate brain excitability within crucial brain circuits, the L-glutamate system, which represents the primary excitatory neurotransmitter system in emotion and cognition circuits, is increasingly considered to play an important role in mental disease etiology and persistence. Several lines of evidence from human clinical studies link dysfunction in the L-glutamate system to the pathogenesis of psychiatric disorders (Cortese and Phan, 2005). For instance, changes in glutamate levels have been found in plasma, cerebrospinal fluid (CSF), and in the brain of patients suffering from mood and anxiety disorders (Levine et al., 2000; Mauri et al., 1998; Mitani et al., 2006). Interestingly, recent postmortem studies showed significant increases in glutamate levels in the frontal cortex and dorsolateral prefrontal cortex of depressed and bipolar patients, respectively (Hashimoto et al., 2007; Lan et al., 2009). Furthermore, various clinical neuroimaging studies have consistently demonstrated volumetric changes in brain regions, in which glutamatergic neurons predominate, such as the hippocampus, amygdala and several cortical regions (Lorenzetti et al., 2009).

The L-glutamate neurotransmitter system of the emotion and cognition circuitry of mammalian brains is composed of a large diversity of genetically regulated factors, including a group of vesicular, glial and synaptic glutamate transporters (Sheldon and Robinson, 2007) as well as two families of glutamate receptors: ligand-gated ionotropic glutamate receptors (iGlu) comprising (2R)-2-(methylamino)butanedioic acid (NMDA)-, 2-amino-3-(5-methyl-3-oxo-2,3-dihydro-1,2-oxazol-4-yl)propanoic acid (AMPA)- and (2S,3S,4S)-3-(carboxylatomethyl)-4-(prop-1-en-2-yl)pyrrolidine-2-carboxylate (kainate, KA)-receptors (Nakanishi, 1992; Planells-Cases et al., 2006; Willard and Koochekpour, 2013; Xiao et al., 2001), and the G protein-coupled metabotropic glutamate receptor (mGlu) subtypes -1 to -8 (mGlu1-8; Conn, 2003; Nicoletti et al., 2011; Pinheiro and Mulle, 2008; Willard and Koochekpour, 2013).

Throughout the last three decades, several drug discovery efforts were made targeting iGlu receptors, with preclinical and clinical data demonstrating NMDA and AMPA receptors to be promising targets in controlling cognitive and emotional changes observed in stress-related disorders (Boyce-Rustay and Holmes, 2006; Graybeal et al., 2012; Kim et al., 1996; Magariños and McEwen, 1995). In this regard, a major breakthrough came from clinical studies using the NMDA receptor antagonist ketamine by showing clinical efficacy in

treatment-resistant depression (TRD) and major depressive disorder (MDD) patients. Interestingly, ketamine administered intravenously showed strong decreases in the Hamilton Depression Rating Scale (HDRS) analysis, with an improvement observed 2 h after infusion that remained significant for over 1 week (Berman et al., 2000; Zarate et al., 2006). However, this iGlu receptor-based strategy is not devoid of limitations and risks, as ketamine administration has also been shown to be associated with cognitive and dissociative adverse effects, which thus limits ketamine's widespread application for the treatment of mood disorders (Pilc et al., 2008; Witkin et al., 2007). In contrast, therapeutic strategies targeting mGlu receptors represent a more subtle alternative in regulating excitatory (and possibly inhibitory) neurotransmission and are therefore considered to have a more favorable side-effect profile than ligand-gated ion channel modulation (Cartmell and Schoepp, 2000; Niswender and Conn, 2010). Indeed, signaling via mGlu receptors is slower and longer lasting than via iGlu receptors, allowing fine-tuning of glutamate regulation and its cellular responses, which could eventually avoid the adverse effects associated with direct modulation of iGlu receptors. In addition to that, growing evidence gives rise to mGlu-based compounds to be effective in regulating iGlu receptor signaling, further emphasizing the modulatory potential of mGlu receptors.

2.1 Glutamate signaling via mGlu receptors in the CNS

The existence of neuromodulatory glutamate receptors, namely the mGlu receptors, provides a mechanism by which binding of glutamate, in contrast to the fast synaptic responses mediated by iGlu receptors, slowly modulates cell excitability, synaptic neurotransmission and plasticity. mGlu receptor-induced modulation occurs via second messenger signaling pathways and their interactions with ion channels (C. H. Kim et al., 2008; Rondard and Pin, 2015; Willard and Koochekpour, 2013; Yin and Niswender, 2014). According to sequence homology, second messenger coupling and pharmacological properties, the mGlu receptor family is subdivided into three groups. The group I members, mGlu1 and mGlu5, are coupled to $G_{q/11}$ proteins and primarily elevate {[(1R,2S,3R,4R,5S,6R)-2,3,5-trihydroxy-4,6-bis(phosphonooxy)cyclohexyl]oxy}phosphonic acid (IP_3), diacylglycerol (DAG), and Ca^{2+} signal transduction. In general, these receptor subtypes function to enhance glutamate-mediated postsynaptic excitation (Abe et al., 1992; Bordi and Ugolini, 1999; Cartmell and Schoepp, 2000; S. J. Kim et al., 2008; D. Youn, 2014; D.-H. Youn, 2014). In contrast,

group II (mGlu2 and mGlu3) and group III (mGlu4, -6, -7, and -8) receptors inhibit adenylyl cyclase activity and other effector proteins via coupling to $G_{i/o}$ proteins and thereby negatively modulate excitatory neurotransmitter efflux and neuronal excitability upon activation (Cartmell and Schoepp, 2000; Kintscher et al., 2012; Lavreysen and Dautzenberg, 2008; McMullan et al., 2012; Mercier and Lodge, 2014; Schoepp, 2001; Tang et al., 2013). Interestingly, various mGlu receptors are expressed in both neurons and glial cells of the CNS, as well as in peripheral tissue like the enteric nervous system or adrenal gland cells (Julio-Pieper et al., 2011). In neurons, group I receptors show predominantly postsynaptic location and modulate cell excitability, while group II and III members are mainly expressed at the presynapse and are involved in regulating neurotransmitter release, mostly inhibiting release (Conn and Pin, 1997; Swanson et al., 2005). As they are members of class C GPCR, all mGlus are characterized by a large extracellular N-terminal "Venus flytrap domain" (VFTD), which is known to serve as the orthosteric ligand binding site and shows abundant homology between the different mGlu receptor subtypes. The binding site for allosteric modulators of the mGlus is located topographically distinct within the transmembrane domain (Christopoulos and Kenakin, 2002; Flor and Acher, 2012; Gregory et al., 2013; Nickols and Conn, 2014; Wood et al., 2011; Wu et al., 2014). As the allosteric binding site contains a higher level of sequence diversity between the receptor subtypes, allosteric ligands typically show greater subtype selectivity (Conn et al., 2009a, 2009b). Importantly, the widespread distribution of mGlu subtypes suggests that these modulatory receptors have the ability to participate in a broad array of physiological functions throughout the CNS and may represent suitable targets for therapeutic intervention in a variety of CNS disorders. Thus, the therapeutic potential of the mGlu receptors is increasingly receiving attention as possible treatment strategies for CNS diseases such as Parkinson's disease (PD), Fragile X syndrome (FXS), schizophrenia, addiction and in particular depression and anxiety-related disorders (Herman et al., 2012; Nicoletti et al., 2015; Picconi and Calabresi, 2014; Pomierny-Chamioło et al., 2014; Scharf et al., 2014).

2.1.1 Group I mGlu receptors: neurobiochemistry and distribution

In general, the group I members mGlu1 and mGlu5 couple to $G_{q/11}$ proteins and activate phospholipase C, a process that results in the formation of IP_3 and DAG (Figure 3). This classical pathway leads to intracellular Ca^{2+} mobilization and activation of protein kinase C

(PKC). Apart from this, group I receptors can also activate a range of further downstream effectors, most notably proteins involved in synaptic plasticity, such as mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK) and a mammalian target of rapamycin (mTOR) (Fieblinger et al., 2014; Pałucha-Poniewiera et al., 2014b; Zhang et al., 2015). With respect to their distribution, expression of both mGlu1 and mGlu5 primarily overlaps within brain regions implicated in mood disorders (Witkin et al., 2007). However, there are also distinct differences, with mGlu1's expression being abundant in cerebellum, olfactory bulb, the CA3 region of the hippocampus, in thalamus, dentate gyrus and substantia nigra, whereas mGlu5 is highly expressed in telencephalic regions, CA1 and CA3 regions of the hippocampus, septum, basal ganglia, striatum, amygdala and nucleus accumbens (Ferraguti and Shigemoto, 2006; Pilc et al., 2008). In addition, mGlu5 is also expressed in glial cells (Figure 3), particularly in astrocytes, where its expression has been highlighted with respect to a number of potential physiological roles, e.g. neuroprotection (Bruno et al., 2001a, 2001b, 1999; D'Antoni et al., 2011; Verkhratsky and Kirchhoff, 2007; Wierońska and Pilc, 2009).

2.1.2 Group II mGlu receptors: neurobiochemistry and distribution

In contrast to group I, the group II members mGlu2 and mGlu3 are coupled predominantly to $G_{i/o}$ proteins, negatively modulating adenylyl cyclase activity and directly regulating ion channels and other downstream signaling components via the release of the $G_{\beta\gamma}$ subunit. In addition, group II mGlus also couple to MAPK and phosphatidyl inositol 3- (PI3-) kinase pathways which are implicated in synaptic plasticity (D'Onofrio et al., 2001; Lin et al., 2014; Niswender and Conn, 2010). Both mGlu2 and mGlu3 are localized presynaptically in rather preterminal than terminal axonal portions, distant from the active zone of neurotransmitter release, where they are potentially activated by synaptic glutamate spillover (Tamaru et al., 2001). mGlu2 mRNA is observed to be highly expressed in pyramidal neurons in the enthorhinal and parasubicular cortical regions and in granule cells of the dentate gyrus (Hayashi et al., 1993; Ohishi et al., 1993a). In contrast, mGlu3 mRNA is highly expressed in neurons of the cerebral cortex and the caudate-putamen and in the granule cells of the dentate gyrus (Ohishi et al., 1993b; Tanabe et al., 1993). In addition, the mGlu3 receptor subtype (Figure 3) is also prominently expressed in glial cells throughout the whole brain, and its activation provides robust neuroprotection *in vitro* and *in vivo* (Battaglia et al., 2014; Ciceroni et al., 2010; Durand et al., 2014, 2013).

2.1.3 Group III mGlu receptors: neurobiochemistry and distribution

Group III represents the largest family of mGlu receptors and comprises the subtypes mGlu4, mGlu6, mGlu7 and mGlu8. Like group II, group III members are predominantly expressed presynaptically (Shigemoto et al., 1997; Figure 3) where they regulate neurotransmitter release (Antflick and Hampson, 2012; de Rover et al., 2008; Grueter and Winder, 2005; Panatier et al., 2004; Pinheiro and Mulle, 2008; Woodhall et al., 2007). By coupling to $G_{i/o}$ proteins, their activation inhibits cAMP formation and indirectly affects synaptic transmission and neurotransmitter release by modulating membrane Ca^{2+}- and K^{+}-channels (Lavreysen and Dautzenberg, 2008; Mercier and Lodge, 2014; Pin and Duvoisin, 1995). As with group II mGlu receptors, the group III subtypes also couple to other signaling pathways, including MAPK and PI3-kinase, providing further complexity to the mechanisms by which they regulate synaptic transmission (Iacovelli et al., 2014, 2004, 2002). Except for the mGlu6 receptor subtype, whose expression is restricted to the retina, all other group III mGlus are widely expressed throughout the mammalian brain and also in peripheral tissue (Julio-Pieper et al., 2011; Pilc et al., 2008). In detail, in the CNS the mGlu4 receptor subtype is highly expressed in the cerebellum, the olfactory bulb and thalamus as well as in the hippocampus, the cerebral cortex and basal ganglia in pre- and post-synaptical position (Corti et al., 2002; Kinoshita et al., 1996; Shigemoto et al., 1997). In addition, widespread peripheral mGlu4 expression has been shown also in the pancreas, adrenal glands and gastrointestinal tract (Akiba et al., 2009; Brice et al., 2002; Chang et al., 2005; Sarría et al., 2006). The mGlu7 receptor is highly localized in the presynaptic active zone and abundantly expressed in brain regions such as neocortex, hippocampus, amygdala, locus coeruleus, thalamus and hypothalamus (Kinoshita et al., 1998; Kosinski et al., 1999; Ngomba et al., 2011). In the periphery, mGlu7 expression has been reported in the adrenal glands, the colon and stomach, among other areas (Julio-Pieper et al., 2010; Nakamura et al., 2010; Scaccianoce et al., 2003). The mGlu8 receptor is found predominantly in the CNS in presynaptic terminals in the olfactory bulb, hippocampus, cerebellum and cortical areas (Ferraguti and Shigemoto, 2006), but also in peripheral tissue such as pancreas and testis (Julio-Pieper et al., 2011). Interestingly, general expression levels of mGlu8 receptors seem considerably lower than those of mGlu4 and mGlu7 (Niswender and Conn, 2010).

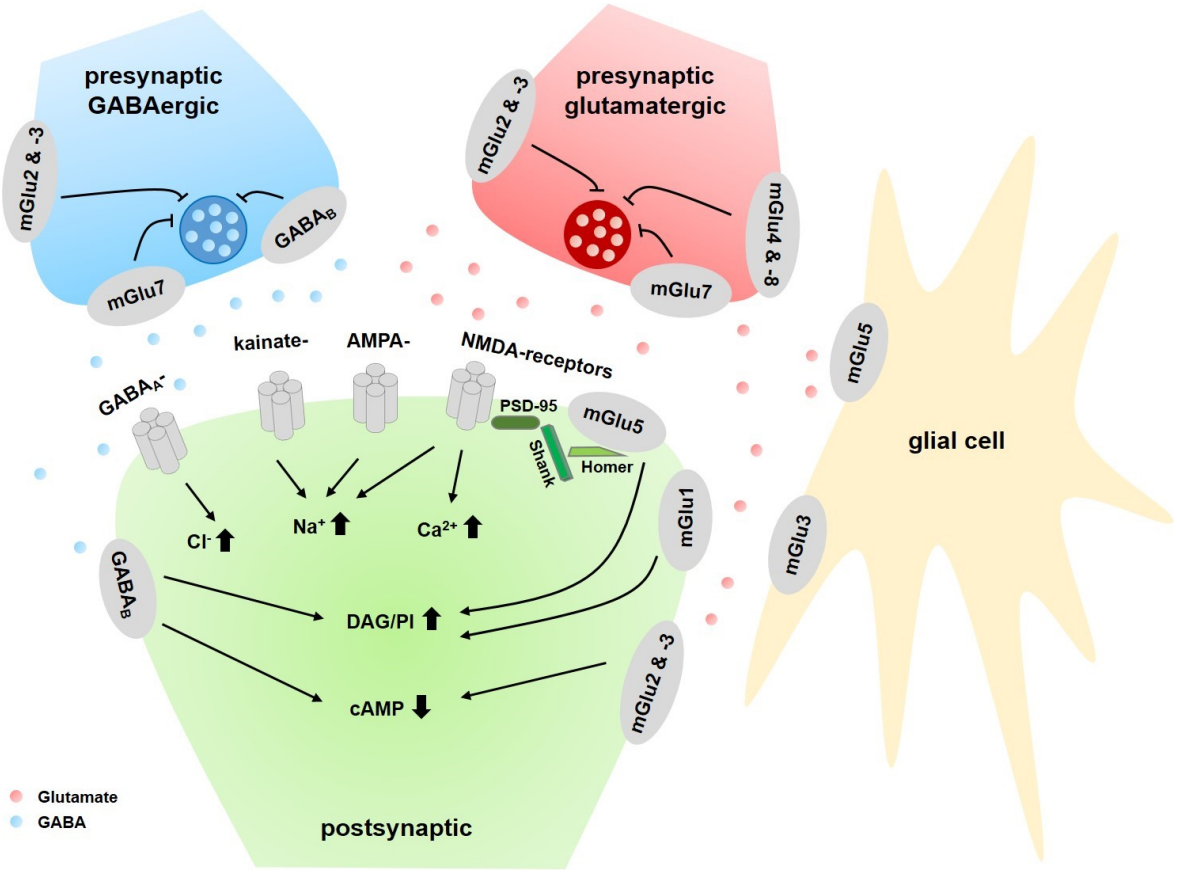


Figure 3. Schematic representation of mGlu receptors at the synapse. In general, group I mGlu subtypes are localized postsynaptically, whereas group II and III receptors are localized mainly in presynaptic locations. While the mGlu7 receptor subtype is localized in the active zone, mGlu subtypes 2, 3, 4, and 8 are generally found in perisynaptic locations on the presynapse. Group II and III receptors modulate the release of glutamate (right, red circles) or 4-aminobutanoic acid, GABA (left, blue circles). At the postsynaptic terminal, the ionotropic (2R)-2-(methylamino)butanedioic acid (NMDA)-, 2-amino-3-(5-methyl-3-oxo-2,3-dihydro-1,2-oxazol-4-yl)propanoic acid (AMPA)- and (2S,3S,4S)-3-(carboxylatomethyl)-4-(prop-1-en-2-yl)pyrrolidine-2-carboxylate (kainate, KA)-receptors respond to glutamate with increases in intracellular sodium or calcium, promoting cell excitability. Group I mGlus signal via $G_{q/11}$ proteins to increase diacylglycerol (DAG) and phosphatidylinositol (PI). Importantly, mGlu5 and NMDA receptors are closely linked to each other via Shank, Homer and PSD-95 (postsynaptic density-95) proteins. Postsynaptic mGlu2/3 and $GABA_B$ receptors couple to cAMP inhibition. Instead, $GABA_A$ chloride channels modulate intracellular chloride levels. Expression of mGlu3 and mGlu5 on glial cells has emerged as another key site for regulation of synaptic activity, however, the consequences of receptor activation on these cells and the exact signaling pathways are presently not well understood. Adapted from Peterlik et al. (2016a).

2.2 Dysregulation of brain mGlu receptor gene expression in chronic stress models – state of the art

Recent findings clearly have sparked interest in neurobiological systems that were previously little explored in mood disorders, such as the glutamatergic system. In particular the clinical findings with ketamine have inspired new lines of preclinical research to explore the glutamate system in more detail, including modulatory receptors that could be targeted to achieve better side-effect profiles (Maeng and Zarate, 2007), and to investigate the underlying neural mechanisms. To my knowledge, only few studies have dealt with chronic stress and the involvement of mGlu receptors in the manifestation of stress-induced changes. But some promising results have already emerged. Wierońska et al. (2001) addressed changes of mGlu5 expression in response to CMS exposure and reported an increase of mGlu5 protein expression in CA1 and a decrease in CA3 of the rat hippocampus. Furthermore, O'Connor and co-workers (2013a) found no changes of hippocampal group III mGlu receptor mRNA expression upon either chronic immobilization stress or chronic social defeat and concluded that hippocampal group III mGlu receptors may not be involved in the manifestation of behavioral and physiological changes observed in these models. However, early-life stress induced by maternal separation specifically reduced the expression of mGlu4 mRNA in the hippocampus, whereas mGlu7 and mGlu8 mRNA remained unaffected (O' Connor et al., 2013). Taken together, they could demonstrate that there were only very few, but selective changes to group III mGlu receptors under early-life stress conditions. These findings ask for further research efforts to study mGlu receptors as potentially important players in chronic stress-induced pathology.

2.3 Current knowledge of mGlu receptor genetic and pharmacological modulation in acute and chronic stress

A possible aim of pharmacological intervention targeting glutamate neurotransmission in stress-related disorders could be that excessive glutamate exposure in specific brain areas should be blocked, whereas normal glutamatergic neurotransmission should be kept unaffected. New ways of fine-tuning the glutamatergic system are now emerging via the pharmacological modulation of mGlu receptor subtypes (Bergink et al., 2004; Pilc et al., 2008; Swanson et al., 2005; Witkin et al., 2007). The wide functional diversity and distinct distribution patterns of mGlu receptor subtypes provide an opportunity for selectively

targeting individual mGlu receptor subtypes in order to attempt the development of novel treatment strategies for emotional disorders. A large body of preclinical studies suggests that ligands for specific mGlu subtypes have potential in multiple mood disorders, including anxiety disorders and depression. More recently, data from clinical studies with mGlu subtype-selective ligands are beginning to emerge and are providing remarkable clinical efficacy of some of these compounds, which I discuss below. In doing so, I will elucidate the role of group I and III receptors in stress physiology and especially bring into focus the recent efforts on modulation of mGlu5 and mGlu7.

2.3.1 Role of group I mGlu receptors in stress physiology – focus on mGlu5 functional blockade

Group I mGlu receptors are broadly distributed within the peripheral and central nervous system and are expressed at post- and perisynaptic sites in several areas implicated in anxiety and emotional processing, and there is evidence for the involvement of particularly the mGlu5 subtype in the pathophysiology of different emotional and somatic disorders. For example, human post-mortem studies reported specific reductions of total mGlu5 mRNA and protein levels in the lateral cerebellum (S H Fatemi et al., 2013; S Hossein Fatemi et al., 2013) and prefrontal cortex (Deschwanden et al., 2011) in MDD patients (Newell and Matosin, 2014). Furthermore, a study by Wierońska et al. (2001) showed an increase of mGlu5 expression in CA1 and a decrease in CA3 of rat hippocampus in response to CMS, supporting the involvement of mGlu5 in the pathophysiology of mood disorders in response to chronic stress. The mGlu5 receptor subtype functionally interacts with NMDA receptors by indirect physical and positive feedback linkage via a variety of intracellular mechanisms including Homer, Shank and PSD-95 proteins (Collett and Collingridge, 2004; Homayoun et al., 2004; Pignatelli et al., 2014; Stoop et al., 2003; Tu et al., 1999; Turle-Lorenzo et al., 2005; Wagner et al., 2014; Figure 3). This close functional association and positive reciprocal regulation between mGlu5 and NMDA makes the mGlu5 receptor subtype an attractive target for the indirect modulation of NMDA receptor function, which is known to be dysregulated in a variety of neuropsychiatric pathologies including mood disorders (Geddes et al., 2011; Hashimoto, 2013; Tokita et al., 2012; Weickert et al., 2013). Importantly, as pharmacological activation of mGlu5 is shown to cause neurotoxicity and neurodegeneration (Battaglia et al., 2004; Bruno et al., 2001a; Nicoletti et al., 1999), the activating mode of action will not be considered in the context of

mGlu5's potential therapeutic application. In contrast, pharmacological blockade of mGlu5 function has recently emerged to be one of the most promising and quite advanced therapeutic strategies for the treatment of psychiatric conditions (Jaeschke et al., 2015; Li et al., 2013; Lindemann et al., 2015, 2011; Michalon et al., 2014, 2012; Tian et al., 2015; Wagner et al., 2014). This approach has demonstrated anti-stress efficacy in a number of animal and human studies and studies using mGlu5-deficient mice elegantly support the functional role of mGlu5 fear learning (Lu et al., 1997; Xu et al., 2009), acute stress physiology (J Brodkin et al., 2002) and depression-related behaviors (Li et al., 2006).

3-(3-chlorophenyl)-1-(1-methyl-4-oxo-5H-imidazol-2-yl)urea (fenobam), a compound shown to be a clinically active anxiolytic already in the early 1980s (Lapierre et al., 1982; Pecknold et al., 1982), was recently described to exert its pharmacological effects via inverse receptor agonist activity at the mGlu5 receptor (Porter et al., 2005). Moreover, allosteric blockade of mGlu5 with the prototypical allosteric receptor antagonist 2-methyl-6-(2-phenylethynyl)pyridine (MPEP) showed broad anxiolytic and antidepressant-like profiles in acute rodent animal models (Gasparini et al., 2008; Li et al., 2006; Liu et al., 2012; Spooren et al., 2000; Tatarczyńska et al., 2001). Li et al. (2006) reported that administration of MPEP and the tricyclic antidepressant (TCA) imipramine resulted in a synergistic antidepressant-like effect in the FST and that this effect was even persistent after sub-chronic treatment (once daily, for five consecutive days). A further study revealed that MPEP remained equally active in reducing the SIH response in mice after sub-chronic dosing for five consecutive days, with comparable efficacy as after acute administration (Nordquist et al., 2007). Those studies provided the first evidence that longer-term administration of mGlu5 blockers may have the potential to ameliorate stress-induced pathophysiology. Moreover, MPEP's anxiolytic activity could be confirmed in a battery of further acute animal models, such as the EPM, Vogel conflict- and marble burying tests and the fear-potentiated startle paradigm (Jesse Brodkin et al., 2002; Kłodzińska et al., 2000; Pilc et al., 2002; Spooren et al., 2000; Tatarczyńska et al., 2001). Another selective mGlu5 receptor antagonist, 3-[2-(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP), turned out to be a good pharmacological tool to confirm antistress activity in several behavioral models (Cosford et al., 2003; Klodzinska et al., 2004; Molina-Hernández et al., 2006; Pałucha-Poniewiera et al., 2014a; Pomierny-Chamioło et al., 2010; Tichá et al., 2011). MTEP showed activity in the FST, TST and olfactory bulbectomy (OB) model (Belozertseva et al., 2007; Pałucha et al., 2005). In the latter, repeated administration of MTEP attenuated the OB-related hyperactivity of rats in

the open field test, a finding that resembles the action of typical antidepressants in the OB model of depression (Pałucha et al., 2005). Obviously, both MPEP and MTEP have been studied in a wide range of preclinical animal models for different therapeutic indications, however, both compounds are not suitable drug candidates for clinical development due to pharmacokinetic constraints (Lindsley and Emmitte, 2009). Furthermore, acute administration of the recently discovered mGlu5-selective negative allosteric modulator (NAM) 2-{2-[2-(difluoromethoxy)-5-({5H,6H,7H-pyrrolo[3,4-b]pyridin-6-yl}carbonyl)phenyl]ethynyl}pyridine (GRN-529) showed dose-dependent efficacy across a broad battery of animal models, including the FST and TST, anxiety tests (attenuation of SIH response and increased punished crossings in the four plate test) and pain models (reversal of hyperalgesia due to sciatic nerve ligation or inflammation) (Hughes et al., 2013). Another novel and highly selective mGlu5 receptor antagonist, methyl(3aR,4S,7aR)-4-hydroxy-4-[2-(3 methylphenyl)ethynyl]-3,3a,5,6,7,7a-hexahydro-2H-indole-1-carboxylate (AFQ056, mavoglurant), showed an even improved pharmacokinetic profile in rodents and better efficacy in SIH tests in mice as compared to the prototypic mGlu5 antagonist MPEP (Vranesic et al., 2014). Interestingly, efficacy of AFQ056 has been reported also in L-dopa-induced dyskinesia in Parkinson's disease and Fragile X syndrome in proof-of-principle clinical studies (Gomez-Mancilla et al., 2014; Petrov et al., 2014; Rascol et al., 2014; Vranesic et al., 2014). Recently, the mGlu5-selective NAM 2-chloro-4-{[1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl]ethynyl}pyridine (basimglurant) turned out to be very promising in a phase II clinical study as an adjunctive therapy in MDD patients by demonstrating safety and efficacy using multiple read-outs. This study showed that a 6-week double-blind treatment of basimglurant *vs.* placebo reached significant improvements in patient-rated Montgomery-Asberg depression rating scale (MADRS) results, in remission assessment and further ratings (Jaeschke et al., 2015; Lindemann et al., 2015; Quiroz et al., 2015). The consistency of the efficacy findings combined with good tolerability warrants further investigation with basimglurant in depressive disorders. Furthermore, the recently discovered mGlu5 NAM 2-chloro- 4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4-yl]ethynyl]pyridine (CTEP), a compound chemically similar to basimglurant and optimized for utility in rodent studies, was shown to be active in acute rodent models, such as the SIH test in mice and the Vogel conflict test in rats (Lindemann et al., 2011). CTEP is the first reported mGlu5 inhibitor with both, very long half-life of approximately 18 h and high oral bioavailability in rodents, classifying as

useful pharmacological tool for long-term treatment. CTEP thus allows the exploration of the full therapeutic potential of mGlu5 inhibition for indications requiring chronic receptor blockade. Indeed, Michalon et al. (2014, 2012) found out that chronic treatment with CTEP in a mouse model of Fragile X rescued learning and memory deficits, elevated locomotor activity and increased spine density, suggesting that this mGlu5 NAM treatment may be effective in correcting multiple neurological symptoms (Lindemann et al., 2015). Furthermore, a very recent study reported that chronic administration of CTEP was able to alleviate some CSDS-induced depressive-like symptoms, such as reduced locomotion and an anhedonic phenotype, while lacking protective effects on selected physiological parameters (Wagner et al., 2014). However, the stress-protective relevance of mGlu5 particularly under permanent chronic psychosocial stress conditions with respect to a broader range of pathologies has not been addressed so far.

2.3.2 Role of group III mGlu receptors in stress physiology – focus on mGlu7 functional blockade

Group III mGlu receptors have received somewhat less attention, mostly due to the obvious paucity of pharmacological tools available to study them (Flor and Acher, 2012; Lavreysen and Dautzenberg, 2008; Mercier and Lodge, 2014; Schoepp et al., 1999). Nevertheless, they are thought to be involved in a number of disease states and physiological conditions, consistent with their role in the regulation of both glutamatergic and GABAergic neurotransmission throughout the brain (Drew et al., 2008; Farazifard and Wu, 2010; Guimarães-Souza and Calaza, 2012; Jang, 2014; Macinnes and Duty, 2008; Semyanov and Kullmann, 2000; Summa et al., 2013). Much of our current knowledge still relies on studies performed by direct central application of compounds and on the characterization of genetically manipulated animals under basal and under stress conditions. For instance, Tatarczyńska et al. (2002) found out that intraventricular injection of the group III mGlu receptor agonist (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I) produced both anxiolytic- and antidepressant-like effects. Its anxiolytic action was shown in both the SIH and EPM tests in mice, and in the Vogel conflict test in rats. Its antidepressant-like action was evaluated in the FST (Stachowicz et al., 2009; Tatarczyńska et al., 2002). Interestingly, another study showed that the antidepressant-like effects of centrally applied ACPT-I could be reversed by the group III mGlu receptor antagonist 2-amino-2-cyclopropyl-2-(4-phosphonophenyl)acetic acid

(CPPG) (Pałucha et al., 2004). However, these compounds are not subtype-selective, as they act at all members of group III receptors, making it impossible to allocate these effects to a specific receptor subtype. Addressing the influence of mGlu4, Kłak et al. (2007) could show that the combined administration of the mGlu4-selective PAM 7-hydroxyimino-N-phenyl-1,7a-dihydrocyclopropa[b]chromene-1a-carboxamide (PHCCC) and a non-effective dose of ACPT-I produced antidepressant-like efficacy in the rat FST. A further study demonstrated that administration of PHCCC into the basolateral amygdala resulted in dose-dependent anti-conflict effects in the rat Vogel conflict test, indicating that positive allosteric modulation of mGlu4 receptors may be a useful therapeutic approach for anxiety (Stachowicz et al., 2004). More recently, the peripheral use of (1R,2S)-2-[(3,5-dichlorophenyl)carbamoyl]cyclohexane-1-carboxylic acid (VU0155041), an mGlu4 PAM (Niswender et al., 2008), demonstrated anxiolytic action in the elevated zero maze (Duvoisin et al., 2011). In addition, the novel mGlu4 PAMs (1S,2R)-2-[(aminooxy)methyl]-N-(3,4-dichlorophenyl)cyclohexane-1-carboxamide (Lu AF21934) and 4-methyl-N-[5-methyl-4-(1H-pyrazol-4-yl)-1,3-thiazol-2-yl]pyrimidin-2-amine (ADX88178) have been reported to induce anxiolytic-like effects in acute rodent models including SIH, four-plate and marble-burying test, and also to be active in multiple models of Parkinson's disease (Kalinichev et al., 2014; Sławińska et al., 2013). Interestingly, mGlu4-deficient mice exerted increased measures of anxiety in acute models, including the open field and elevated zero maze, and impaired sensorimotor function on the rotarod test (Davis et al., 2012). Consistent with this, they also showed enhanced amygdala-dependent cued fear conditioning (Davis et al., 2013). Similar to these findings, mice lacking mGlu8 showed higher measures of anxiety as compared to control animals (Duvoisin et al., 2005; Linden et al., 2002) and when exposed to novel, aversive environments, they exhibit greater neuronal activation in stress-related brain regions (Linden et al., 2003). These studies suggest enhanced reactivity to stressors in mice deficient for mGlu4 or mGlu8. To go on with mGlu8, acute pharmacological stimulation with its agonist 4-[(1S)-1-amino-2 hydroxy-2-oxoethyl]phthalic acid (DCPG) reduced innate anxiety in the open field and EPM tests (Duvoisin et al., 2010) and reduced the expression of contextual fear without affecting the acquisition and expression of cued fear (Fendt et al., 2013). Furthermore, 2-amino-2-(4-phosphonophenyl)acetic acid (RS-PPG), an mGlu8 receptor-preferring agonist, induced dose-dependent antidepressant-like effects in the FST after central administration (Pałucha et al., 2004). Moreover, the mGlu8-selective PAM 2-{[(4-bromophenyl)methyl]sulfanyl}-N-[4-(butan-2-yl)phenyl]acetamide (AZ12216052)

reduced measures of anxiety in the open field and EPM tests (Duvoisin et al., 2010). However, as this anxiolytic effect was still present in mGlu8-KO mice, the effect of AZ12216052 on measures of anxiety likely involves molecular targets other than mGlu8, too (Duvoisin et al., 2011). Nevertheless, the behavioral data so far suggest that mGlu4 and mGlu8 receptor activation may render anxiolytic, anti-stress as well as antidepressant-like effects (Raber and Duvoisin, 2014).

With respect to the mGlu7 subtype, the most widely distributed of the presynaptic mGlu receptors, clear evidence for its important role in fear and stress physiology comes from studies using mice with genetic ablation of mGlu7 (Cryan et al., 2003; Fendt et al., 2013; Masugi et al., 1999). These mice are characterized by reduced amygdala-dependent conditioned fear and aversion. Moreover, they display an anti-depressant and anxiolytic-like phenotype in acute behavioral tests for despair and innate anxiety (Cryan et al., 2003; Masugi et al., 1999). In addition, mGlu7-deficient mice have increased hippocampal brain-derived neurotrophic factor (BDNF) protein levels and show an upregulated GR-dependent feedback suppression of the HPA axis (Mitsukawa et al., 2006), further supporting mGlu7's critical role in stress physiology. Regarding pharmacological modulation, only a few systemically active allosteric modulators for mGlu7 were identified so far, yielding interesting results in acute behavioral paradigms. For instance, the first mGlu7-selective allosteric agonist N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082) was shown to elevate basal plasma ACTH and CORT levels (Mitsukawa et al., 2005), displayed mGlu7-dependent antidepressant-like activity (Bradley et al., 2012; Palucha et al., 2007), and modulated acquisition and extinction of conditioned fear (Fendt et al., 2013, 2008; Toth et al., 2012). At a first glance, these effects seem to be in contradiction with the anxiolytic- and antidepressant-like behavioral changes observed in mice lacking mGlu7 or after siRNA-mediated knockdown of the receptor (O'Connor et al., 2013b). However, AMN082 has been shown also to induce rapid and lasting mGlu7 internalization, a form of functional antagonism and a possible mechanism for the drugs anxiolytic- and antidepressant-like activity (Pelkey et al., 2007). A further explanation would be that AMN082 not only binds to mGlu7 but with weaker affinity also to monoamine transporters, similar to its primary metabolite N-benzhydrylethane-1,2-diamine (Met-1), which inhibits serotonin and norepinephrine reuptake transporters with a physiologically relevant affinity (Sukoff Rizzo et al., 2011). Altogether, these findings may explain the seemingly conflicting results obtained using AMN082 *in vivo*. Similar to AMN082, two systemically active mGlu7 NAMs have also yielded divergent results in behavioral tests,

despite displaying very similar pharmacological properties *in vitro*. 6-(2,4-dimethylphenyl)-2-ethyl-4,5,6,7-tetrahydro-1,3-benzoxazol-4-one (ADX71743) was shown to have robust anxiolytic effects in the EPM and the marble burying test (Kalinichev et al., 2013). In contrast, 6-(4-methoxyphenyl)-5-methyl-3-(pyridin-4-yl)-4H,5H-[1,2]oxazolo[4,5-c]pyridine-4-one (MMPIP) was reported to have only weak anxiolytic activity but reversed antidepressant-like effects of AMN082 in rats (O'Connor and Cryan, 2013; Pałucha-Poniewiera and Pilc, 2013). In addition, MMPIP was reported to impair cognitive performances in the object recognition and the object location test (Hikichi et al., 2010). Like AMN082, both NAMs likely act via allosteric sites in more lipophilic domains and, thus, may be associated with desirable off-target effects. Furthermore, allosteric ligands have been shown to act in a context-dependent manner and not to necessarily affect all downstream signaling pathways of a given GPCR (Niswender et al., 2010). The recent discovery and characterization of the mGlu7 antagonist 7-hydroxy-3-(4-iodophenoxy)-4Hchromen-4-one (XAP044) may have provided a step forward in understanding mGlu7's function. XAP044 was identified as the first mGlu7-selective full antagonist that binds to the receptor's VFTD, a mechanism alternative to binding to allosteric sites.

In summary, pharmacological blockade of mGlu7 may represent the most promising mode of action in attempting to reverse stress-related pathophysiological states in psychiatric illness. In line with this, human genetic studies with depressed siblings and recurrent MDD patients pointed at GRM7 (the gene coding for mGlu7 receptors) as a gene potentially involved in human depression (Hamilton, 2011; Shyn et al., 2011). Taken together, considerable progress has been made in recent years in increasing our understanding of particularly the mGlu7 receptor within the CNS, and has remarkably revealed key roles for this receptor subtype in acute stress, fear- and depression-related behavior, thereby emphasizing the therapeutic potential of group III-directed ligands and asking for future studies under chronic stress conditions.

3. Concluding introductory remarks

Encouraging evidence has emerged from both preclinical and clinical research in recent years, supporting key roles for the brain glutamatergic neurotransmitter system in the physiology of psychiatric disorders. However, only little is known about the contribution

of the glutamate system to the pathophysiology following chronic psychosocial stress, which is the most acknowledged risk factor for emotion disorders, such as anxiety and depression (Caspi et al., 2003; Cryan and Holmes, 2005; Cryan and Slattery, 2007; de Kloet et al., 2005; Hammen, 2005; Schneiderman et al., 2005; Virtanen and Kivimäki, 2012). Moreover, current drug discovery efforts targeting the mGlu receptors led to the identification of pharmacological tools with promising efficacy in psychiatric conditions (Conn et al., 2009a; Lavreysen and Dautzenberg, 2008; Mercier and Lodge, 2014; Nicoletti et al., 2015; Swanson et al., 2005). The potential of these pharmacological tools in the manifestation of physiological and behavioral consequences of chronic psychosocial stress still needs to be investigated. On a preclinical basis, the CSC paradigm might be an appropriate animal model, as it utilizes chronic psychosocial stressor exposure and results in decreased GC signaling and concomitant somatic and affective pathologies, such as an overall proinflammatory and cancer-prone phenotype and an anxiogenic and substance abuse phenotype (Füchsl et al., 2014; Langgartner et al., 2015; Peters et al., 2013, 2012; Reber et al., 2007; Schmidt et al., 2010; Uschold-Schmidt et al., 2013, 2012). All these consequences are relevant for the development of somatic, e.g. gastrointestinal, and/or psychiatric disorders and the question whether mGlu receptors have the potential to modify these consequences is of great interest.

The recent discoveries of CTEP and basimglurant, two compounds with markedly improved pharmacokinetic and safety profiles as compared to previous mGlu5 NAMs, such as MPEP and MTEP, may be most suitable to study the long-term effects of mGlu5-blockade *in vivo* following chronic stress exposure. Most notably, CTEP's sufficiently long half-life amenable for once daily administration in rodents and its high *in vivo* potency to achieve a low application dose makes it a suitable compound for chronic application and for the study of mGlu5's role in chronic stress conditions. Indeed, Wagner et al. (2014) demonstrated that sustained mGlu5 receptor blockade via sub-chronic CTEP administration was able to recover CSDS-induced behavioral alterations. The finding, that CTEP did not have any stress-protective effects on selected physiological parameters, requires further investigation. A potentially profitable approach in this regard may be to investigate whether chronic CTEP administration can attenuate the various physiological, immunological, and also behavioral consequences of chronic psychosocial stress induced by CSC exposure. Such studies may suggest future therapeutic avenues for mGlu5 NAMs in chronic clinical conditions, including somatoform psychiatric disorders.

The mGlu7 receptor is also a promising target in chronic stress physiology, but the question still remains, which mechanism of action – its activation or its blockade – might be effective in ameliorating the detrimental consequences of chronic psychosocial stress. Considerable progress has been made with the discovery of the mGlu7 agonist AMN082 (Mitsukawa et al., 2005) and the mGlu7 NAM ADX71743 (Kalinichev et al., 2013), two compounds that have shown robust efficacy in animal models for anxiety and depression, but have not yet been investigated in any context of chronic or even psychosocial stress. Of note, the recent discovery of the first orthosteric-like mGlu7 antagonist XAP044 (Gee et al., 2014), might provide an additional and suitable tool compound to elucidate the role of mGlu7 in chronic psychosocial stress conditions, at least in rodents. Moreover, the use of mice lacking mGlu7 might help to dissect the role of mGlu7 in chronic psychosocial stress conditions and to suggest which mode and direction of mGlu7 modulation might be preferable.

Taken together, there is emerging evidence that makes it worthwhile to further investigate in detail the potentially beneficial roles of especially mGlu5 and mGlu7 subtype-selective modulation in the context of chronic psychosocial stress and to provide a better understanding of the neural mechanisms involved and regulated by mGlu receptors. Besides providing fundamental neurophysiological insights, these investigations will hopefully stimulate drug development towards mGlu5- and mGlu7-targeted therapies aiming at the large panel of human chronic stress-induced disorders.

4. Aims of the present thesis

The present PhD thesis can be separated into two major parts:

1.) *In vivo* characterization of the first orthosteric-like mGlu7-selective antagonist XAP044.

2.) Investigation of molecular changes within the L-glutamate system in response to chronic psychosocial stress in mice, with a focus on mGlu receptors. Detailed analysis of potentially beneficial roles of selective mGlu5 and mGlu7 receptor subtype modulation on chronic psychosocial stress.

Ad 1.)

A very recent study paved the way in advancing our understanding of mGlu7 function and its therapeutic implication. Gee et al. (2014) presented XAP044, the first mGlu7-selective full antagonist that blocks the receptor's signaling pathways via binding to its hydrophilic VFTD close to the L-glutamate binding site. Thus, XAP044 presumably acts via a novel site compared to the allosteric ligands described above (AMN082, ADX717343, MMPIP). Moreover, XAP044 was shown to selectively block long-term potentiation (LTP) in the lateral amygdala of wildtype but not of mGlu7-deficient mice, further substantiating a role for mGlu7 in the cellular physiology of the fear and emotion circuitry. It was part of the present PhD thesis to characterize XAP044 *in vivo* in a broad battery of acute stress tests for innate anxiety, depression and conditioned fear tests in mice and thus to demonstrate a new mechanism of mGlu7 receptor binding to have potential therapeutic usefulness.
The results presented here contributed to and are part of the study published by Gee et al. (2014) in the *Journal of Biological Chemistry*.

Ad 2.)

The L-glutamate system has often been addressed in the context of acute stress, fear- and depression-related behavior. A number studies provide striking evidence that both the mGlu5 and mGlu7 subtypes are crucially involved in the regulation of physiological processes associated with acute stress, fear and depression-related behaviors as well as in the regulation of neuroendocrine function. However, only little is known about the role these receptors play in chronic psychosocial stress, the most acknowledged risk factor for emotion and somatoform disorders. Therefore, at least two main questions emerged: i) what are the molecular changes that occur within the mGlu receptor system in response to chronic psychosocial stress? ii) Is it possible to ameliorate the chronic stress-induced detrimental behavioral, physiological and immunological consequences by genetic and pharmacological modulation of the mGlu5 and mGlu7 receptor subtypes? These questions were addressed by using the CSC paradigm, an animal model that utilizes chronic psychosocial stressor exposure and that results in concomitant affective and somatic alterations. Given the crucial role of mGlu5 and mGlu7 in the regulation of acute stress and fear as well as of neuroendocrine function, robust alterations were expected in gene expression levels of both receptor subtypes after CSC exposure in brain regions involved in the control of behavioral and physiological stress responses. Therefore, potential effects of CSC were assessed on mGlu receptor mRNA expression particularly in the prefrontal

cortex (PFC), the hypothalamus (HT) and the hippocampus (HC). Furthermore, functional inhibition of mGlu5 and mGlu7 was predicted to result in a decreased vulnerability to chronic psychosocial stress, *i.e.* lower levels of anxiety and relief of physiological and immunological maladaptations. To this end, conventional mGlu7 knockout (KO) mice and corresponding wildtype (WT) mice exposed CSC were assessed with respect to selected stress-related behavioral parameters, HPA axis functionality and immune function. On the other hand, to reveal a potential stress-protective role of mGlu5 inhibition, conventional mGlu5 KO exposed to CSC were analyzed with respect to the same parameters as above and compared to corresponding WT mice. Moreover, to extend the findings from the mGlu5 KO and to obtain independent evidence for mGlu5 receptor inhibition to be stress-protective, CTEP was continuously infused via micro-osmotic pumps in mice during CSC exposure and selected parameters were analyzed after 19 days of CSC in these mice.

Material and Methods

The Material and Methods section includes chapters taken and adapted from one joint first author publication and submitted first author manuscripts:

1.) Gee, C.E.*, **Peterlik, D.***, Neuhäuser, C., Bouhelal, R., Kaupmann, K., Laue, G., Uschold-Schmidt, N., Feuerbach, D., Zimmermann, K., Ofner, S., Cryan, J.F., van der Putten, H., Fendt, M., Vranesic, I., Glatthar, R., Flor, P.J., 2014. Blocking metabotropic glutamate receptor subtype 7 (mGlu7) via the Venus flytrap domain (VFTD) inhibits amygdala plasticity, stress, and anxiety-related behavior. *J. Biol. Chem.* 289, 10975–87. doi:10.1074/jbc.M113.542654.* **Both authors contributed equally to this work.** (*J. Biol. Chem.* permits the author to include published journal articles in full or in part in the author's dissertation)

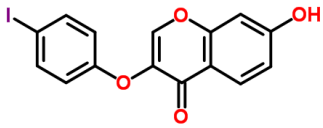
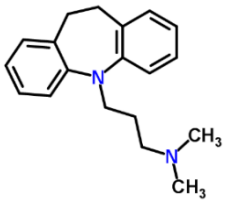
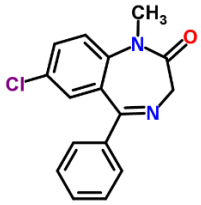
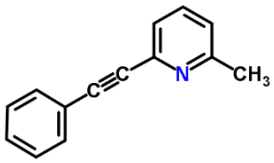
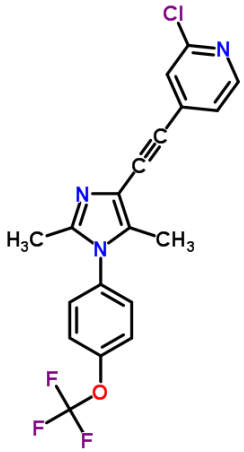
2.) **Peterlik, D.**, Stangl, C., Bauer, A., Bludau, A., Keller, J., Grabski, D., Killian, T., Schmidt, D., Zajicek, F., Jaeschke, G., Lindemann, L., Reber, S.O., Flor, P.J., Uschold-Schmidt, N., 2016b. Blocking Metabotropic Glutamate Receptor Subtype 5 Relieves Maladaptive Stress Consequences Induced by Chronic Male Subordination. *Brain. Behav. Immun.*, doi:10.1016/j.bbi.2016.08.007, *in press.* (*Brain. Behav. Immun.* permits the author to include published journal articles in full or in part in the author's dissertation)

3.) **Peterlik, D.**, Stangl, C., Bludau, A., Grabski, D., Strasser, R., Schmidt, D., Flor, P.J., Uschold-Schmidt, N., 2016c. Relief from detrimental consequences of chronic psychosocial stress in mice deficient for the metabotropic glutamate receptor subtype 7. *Neuropharmacology*, doi:10.1016/j.neuropharm.2016.04.036, *in press.* (*Neuropharmacology* permits the author to include published journal articles in full or in part in the author's dissertation)

Peterlik D. is responsible for design and performance of the experiments, data collection, analysis and interpretation and writing of the first drafts of the manuscripts.

1. Material

1.1 Drugs

Drug	Chemical structure		Manufacturer
XAP044		http://www.chemspider.com/Chemical-Structure.4558326.html?rid=bf8d5965-13d8-47fb-b100-460b3e813d63	Novartis Pharma AG (Basel, Switzerland)
Imipramine		http://www.chemspider.com/Chemical-Structure.3568.html?rid=870b2d98-2cdb-4bd0-8f5f-260882edb9cb	Sigma-Aldrich (Deisenhofen, Germany)
Diazepam		http://www.chemspider.com/Chemical-Structure.2908.html?rid=671408ba-819e-4757-bc8f-7e8a2519dd5b	Ratiopharm GmbH (Ulm, Germany)
MPEP		http://www.chemspider.com/Chemical-Structure.2291589.html?rid=5bb4801b-8774-475f-86a2-9dcefb25ea73&page_num=0	Novartis Pharma AG (Basel, Switzerland)
CTEP		http://www.chemspider.com/Chemical-Structure.9821562.html?rid=bd3aa80f-3ed3-4477-9e76-21d53a6c1811	F. Hoffmann-La Roche Ltd. (Basel, Switzerland)

1.2 RNA processing, reverse transcription and quantitative PCR (qPCR)

Application	Product	Company
RNA isolation	Trizol reagent (peqGOLD Trifast)	Peqlab (Erlangen, Germany)
	Chloroform	Acros organics (Geel, Belgium)
	Isopropanol	Merck (Darmstadt, Germany)
	Ethanol	Mallinckrodt Baker (Griesheim, Germany)
	Glycogen (20 mg/ml)	Thermoscientific (Braunschweig, Germany)
	DEPC (for 0.1 % DEPC water)	Carl Roth (Karlsruhe, Germany)
RNA measurement	Spectrophotometer ND-100	Peqlab (Erlangen, Germany)
Reverse transcription	dNTPs mix (10 mM)	Carl Roth (Karlsruhe, Germany)
	Oligo (dT) primer	NEB (Frankfurt, Germany)
	DEPC (for 0.1 % DEPC water)	Carl Roth (Karlsruhe, Germany)
	FS buffer (5x)	Invitrogen (Karlsruhe, Germany)
	$MgCl_2$ (50 mM)	Invitrogen (Karlsruhe, Germany)
	DTT (0.1 M)	Invitrogen (Karlsruhe, Germany)
	RNase Out (40 U/µl)	NEB (Frankfurt, Germany)
	SuperScript® III RT (200 U/µl)	Invitrogen (Karlsruhe, Germany)
	RNase H (5.000 U/ml)	NEB (Frankfurt, Germany)
qPCR	SYBR®Green (fast, Master Mix)	Applied Biosystems (Darmstadt, Germany)
	7500 Fast Real-Time PCR System	Applied Biosystems (Darmstadt, Germany)

1.3 Oligonucleotides

The following oligonucleotides were used as primers for gene expression analysis via qPCR. All primers were designed using Primer Express 3.0 software (Applied Biosystems, Darmstadt, Germany) and were purchased from Metabion (Martinsried, Germany).

Gene	5'-3' Sequence
GAPDH forward	TGTGTCCGTCGTGGATCTGA
GAPDH reverse	CCTGCTTCACCACCTTCTTGA
mGlu2 forward	CGTGTCCGTCAGCCTCAGT
mGlu2 reverse	TGGCTCACCACGACGTTCTTCTG
mGlu3 forward	TGTGATGGTGTCTGTGTGGCT
mGlu3 reverse	GTTTCCCGCTTCTCTGGCA
mGlu5 forward	TGTGTACCTTCTGCCTCATTGC
mGlu5 reverse	GGAGAGAGACCGATGCCAATT
mGlu7 forward	GCAGAAGGAGCCATCACCAT
mGlu7 reverse	GTCCGGGATGTGAAGTAAGCA

1.4 Blood hormone level analysis and adrenal ACTH stimulation *in vitro*

Application	Product	Company
Blood sampling	EDTA-coated tubes	Sarstedt (Nürnbrecht, Germany)
Adrenal ACTH stimulation *in vitro*	DMEM/F-12	Life Technologies (Darmstadt, Germany)
	BSA	Sigma-Aldrich (Deisenhofen, Germany)
	ACTH 1-24 (100 nM)	Sigma-Aldrich (Deisenhofen, Germany)
	Saline (0.9 % NaCl)	Braun Melsung AG (Melsung, Germany)
Plasma ACTH measurement	ACTH ELISA Kit	IBL International (Hamburg, Germany)
Plasma and adrenal supernatant CORT measurement	CORT ELISA Kit	IBL International (Hamburg, Germany)

1.5 Analysis of immunological parameters

Application	Product	Company
Mes LNC isolation and stimulation *in vitro* with anti-CD3/anti-CD28	Cell strainer (70 µm)	Becton Dickinson Biosciences (Heidelberg, Germany)
	RPMI 1640	Sigma-Aldrich (Deisenhofen, Germany)
	Fetal bovine serum (FBS) superior	Biochrom AG (Berlin, Germany)
	Penicillin	PAA laboratories GmbH (Cölbe, Germany)
	Streptomycin	PAA laboratories GmbH (Cölbe, Germany)
	β-mercaptoethanol	PAA laboratories GmbH (Cölbe, Germany)
	anti-CD3 antibody	BD Biosciences (Heidelberg, Germany)
	anti-CD28 antibody	eBioscience GmbH (Frankfurt, Germany)
IFN-γ measurement	IFN-γ ELISA Kit	BioLegend (London, England)
Colon processing/staining	Embedding cassettes	Simport Plastics Ltd. (Bernard-Pilon, Beloeil QC, Canada)
	Harris haematoxylin	Sigma-Aldrich (Deisenhofen, Germany)
	Eosin Y solution	Sigma-Aldrich (Deisenhofen, Germany)
	Microscope slides (SuperFrost)	Gerhard Menzel GmbH (Braunschweig, Germany)
	Mounting solution, Roti Histokitt II	Carl Roth (Karlsruhe, Germany)

1.6 Chemicals, enzymes, reagents and technical equipment

Chemicals, enzymes and reagents used in this thesis were obtained from Beckman Coulter (Krefeld, Germany), Biomol (Hamburg, Germany), Bio-Rad (München, Germany), Braun (Melsungen, Germany), Carl Roth GmbH (Karlsruhe, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Deisenhofen, Germany). Cell culture materials, general plastic material and other equipment were purchased from BD Biosciences (Heidelberg, Germany), Beckman Coulter (Krefeld, Germany), Eppendorf AG (Hamburg, Germany), Leica Microsystems (Wetzlar, Germany), Sarstedt (Nürnbrecht, Germany), Thermo Scientific (Rockford, USA) and VWR GmbH (Darmstadt, Germany).

1.7 Software

Application	Product	Company
Text and data processing	Microsoft Office 2013	Microsoft
Graph plotting	SigmaPlot 11.0	Systat software
Reference management	Mendeley 1.15 (freeware)	Chip.de/downloads
EPM analysis	Plusmaze (DOS program, ©Ernst Fricke, 1993)	Ernst Fricke
ELISA analysis	FLUOstar OPTIMA	BMG Labtech GmbH (Offenbach, Germany)
Statistical analysis	SPSS 22.0	IBM

2. Methods

2.1 Animals

The *in vivo* effects of XAP044 were assessed in the TST, the EPM and the cued fear conditioning test in individually housed male C57BL/6 WT mice (Charles River, Sulzfeld, Germany; see Results, 1).

The effects of acute and sub-chronic CTEP treatment on SIH response (Results, 4.7) were assessed also in individually housed male C57BL/6 WT mice (Charles River, Sulzfeld,

Germany). For investigating the consequences of 19 days of CSC exposure on mGlu receptor mRNA expression, male C57BL/6 WT mice (Charles River, Sulzfeld, Germany) weighing 19-22 g and exposed to 19 days of CSC were used (Results, 2).
For studying the influence of mGlu7 genetic ablation on the consequences of 19 days of CSC (Results, 3.1-3.4), male C57BL/6 mGluR7 KO mice (generated as described previously from E14 (129/Ola) embryonic stem cells; Sansig et al., 2001) and their male WT littermates (from C57BL/6 heterozygous breeding pairs in the animal facility of the University of Regensburg) were used. Aged 6-8 weeks and weighing 19-22 g, these experimental mice were individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) for one week before the start of CSC exposure.
For studying the influence of genetic and pharmacological inhibition of mGlu5 the consequences of 19 days of CSC (Results, 4), conventional C57BL/6 mGlu5 KO mice and C57BL/6 mice chronically infused with CTEP were used in combination with the CSC paradigm. Here, male C57BL/6 mGlu5 KO mice and their male WT littermates (from C57BL/6 heterozygous breeding pairs in the animal facility of the University of Regensburg), and male C57BL/6 WT mice (Charles River, Sulzfeld, Germany), aged 6-8 weeks and weighing 19-22 g were used as experimental mice and individually housed in standard polycarbonate mouse cages (16 x 22 x 14 cm) for at least one week before starting the CSC procedure. Male CD1 mice weighing 30-35 g from our own breeding were used as dominants.

In all experiments, mice were kept under standard laboratory conditions (12 h light/dark cycle, lights on at 0600 h, 22 °C, 60% humidity) with free access to tap water and standard mouse diet (ssniff Spezialdiäten GmbH, Soest, Germany). All experimental protocols were approved by the Committee on Animal Health and Care of the local government and conformed to the international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

2.2 Drug treatment and surgical procedure

Acute application of XAP (i.p.)

XAP044 (identified by high throughput random drug screening and follow-up chemical efforts; Gee et al., 2014), MPEP, diazepam, and imipramine were dissolved in 10% Neoral® vehicle (Novartis Pharma AG, Basel, Switzerland) and diluted in 0.4%

methylcellulose (AMIMED, Allschwill, Switzerland). Either vehicle (10% Neoral® vehicle, 0.4% methylcellulose), XAP044 (10 and 60 mg/kg), imipramine (20 mg/kg), diazepam (1 mg/kg) or MPEP (30 mg/kg) were administered *i.p.* at a volume of 10 ml/kg body weight 30 min prior to respective *in vivo* testing. Imipramine and diazepam served as positive controls for the TST and the EPM test, respectively. MPEP was used as positive control in the cued fear conditioning experiment.

Chronic application of CTEP via micro-osmotic pumps

To achieve permanent receptor saturation during CSC exposure, CTEP (F. Hoffmann-La Roche, Basel, Switzerland) was administered chronically via Alzet® micro-osmotic pumps (pumping rate: 0.11 µl/h, Alzet®, Model 1004, Cupertino, USA). Compound release initiated one week prior starting the CSC paradigm (day -6) in order to establish a stable baseline receptor occupancy and continued until the end of chronic stressor exposure (day 20). Micro-suspensions of CTEP were prepared in vehicle (VEH, polyethyleneglycol 400 (PEG), Sigma Aldrich, Steinheim, Germany) and sonicated (3 x 1.5 min) to ensure a continuous substance release of 0.05, 0.5 or 2 mg/kg/day. As reported by Peters et al. (2014), a micro-osmotic pump was implanted *s.c.* in the abdominal region through a 1 cm long incision at the lower neck of the mouse under isoflurane anesthesia (Baxter, GmbH, Germany). To avoid post-surgical infections, each mouse received 100 µl of antibiotics (*s.c.*, Baytril® 2.5% Bayer Vitral GmbH, Leverkusen, Germany), followed by wound treatment with betaisodona (Mundipharma GmbH, Limburg, Germany).

Using micro-osmotic pumps for this purpose was of particular advantage (in terms of animal welfare and experimental convenience), as multiple injections associated with frequent manipulation of animals have been shown to alter the animal's phenotype by affecting behavioral and other parameters. For example, vehicle or sham injections were associated with impairment of the animal's behavior in the EPM test (Lapin, 1995). Moreover, preliminary studies in our group revealed that repeated (daily) *i.p.* injections of vehicle (0.5% methylcellulose) resulted in an increased adrenal and decreased thymus weight when compared to non-injected controls (unpublished data) and thus demonstrated that daily *i.p.* injections induced stress effects interfering with the effects of CSC procedure. On this background, chronic administration of CTEP via micro-osmotic pumps offered the advantage of not requiring daily animal manipulation and the risk of impairing sensitive CSC-related parameters. Of note, it was recently demonstrated that *i.c.v.*

administration of drugs via micro-osmotic pump is well compatible with the CSC procedure in mice (Peters et al., 2014).

Acute and sub-chronic application of CTEP (i.p.)

To verify and to extend (with respect to the route and duration of administration) the anxiolytic properties of oral CTEP reported by Lindemann et al. (2011), the effect of acute and sub-chronic CTEP treatment on the SIH response in naïve C57BL/6 mice was assessed. Here, SIH measurement took place 1 h following a single *i.p.* injection (acute), or on day 16 and day 24 following 8 and 12 *i.p.* injections every 48 h (sub-chronic), respectively. CTEP doses were either 0.1, 0.5 or 2 mg/kg with a volume of 2.5 ml/kg body weight.

2.3 Acute behavioral tests

2.3.1 The TST

The TST was carried out in a gray Plexiglas box containing three compartments separated by gray Plexiglas walls, each compartment measuring 38 x 38 x 80 cm, between 0800 h and 1100 h. To evaluate the effects of systemic XAP044 administration on depressive-like behavior, mice were injected *i.p.* with vehicle (10% Neoral® vehicle, 0.4% methylcellulose), imipramine (20 mg/kg) as positive control, or XAP044 (10 and 60 mg/kg). After 30 min they were individually suspended by the tail to a vertical ring-stand bar (distance from floor: 40 cm) using adhesive tape (distance from tip of tail: 2 cm). Typically, the animals demonstrated several phases of escape-oriented behavior with temporally increasing periods of immobility. A 6-min test session was employed, which was videotaped and subsequently analyzed for the time the mice spent immobile (Results, 1.1) by a trained observer blind to the treatment.

2.3.2 Cued fear conditioning and fear expression

To assess conditioned freezing response in mice, a computerized fear-conditioning system (TSE, Bad Homburg, Germany) was used as described previously (Fendt et al., 2013; Toth et al., 2012). The cued fear experiments were performed in two different contexts, A and B, that differed in visual, tactile, and olfactory cues. On day 1, fear acquisition was assessed in context A, which consisted of a transparent perspex box (45 x 45 x 40 cm) with a transparent lid. The floor was made up of 45 x 0.4 m stainless steel bars set 0.5 cm apart,

which served as conductors of mild foot shocks. The boxes were located in a wooden chamber to reduce external noise and visual stimulation, provided with loudspeakers for the acoustic stimuli, and ventilation fans producing a low background noise. Illumination (300 lx for context A and 20 lx for context B) was provided by four white LEDs. To evaluate the effects of systemic XAP044 administration on conditioned freezing response, mice were individually placed into the conditioning chamber (context A) and, after a 5-min adaptation period, exposed to five CS-US pairings 30 min after *i.p.* injection of vehicle (10% Neoral® vehicle, 0.4% methylcellulose), MPEP (30 mg/kg) as positive control, or XAP044 (10 and 60 mg/kg). The conditioned stimulus (CS) was a white noise (80 db, 8 kHz, 30 s), which co-terminated with a mild electric food shock (US, 0.6 mA, 2 s) with a 2 min inter-trial interval (Results, 1.3). After the CS-US pairings, mice were left in the conditioning chamber for 5 min before they were returned to their home cage. The conditioning chamber was cleaned with a neutral smelling detergent before each trial. On day 2, fear expression was evaluated in context B, which consisted of a black box (45 x 45 x 40 cm) with a smooth floor. After a 5 min adaptation period, the animals received two CS presentations (80 db, 8 kHz, 30 s) with an inter-stimulus interval of 30 s (Results, 1.3). Context B was cleaned with a lemon-scented detergent before each trial. Animal movement was detected with infrared sensors. In both the fear acquisition and the fear expression phases, the time spent freezing (immobility, no infrared beam crosses for more than 1 s) relative to the total time (set to 100%) was automatically recorded and defined as freezing response (%) (Fanselow, 1980).

2.3.3 The EPM test

The EPM consisted of two bight, open (130-140 lx, 6 cm x 30 cm) and two dark, closed (10-20 lx, 6 cm x 30 cm x 17 cm) arms radiating from a central platform (6 cm x 6 cm) to form a plus-shaped figure elevated 130 cm above the floor. The open arm edges were 0.3 cm in height to prevent mice from falling. Each mouse was placed on the central platform facing a closed arm (CA). The maze was cleaned thoroughly before each test. EPM testing period was 5 min and took place between 0800 h and 1100 h. Animal movement on the EPM was monitored by a camera and subsequently analyzed using the program Plus-maze (DOS program, Ernst Fricke, 1993, Munich, Germany) by an observer blind to the animal's treatment/genotype/housing condition. The percentage of time spent on open arms of the EPM was used as a measure of anxiety. In addition, the number of closed-arm

entries was taken as an indicator for locomotor activity. In all experiments, mice were transported to the EPM room one day prior to testing.

To evaluate the effects of systemic XAP044 administration on innate anxiety-related behavior, each mouse was tested 30 min after *i.p.* injection of vehicle (10% Neoral® vehicle, 0.4% methylcellulose), diazepam (1 mg/kg) as positive control, or XAP044 (10 and 60 mg/kg) (Results, 1.2).

In order to assess genotype- (Results, 3 and 4) or treatment-specific (Results, 4) effects of CSC on innate anxiety-related behavior, mice were tested on the EPM day 18 of CSC exposure. After testing, CSC mice were put back in their respective CSC colony and SHC mice were kept single-housed. Different to what was previously described (Füchsl et al., 2014; Peters et al., 2014; Reber et al., 2007), day 18 instead of day 19 of CSC was chosen for assessing innate anxiety-related behavior on the EPM in the present thesis, as mice were additionally tested for their physiological anxiety in the more stressful SIH test on day 19.

2.3.4 The SIH test

The SIH test was performed as described elsewhere (Gee et al., 2014; O'Connor et al., 2013b; Peterlik et al., 2016a) between 0800 h and 1000 h in a room different from where the animals were housed before in order to avoid any confounding influence of housing on test performance and vice versa. Rectal temperature was recorded twice at T1 and T2 (15 min later) using a digital thermometer (2.5 mm diameter, Amarell GmbH and Co KG, Kreuzwertheim, Germany). In order to avoid any rectal injuries the thermometer was covered with vaseline before inserting it 1.5 cm into the rectum. Recording of T1 indicated baseline temperature and served as stressor at the same time. Recording of T2 allowed determination of the SIH response defined as the difference between T2 and T1. SIH measurement was performed on day 19 of CSC exposure in CSC and SHC mice (Results, 4.1) or after acute and sub-chronic treatment (Results, 4.7) in naïve mice, with all mice individually housed between the two temperature recordings at T1 and T2. In CSC-related experiments, SIH measurement was established in order to complement potential effects of CSC on physiological anxiety, in addition to the well characterized effects on (innate) anxiety-related behavior (Langgartner et al., 2015; Reber et al., 2007; Slattery et al., 2012).

2.4 The CSC paradigm

The CSC paradigm was conducted as described previously (Langgartner et al., 2015; Peterlik et al., 2016a; Reber et al., 2007). Briefly, experimental mice were assigned to the SHC or CSC group in a genotype- , treatment- as well as weight-matched manner. Four CSC mice of the same genotype or the same treatment were housed together with a dominant male for 19 consecutive days in order to induce chronic psychosocial stress. To avoid habituation during chronic stressor exposure, each dominant male was replaced by a novel one on days 8 and 15 (Figure 2a). As appropriate controls, SHC mice were used (Singewald et al., 2009), in line with previous studies demonstrating single-housing to be less stressful in male mice as compared to group housing (Bartolomucci et al., 2003; Chourbaji et al., 2005; Gasparotto et al., 2005). SHC mice remained undisturbed in their home cage except for change of bedding once a week. On day 18 and 19 of CSC, animals' innate and physiological anxiety were assessed on the EPM (day 18) and in the SIH test (day 19), respectively. After testing, stressed mice were placed back into their respective CSC colony and SHC mice remained single-housed. On day 20, all mice were rapidly decapitated between 0800 h and 1100 h and trunk blood was collected for quantification of plasma CORT and plasma ACTH levels, and pituitary, adrenal, thymus and spleen weights, as well as the *in vitro* ACTH responsiveness of adrenal explants were assessed. In addition, the histological damage score of the colon, the number of viable mesenteric lymph node cells (mesLNC) and anti-CD3/anti-CD28-stimulated IFN-γ production of mesLNC *in vitro* were determined (detailed description see below).

2.5 Trunk blood sampling

To determine the effects of CSC exposure on basal morning plasma ACTH and CORT concentrations, mice were rapidly killed by decapitation under CO_2 anesthesia within 3 minutes after entering the animal room between 0800 h and 1100 h on day 20. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nuembrecht, Germany) on ice and centrifuged at 4 °C (5000 rpm for 5 min). Plasma was isolated and samples were stored at -20 °C until assayed.

In addition, the concentration of CTEP in plasma (day 20) of CSC and SHC animals treated with CTEP at different doses (Discussion, 4.2) was determined. Analysis was performed by F. Hoffmann-La Roche Ltd. (Basel, Switzerland) using a combined high-

performance liquid chromatography/mass spectrometry (HPLC/MS) method as described in Lindemann et al. (2011).

2.6 Determination of body and organ weight and brain dissection

On day 20, mice were weighed immediately before decapitation to assess the effects of CSC on body weight. Afterwards, the pituitary, left and right adrenal glands, thymus and spleen of each animal were removed, pruned from fat and weighed separately. In addition, the sum of left and right absolute adrenal weights was calculated for each animal. Until all mice were killed and all adrenals removed, the latter were stored in ice-cold DMEM (DMEM/F-12, Life Technologies, Darmstadt, Germany) containing 0.1% bovine serum albumin (BSA). Values represent absolute measurements (in mg) of respective organs.
The brain was carefully removed from the cranium (with careful removal of the dura mater), followed by immediate dissecting in PFC, HT and HC as described in the following: The PFC was extracted by vertical cutting off 1-2 mm of the frontal part of the brain. Subsequently, the hypothalamus was carefully cut using micro-scissors. At last, the hippocampi were removed after separating the two hemispheres by a median cut of the cortex and pulling out both the left and the right hippocampus by means of forceps and razor blade. All brain regions were immediately stored in Trizol reagent (Peqlab, Erlangen, Germany; for total RNA isolation) or snap-frozen in liquid nitrogen (for mGlu5 protein analysis performed by F. Hoffmann-La Roche Ltd., Basel, Switzerland).

2.7 ACTH stimulation of adrenal explants *in vitro*

Stimulation of adrenal explants with ACTH (100 nM) in vitro was performed as previously described (Füchsl et al., 2014; Peters et al., 2014; Uschold-Schmidt et al., 2012). Briefly, left and right adrenals were stored in ice-cold DMEM/F-12 (Life Technologies, Darmstadt, Germany) containing 0.1% BSA until all mice were killed and adrenals removed. Afterwards, each left and right adrenal gland was cut into two halves each containing cortical and medullary tissue. The halves were weighed and pre-incubated in 200 ml DMEM/F-12 for 4 h (37 °C, 5% CO_2) prior to any further treatment. Culture medium was then replaced, and each half of one adrenal was incubated with medium containing either 0.9% saline (basal) or 0.9% saline plus ACTH (100 nM) for 6 h (37 °C; 5% CO_2). After incubation, supernatants were carefully removed and stored at -20 °C until being analyzed using a commercially available ELISA for CORT (IBL International, Hamburg, Germany).

CORT concentrations were calculated in relation to the weight of the respective adrenal explants (*i.e.* relative CORT secretion). To illustrate the *in vitro* adrenal CORT secretion in relation to the whole organism, relative CORT secretion from the left and right adrenal gland of each mouse was summed up.

2.8 ELISA for CORT and ACTH

Plasma and supernatant samples were analyzed using a commercially available ELISA for CORT (analytical sensitivity < 1.631 nmol/l and intra-assay and inter-assay coefficients of variation (CV) ≤ 6.35%; IBL International, Hamburg, Germany) and ACTH (plasma samples only; analytical sensitivity 0.22 pg/ml and intra-assay and inter-assay CV ≤ 7.1%; IBL International, Hamburg, Germany).

2.9 Isolation and incubation of mesLNC

To determine housing-, genotype- and treatment-specific effects of CSC on the IFN-γ secretion of anti-CD3 and anti-CD28-stimulated mesLNC *in vitro*, mesenteric lymph nodes were isolated from each animal as described previously (Füchsl et al., 2014; Reber et al., 2007). Mesenteric lymph nodes were stored in ice-cold RPMI medium (RPMI 1640, 10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 3 x 10^{-5} M β-mercaptoethanol) until all mice were killed and mesenteric lymph nodes removed. Afterwards, mesenteric lymph nodes were mechanically homogenized and filtered through a cell strainer (70 µm nylon, Falcon; Becton Dickinson Biosciences, Heidelberg, Germany). The cell suspension was centrifuged for 15 min (16000 rpm, 4°C, Allegra X-12R, Beckman Coulter) and supernatant was discarded. In turn, cell pellet was re-suspended in 5 ml RPMI medium and (viable) cell number was assessed using a cell viability analyzer (Vi-Cell XR; Beckman Coulter, Krefeld, Germany). Then, 5 x 10^5 (100 µl) mesLNC were transferred to 2 wells of a 96-well plate pre-coated with anti-CD3 antibody (2.5 µg/ml) and were additionally stimulated with anti-CD28 antibody (0.5 µg/well, 100 µl). After incubation for 24 h (37 °C, 5% CO_2; Results, 4.1) or 48 h (37 °C, 5% CO_2; Results, 3.4 and 4.5), IFN-γ levels were measured in the supernatants of two wells per animal by ELISA (BioLegend, San Diego, USA) and averaged per animal and group.

2.10 Determination of the histological damage score in the colon

The histological damage score of colonic tissue was determined as described previously (Obermeier et al., 2003; Reber et al., 2007). The colon was removed and mechanically cleaned from feces. Afterwards, 1 cm of the distal third was cut longitudinally, laid on a filter paper, and fixed in 5% paraformaldehyde overnight. The next day, the fixed tissue was embedded in paraffin and cut. For each animal two longitudinal 3 µm sections were taken at 100 µm distance and stained with hematoxylin and eosin according to the following staining procedure:

Xylol 2 x 5 min → 100% EtOH 2 x 1 min → 90% EtOH 1 min → 70% EtOH 1 min → distilled H_2O 1 min → hematoxylin 10-15 min → hot H_2O 3 min → cold H_2O 3 min → eosin 0.5-2 min → distilled H_2O brief rinsing → 70% EtOH 2 x 1 min → 90% EtOH 1 min → 100% EtOH 2 x 1 min → Xylol 2 x 5 min

Mice were scored individually, each score representing the mean of the two sections. Histological scoring was evaluated by an investigator blind to housing/treatment/genotype conditions, according to the following scheme:

Epithelium	**Infiltration**
0, normal morphology	0, no infiltration
1, loss of goblet cells	1, infiltrate around crypt basis
2, loss of goblet cells in large areas	2, infiltrate reaching to lamina muscularis mucosae
3, loss of crypts	3, extensive infiltration reaching and thickening of the mucosa with abundant oedema
4, loss of crypts in large areas	4, infiltration of the lamina submucosa

The total histological score of each mouse represents the sum of the epithelium and infiltration score within a range from 0 to 8. The scores were than averaged per animal and group. Respective SHC values were set to 100%.

2.11 Relative quantification of mGlu receptor mRNA via quantitative PCR (qPCR)

The effect of 19 days of CSC on mGlu2 and mGlu3, mGlu5, and mGlu7 mRNA in PFC, HT and HC was determined relative to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in comparison to respective SHC controls (set at 100%). Here, total RNA was isolated using Trizol reagent according to manufacturer's instructions (Peqlab, Erlangen, Germany). RNA was re-suspended in 20 µl of RNase free water and its concentration and quality were analyzed spectrophotometrically (NanoDrop Spectrophotometer, Peqlab, Erlangen, Germany). cDNA was prepared from 500 ng of total RNA in a 20 µl final reverse transcription reaction mixture (using Superscript III; Invitrogen, Karlsruhe, Germany). Quantitative PCR was performed using SYBR® Green Master Mix and an ABI 7500 Fast Sequence Detection System (Applied Biosystems, Darmstadt, Germany), with a cycling profile of 95°C (20 sec), followed by 40 cycles of 95°C (3 sec), 60°C (30 sec). Samples were prepared in triplicates and changes in gene expression were determined according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) by using GAPDH for normalization and SHC as control.

2.12 Receptor saturation analysis of mGlu5

Briefly, analysis of mGlu5 receptor binding sites (Discussion, 2) was performed by F. Hoffmann-La Roche Ltd. (Basel, Switzerland) using a tritiated version of the mGlu5 PET tracer ABP688 ([^{3}H]ABP688; Hintermann et al., 2007) as described elsewhere (Lindemann et al, 2011, 2015).

2.13 Statistics

All data represent the mean + or ± S.E.M. and were analyzed using the software IBM SPSS 22.0.

TST and EPM test data obtained from XAP044 experiments (Results, 1.1, 1.2) were analyzed using a Kruskal-Wallis analysis of variance (ANOVA) followed by a *post hoc* Dunnett's test when appropriate. Fear acquisition and fear expression data (Results, 1.3) were analyzed using repeated measures ANOVA followed by a *post hoc* Fisher's least significant difference (LSD) test when appropriate.

Data obtained from CSC experiments depending on one factor (housing) were analyzed using an independent Student's *t*-test (Results, 2 and 3.1). Data depending on two factors (*i.e.* housing and genotype, housing and treatment, housing and stimulation) were analyzed using two-way ANOVA. Significant main and interaction effects were followed by Bonferroni *post hoc* analysis when appropriate or an independent Student's *t*-test (Results, 3.2-3.4 and 4.1, 4.3-4.6).

Data obtained from acute CTEP experiments depending on one factor (housing or treatment; Results, 4.7) were analyzed using independent Student's *t*-test or one-way ANOVA followed by LSD *post hoc* testing.

Data obtained from sub-chronic CTEP experiments depending on each other (Results, 4.7) were analyzed using repeated measures ANOVA followed by Bonferroni or LSD *post hoc* testing or an independent Student's *t*-test.

In all cases, statistical significance was accepted at $p \leq 0.05$.

Results

The Results section includes chapters taken and adapted from different (joint) first author publications and submitted first author manuscripts:

1.) **Peterlik, D.**, Flor, P.J., Uschold-Schmidt, N., 2016a. The Emerging Role of Metabotropic Glutamate Receptors in the Pathophysiology of Chronic Stress-Related Disorders. *Curr. Neuropharmacol.,* 14(5), 514–39. (*Curr Neuropharmacol* permits the author to include published journal articles in full or in part in the author's dissertation)

2.) Gee, C.E.*, **Peterlik, D.***, Neuhäuser, C., Bouhelal, R., Kaupmann, K., Laue, G., Uschold-Schmidt, N., Feuerbach, D., Zimmermann, K., Ofner, S., Cryan, J.F., van der Putten, H., Fendt, M., Vranesic, I., Glatthar, R., Flor, P.J., 2014. Blocking metabotropic glutamate receptor subtype 7 (mGlu7) via the Venus flytrap domain (VFTD) inhibits amygdala plasticity, stress, and anxiety-related behavior. *J. Biol. Chem.* 289, 10975–87. doi:10.1074/jbc.M113.542654.* **Both authors contributed equally to this work.** (*J. Biol. Chem.* permits the author to include published journal articles in full or in part in the author's dissertation)

3.) **Peterlik, D.**, Stangl, C., Bauer, A., Bludau, A., Keller, J., Grabski, D., Killian, T., Schmidt, D., Zajicek, F., Jaeschke, G., Lindemann, L., Reber, S.O., Flor, P.J., Uschold-Schmidt, N., 2016b. Blocking Metabotropic Glutamate Receptor Subtype 5 Relieves Maladaptive Stress Consequences Induced by Chronic Male Subordination. *Brain. Behav. Immun.*, doi:10.1016/j.bbi.2016.08.007, *in press.* (*Brain. Behav. Immun.* permits the author to include published journal articles in full or in part in the author's dissertation)

4.) **Peterlik, D.**, Stangl, C., Bludau, A., Grabski, D., Strasser, R., Schmidt, D., Flor, P.J., Uschold-Schmidt, N., 2016c. Relief from detrimental consequences of chronic psychosocial stress in mice deficient for the metabotropic glutamate receptor subtype 7. *Neuropharmacology*, doi:10.1016/j.neuropharm.2016.04.036, *in press.* (*Neuropharmacology* permits the author to include published journal articles in full or in part in the author's dissertation)

Peterlik D. is responsible for design and performance of the experiments, data collection, analysis and interpretation and writing of the first drafts of the manuscripts.

1. Acute pharmacological blockade of mGlu7 inhibits depressive-, anxiety-like and fear-related behavior

The role of mGlu7 in anxiety and acute stress-related behavior and physiology is well documented (see Introduction), and several studies investigating mGlu7's role in emotional behavior suggest receptor blockade as a promising mechanism for the treatment of various disorders in man. However, systemically active allosteric ligands so far have yielded only disparate results in behavioral paradigms. In a previous experiment at the Novartis Institutes for BioMedical Research (Basel, Switzerland; under the supervision of Prof. Peter J. Flor) it was already shown that XAP044 treatment dose-dependently reduced the stress-induced rise in rectal body temperature in the mouse SIH test. Importantly, XAP044 did not show any significant effects on basal body temperature at any of the tested doses (10, 30 and 60 mg/kg, data not shown; see Gee et al., 2014). In the present study, the effects of XAP044 were further assessed in a broader range of behavioral tests including the TST, the EPM and the cued fear conditioning test.

1.1 Attenuation of depressive-like behavior in the TST

XAP044 treatment decreased immobility in the TST ($H(3) = 32.2$, $p \leq 0.001$). Both 10 mg/kg ($p \leq 0.01$) and 60 mg/kg ($p \leq 0.001$) of XAP044 significantly reduced the time the mice spent immobile compared to vehicle-treated animals 30 min following *i.p.* injection. Administration of the TCA imipramine (20 mg/kg; $p \leq 0.001$), used as positive control, also reduced the immobility time compared to the vehicle group (Figure 4).

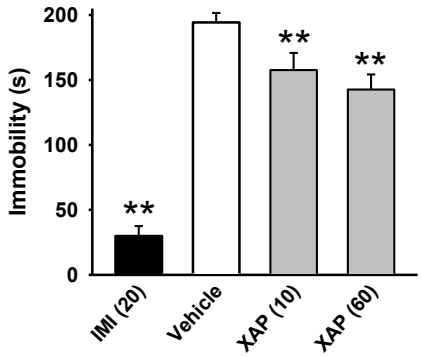


Figure 4. XAP044 attenuates depressive-like behavior in the tail suspension test (TST). Immobility time (s) in the TST 30 min following *i.p.* injection of imipramine (20 mg/kg; positive control), vehicle, or XAP044 (10 and 60 mg/kg) in mice. Data represent the mean + S.E.M.; **, $p \leq 0.01$ *vs.* vehicle group; Kruskal-Wallis ANOVA followed by a *post hoc* Dunnett's test; n = 8-14 per treatment group. IMI, imipramine; XAP, XAP044. Adapted from Gee et al. (2014).

1.2 Relief of innate anxiety in the EPM test

XAP044 treatment decreased innate anxiety-related behavior in the EPM ($H(3) = 19.4$, $p \leq 0.001$; Figure 5). At a dose of 60 mg/kg, the *i.p.*-injected XAP044 significantly increased the ratio of open/total arm entries on the EPM ($p = 0.006$) compared to vehicle-treated mice, indicative of a decrease in anxiety-related behavior (Figure 5a). The same effect was seen in mice treated with the benzodiazepine diazepam (1 mg/kg; $p \leq 0.001$; Figure 5a), used as positive control. Importantly, the number of total arm entries as an indicator of locomotor activity was neither affected by XAP044 nor by diazepam treatment ($H(3) = 3.2$, $p = 0.361$; Figure 5b).

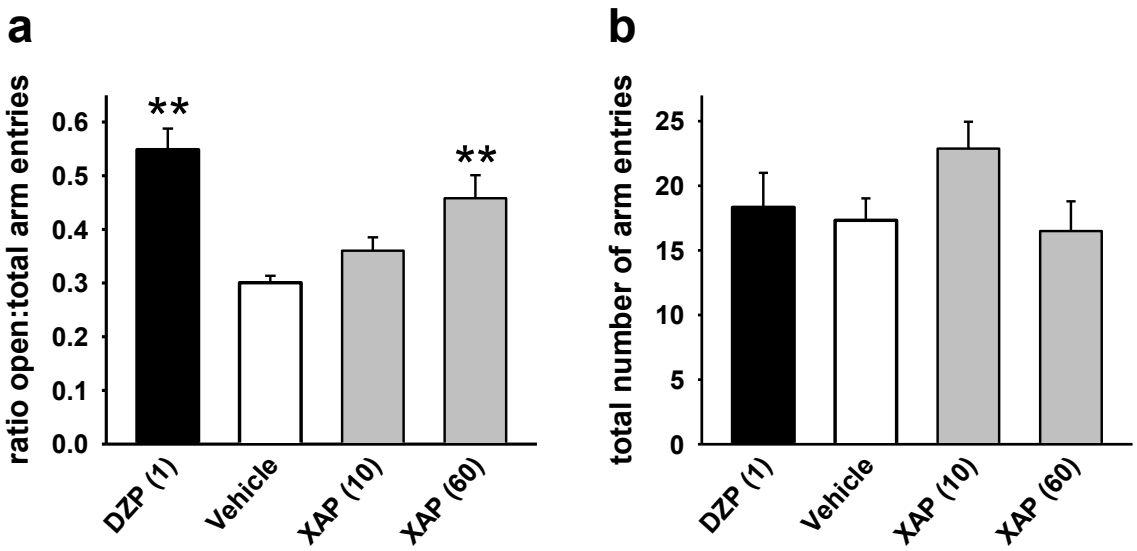


Figure 5. XAP044 relieves innate anxiety in the elevated plus-maze (EPM) test. (a) Ratio open/total arm entries and (b) number of total arm entries on the EPM 30 min following *i.p.* injection of diazepam (1 mg/kg; positive control), vehicle, or XAP044 (10 and 60 mg/kg) in mice. Data represent the mean + S.E.M.; **, $p \leq 0.01$ *vs.* vehicle group; Kruskal-Wallis ANOVA followed by a *post hoc* Dunnett's test; n = 8-14 per treatment group. DZP, diazepam; XAP, XAP044. Adapted from Gee et al. (2014).

1.3 Reduction of cued fear conditioning/learned fear without affecting fear expression

Freezing of the mice during the fear conditioning session on day 1 was found to be significantly affected by the factor treatment ($F_{3,35} = 16.992$, $p \leq 0.001$). Furthermore, the level of freezing increased across CS-US pairings ($F_{5,175} = 154.599$, $p \leq 0.001$). Also, XAP044 injected *i.p.* at a dose of 60 mg/kg resulted in less freezing during the fear

acquisition session compared to the vehicle group, which was statistically significant when CS-US pairings were presented for the third time (p = 0.030; Figure 6a) and a trend was observed at the fifth CS-US pairing (p = 0.077; Figure 6a). This is indicative of delayed short-term fear acquisition by XAP044. As a positive control for delayed fear acquisition, the allosteric mGlu5 antagonist MPEP was used (30 mg/kg), which resulted in a significant reduction of the freezing response across all trials ($p \leq 0.032$ for all pairings). Additionally, fear expression was assessed 24 h following conditioning session. However, neither XAP044 nor MPEP had any effect on the freezing response compared to vehicle-treated animals at CS presentations ($F_{3,35} = 1.040$, p = 0.387; Figure 6b). The effect of XAP044 to delay short-term fear acquisition *in vivo* corresponds well with XAP044's mGlu7-dependent inhibition of LTP in the lateral amygdala (Gee et al., 2014), an assay that is considered an *in vitro* electrophysiological correlate of fear learning.

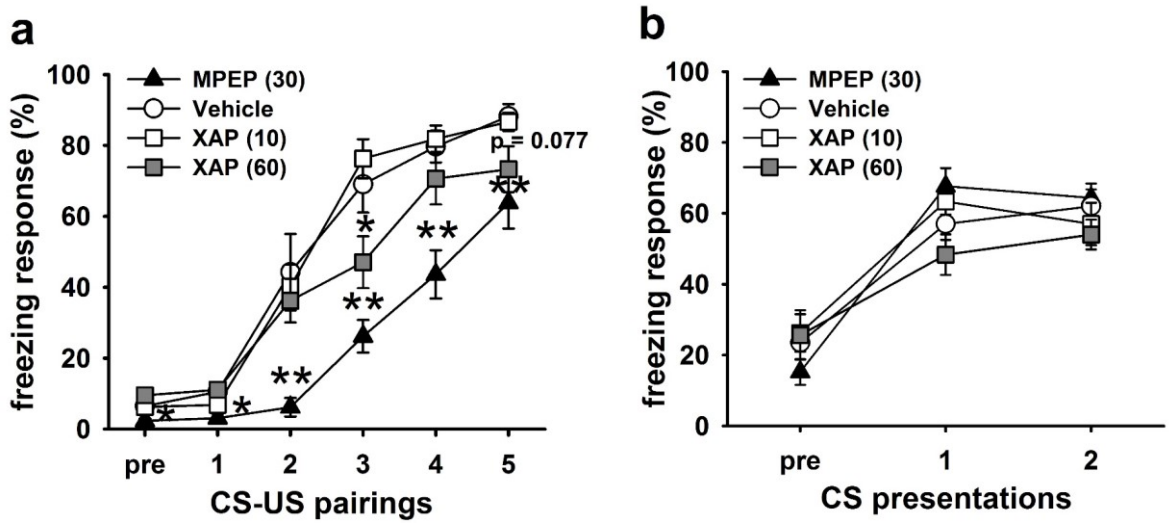


Figure 6. XAP044 reduces freezing during the acquisition session of fear conditioning. (a) Freezing response (% of time during conditioned stimulus presentation) during the acquisition of cued fear starting 30 min following *i.p.* injection of either MPEP (30 mg/kg; positive control), vehicle, or XAP044 (10 and 60 mg/kg) and (b) expression test on learned fear 24 h following conditioning session in mice. Data represent the mean ± S.E.M.; * $p \leq 0.05$; **, $p \leq 0.01$ *vs.* vehicle group; repeated measures ANOVA followed by a *post hoc* LSD test; n = 8-14 per treatment group. CS, conditioned stimulus; US, unconditioned stimulus; XAP, XAP044. Adapted from Gee et al. (2014).

2. Chronic psychosocial stress in mice affects mGlu5 and mGlu7, but not mGlu2/3 mRNA expression in distinct brain regions

Although there is evidence for the involvement of the L-glutamate receptor system, e.g. the mGlu5 and mGlu7 receptors, in acute stress and fear regulation, possible molecular changes within the brain mGlu receptor system in response to chronic stress have been addressed only less so far. To extend and to specify the range of recent reports from chronic stress-induced mGlu receptor gene dysregulation, the gene expression profile of certain mGlu receptors was investigated upon chronic psychosocial stressor exposure in mice. Using the CSC animal model, the consequences of chronic psychosocial stressor exposure on mRNA expression of the mGlu2, -3, -5 and -7 subtypes in the PFC, HT and HC were assessed.

Except for a trend towards upregulated mGlu3 mRNA levels in the HT (t_{16} = -2.042, p = 0.058), no significant CSC-induced changes of mGlu2- or mGlu3 mRNA expression were found in either brain region investigated (Figure 7a, b), indicating that group II mGlu receptors may play a less prominent role in CSC-induced pathophysiology. Next, CSC resulted in a trend towards increased mGlu5 mRNA expression in the HT (t_{16} = -1.931, p = 0.071; see also Peterlik et al. (2016b)), but not in the PFC or in the HC when compared to SHC mice (Figure 7c). In addition, mGlu7 mRNA levels were significantly reduced only in the PFC of CSC compared to SHC mice (HT, t_{17} = 2.456, p = 0.025; Figure 7d; see also Peterlik et al. (2016c)), suggesting that the mGlu7 receptor subtype is potentially involved in PFC-mediated emotional and/or cognitive processes that could be altered by CSC exposure.

However, the present data display only mRNA expression changes of mGlu receptors – and no changes at the protein level. Together, the data at least suggest regulation of specific mGlu receptor subtypes in certain brain regions in response to chronic psychosocial stressor exposure.

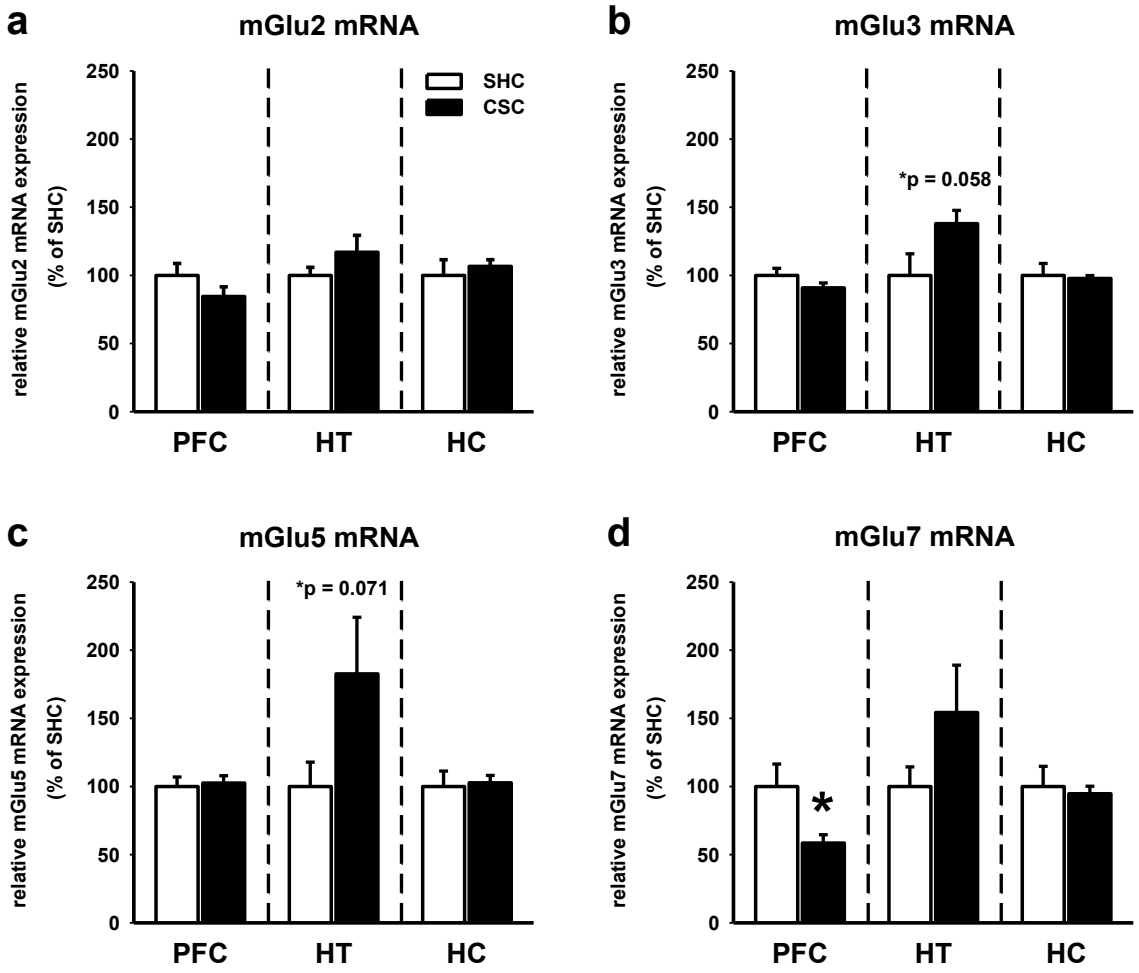


Figure 7. Selective changes in relative mRNA expression of distinct mGlu receptors in the PFC, HT and HC in response to 19 days of CSC. mGlu2, mGlu3, mGlu5 and mGlu7 mRNA levels were assessed in CSC mice relative to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and in comparison to respective SHC mice (set at 100%). No significant effects of 19 days of CSC on (a) mGlu2 and (b) mGlu3 mRNA levels were found in either of the three brain regions. (c) 19 days of CSC resulted in an increase by trend of relative mGlu5 receptor mRNA expression in the HT (p = 0.071), whereas no changes were observed in the PFC and HC (adapted from Peterlik et al. (2016b)). (d) mGlu7 mRNA levels were significantly reduced only in the PFC, but not in the HT and HC, after 19 days of CSC (adapted from Peterlik et al. (2016c)). White bar, SHC (n = 6); black bar, CSC (n = 9-11). Data represent mean + S.E.M. *$p \leq 0.05$ *vs.* respective SHC mice, Student's *t*-test; PFC, prefrontal cortex; HT, hypothalamus; HC, hippocampus.

3. Chronic psychosocial stress-protective phenotype in mice lacking mGlu7

In order to evaluate a functional role of mGlu7 in chronic psychosocial stress-induced affective and somatic consequences, different behavioral, physiological, and immunological parameters were assessed in male mGlu7 KO mice and their WT littermates following 19 days of CSC exposure and compared to SHC mice.

3.1 CSC-induced changes do not differ between C57BL/6J and C57BL/6N mice

Prior to starting experiments with mGlu7 KO mice in the context of CSC, it was essential to show that CSC-induced behavioral and physiological changes described for C57BL/6N WT mice also occur in C57BL/6J mice, the background on which the mGlu7 mutant allele has been backcrossed for more than 14 generations (Cryan et al., 2003; Sansig et al., 2001). Therefore, C57BL/6J WT mice were exposed to 19 days of CSC and compared to SHC littermates. CSC exposure significantly increased innate anxiety on the EPM on day 19 (reduced percentage of time on open arms; $p = 0.026$; Figure 8a) without altering locomotor activity (Figure 8b). Next, selected physiological parameters, exemplified by absolute adrenal and thymus weights, were also affected in response to CSC exposure. In detail, absolute adrenal weight was significantly increased ($p = 0.004$; Figure 8c), whereas absolute thymus weight was significantly decreased ($p = 0.024$; Figure 8d) in CSC compared to SHC mice.

Together, these findings are well in agreement with the published literature on C57BL/6N mice (Langgartner et al., 2015; Reber et al., 2007) and thus provide a solid basis to continue with CSC experiments using KO mice that have been generated on the C57BL/6J background.

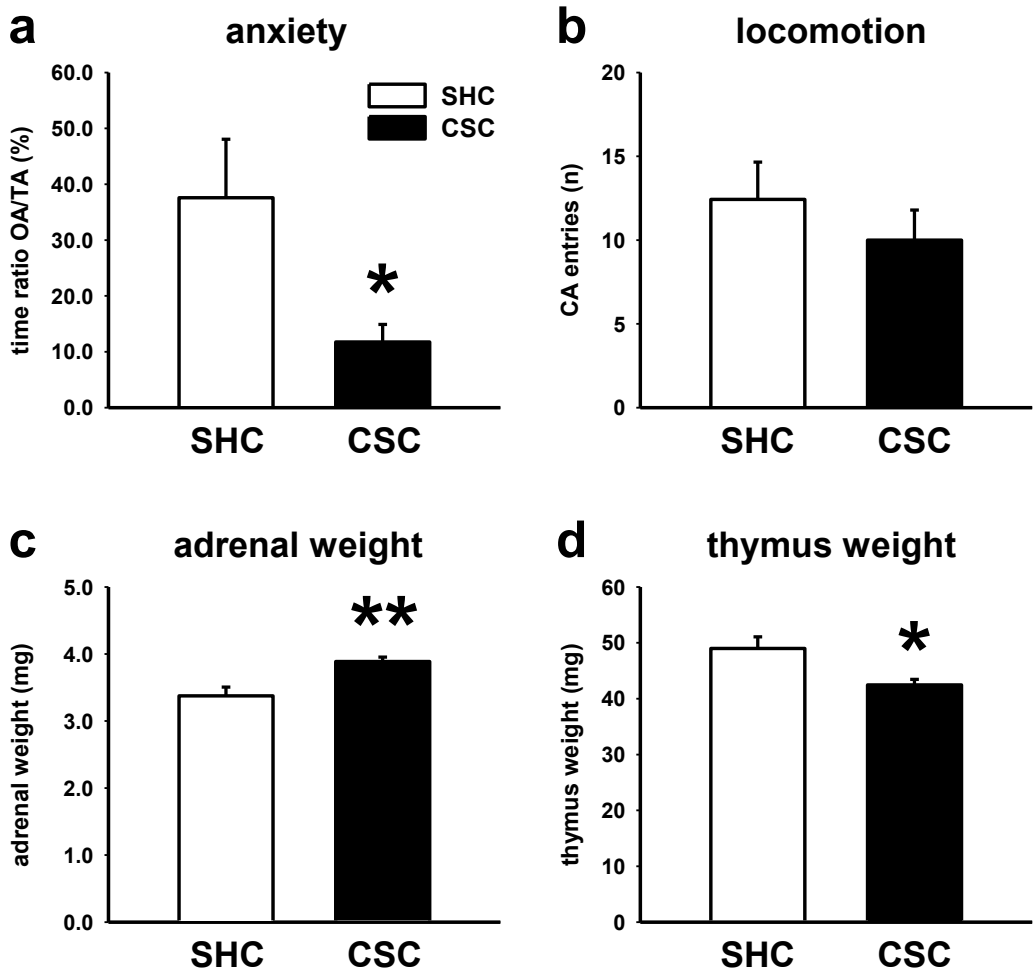


Figure 8. Effects of 19 days of CSC exposure on anxiety-related behavior and selected physiological parameters in C57BL/6J WT mice. In accordance with previously published findings in C57BL/6N mice (Langgartner et al., 2015; Reber et al., 2007), CSC induced an (a) increased anxiety-related behavior, indicated by a reduced percentage of time spent on the open arms (time ratio OA/TA (%) when compared to SHC mice. (b) Importantly, the number of closed arm (CA) entries (n) was comparable between both groups, reflecting an unaffected locomotor activity following CSC exposure. Moreover, (c) absolute adrenal weight (sum of both adrenals; mg) was increased and (c) absolute thymus weight (mg) was decreased in CSC compared to SHC mice. White bar, SHC (n = 7-8); black bar, CSC (n = 6-8). Data represent the mean + S.E.M, Student's t-test; * $p \leq 0.05$, ** $p \leq 0.01$ *vs.* respective SHC mice.

3.2 mGlu7 genetic ablation protects against the CSC-induced anxiety-prone phenotype

mGlu7 KO mice were repeatedly shown to have a stress-protective phenotype with respect to acute stressor exposure. For example, they show a reduced hyperthermic response in the SIH test as well as reduced anxiety-related behavior when tested on the EPM compared to their WT littermates (Callaerts-Vegh et al., 2006; Cryan et al., 2003; Stachowicz et al., 2008). The present study assessed whether mGlu7 genetic ablation also influences the

behavioral outcome of chronic psychosocial stressor exposure, more precisely the CSC-induced anxiety-prone phenotype (Füchsl et al., 2014; Peters et al., 2014; Reber et al., 2007). Assessment of anxiety-related behavior in the EPM test revealed a main effect of the factors housing ($F_{1,88}$ = 13.658, $p \leq 0.001$) and genotype ($F_{1,88}$ = 5.741, p = 0.019), with a significantly decreased percentage of time spent on the open arms in CSC compared to SHC mice of the WT group ($p \leq 0.001$; Figure 9a). This finding is in accordance with the literature and indicative for a CSC-induced increase in anxiety-related behavior. Interestingly, this CSC-induced decreased percentage of time spent on the open arms was absent in the KO group. Bonferroni *post hoc* analysis further revealed a significantly higher percentage of time spent on the open arms in CSC KO compared to CSC WT mice (p = 0.012; Figure 9a). Importantly, the number of CA entries, indicative for locomotor activity, was neither affected by CSC exposure nor by genotype (Figure 9b). Together, the present findings suggest that mice lacking mGlu7 are protected against developing the CSC-induced anxiety-prone phenotype.

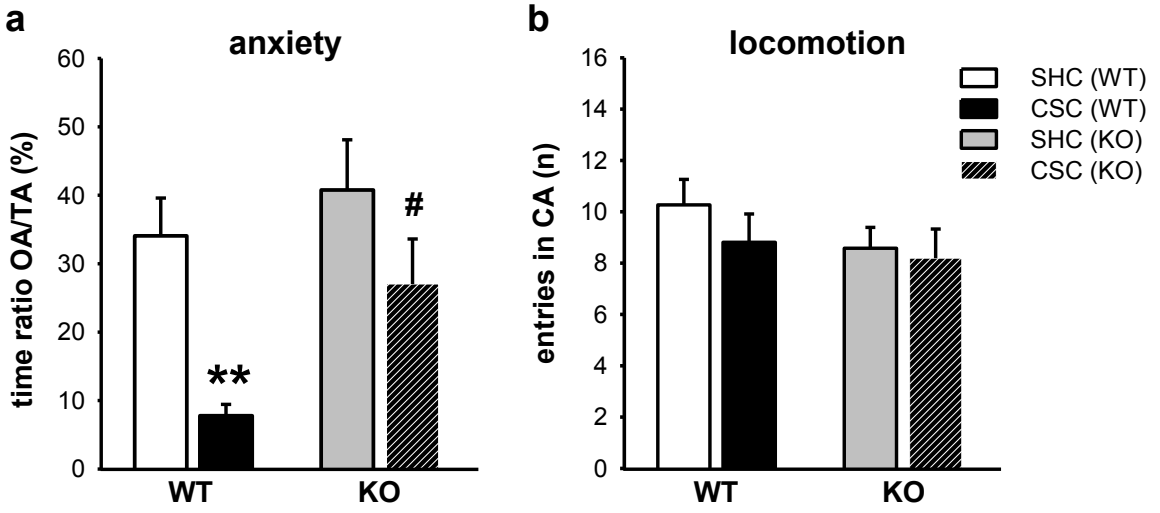


Figure 9. Stress-protective effects of mGlu7 deficiency on the CSC-induced anxiety-prone phenotype. To assess chronic psychosocial stress effects on anxiety-related behavior, SHC and CSC mice of the wildtype (WT) and knockout (KO) group were exposed to the EPM on day 19 of CSC. (a) The percentage of time spent on the open arms (time ratio OA/TA in %) on the EPM was decreased in CSC compared to SHC mice only of the WT group, indicative for an increase in innate anxiety. Moreover, the percentage of time spent on the open arms was significantly higher in CSC KO compared to CSC WT mice. (b) The number of closed arm (CA) entries was neither affected by CSC nor by genotype, indicating unaffected locomotor activity. White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). n = 19-26 per genotype and housing group. Data represent mean + S.E.M. $^{**}p \leq 0.01$ *vs.* respective SHC group; $^{\#}p \leq 0.05$ *vs.* respective WT group; two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016c).

3.3 mGlu7 genetic ablation protects against CSC-induced physiological and neuroendocrine alterations

Exposure to 19 days of CSC has been shown to result also in profound physiological and neuroendocrine changes (Füchsl et al., 2014; Reber et al., 2007; Uschold-Schmidt et al., 2012; Veenema et al., 2008). In the present study, different relevant parameters following CSC exposure were assessed in detail in mGlu7 KO mice and their WT littermates to evaluate a potential involvement of mGlu7 in CSC-induced HPA axis dysfunction and other physiological parameters. First of all, assessing body weight gain revealed no effects of CSC exposure in WT mice, which is in line with the literature (Füchsl et al., 2014; Slattery et al., 2012; Veenema et al., 2008). In addition, no effects were also found in the mGlu7 KO group (Figure 10a). Absolute pituitary weight was found to be dependent on both the factor housing ($F_{1,70} = 4.596$, $p = 0.036$) and the factor genotype ($F_{1,70} = 10.613$, $p = 0.002$), with a significant increase in pituitary weight in WT animals exposed to CSC compared to respective SHC mice ($p = 0.003$). Interestingly, this CSC effect was absent in mGlu7 KO animals. Moreover, pituitary weight was significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Figure 10b). Analysis of absolute adrenal weight also revealed a main effect of both the factor housing ($F_{1,69} = 56.725$, $p \leq 0.001$) and the factor genotype ($F_{1,69} = 14.080$, $p \leq 0.001$). Bonferroni *post hoc* analysis showed a significant increase in weight in CSC compared to SHC mice of the WT group ($p \leq 0.001$). This CSC effect was also present in the KO group, but less pronounced ($p = 0.002$). Therefore, adrenal weight was also significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Figure 10c).

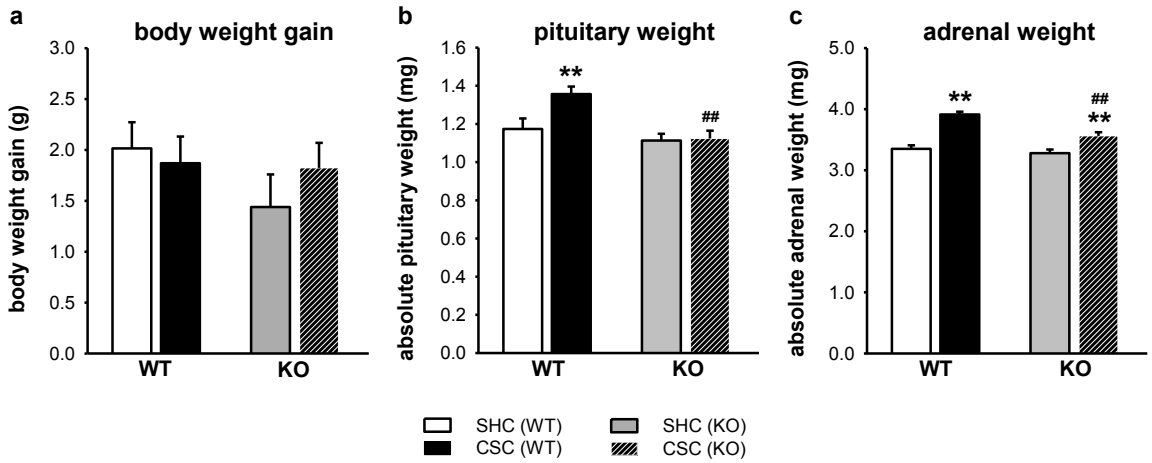


Figure 10. Stress-protective effects of mGlu7 deficiency on CSC-induced physiological alterations. (a) Body weight gain during 19 days of CSC was neither affected by CSC nor by genotype. (b) 19 days of CSC induced a significant increase in absolute pituitary weight in the wildtype (WT) but not in the knockout (KO) group. In addition, pituitary weight was significantly lower in CSC KO compared to CSC WT mice. (c) CSC exposure also induced a significant increase in absolute adrenal weight in WT mice and a less pronounced increase also in KO mice. Hence, adrenal weight was significantly lower in CSC KO compared to CSC WT mice. White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). n = 14-23 per genotype and housing group. Data represent mean + S.E.M. $^{**}p \leq 0.01$ *vs.* respective SHC group; $^{\#\#}p \leq 0.01$ *vs.* respective WT group; two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016c).

Furthermore, basal morning plasma ACTH levels were dependent on the factor housing ($F_{1,68} = 6.462$, $p = 0.013$), with significantly higher basal morning plasma ACTH levels in CSC compared to SHC mice of the WT group ($p = 0.004$). This stress effect was absent in the KO group (Figure 11a). Basal morning plasma CORT levels were neither dependent on the factor housing nor on the factor genotype (Figure 11b). It was further analyzed whether there were potential genotype-dependent effects of CSC exposure on adrenal ACTH responsiveness *in vitro*. In the WT group, there was a main effect of the factors housing ($F_{1,48} = 15.587$, $p \leq 0.001$) and stimulation ($F_{1,48} = 43.881$, $p \leq 0.001$), as well as a housing x stimulation interaction ($F_{1,48} = 11.160$, $p = 0.002$) on adrenal CORT secretion *in vitro*. In contrast, adrenal CORT secretion from explants of mGlu7 KO mice were only dependent on the factor stimulation ($F_{1,44} = 31.576$, $p \leq 0.001$). Adrenal explants from both SHC ($p \leq 0.001$ for each genotype) and CSC (WT: $p = 0.025$; KO: $p \leq 0.001$) mice showed an increased CORT secretion in response to ACTH compared to basal (saline) stimulation. However, adrenal CORT secretion in response to ACTH was significantly lower in CSC

compared to SHC mice of the WT group ($p \leq 0.001$). This CSC-induced attenuation of adrenal *in vitro* ACTH responsiveness was absent in mGlu7 KO mice (Figure 11c).
Together, these findings indicate that mGlu7 genetic ablation protects against developing CSC-induced HPA axis dysfunction.

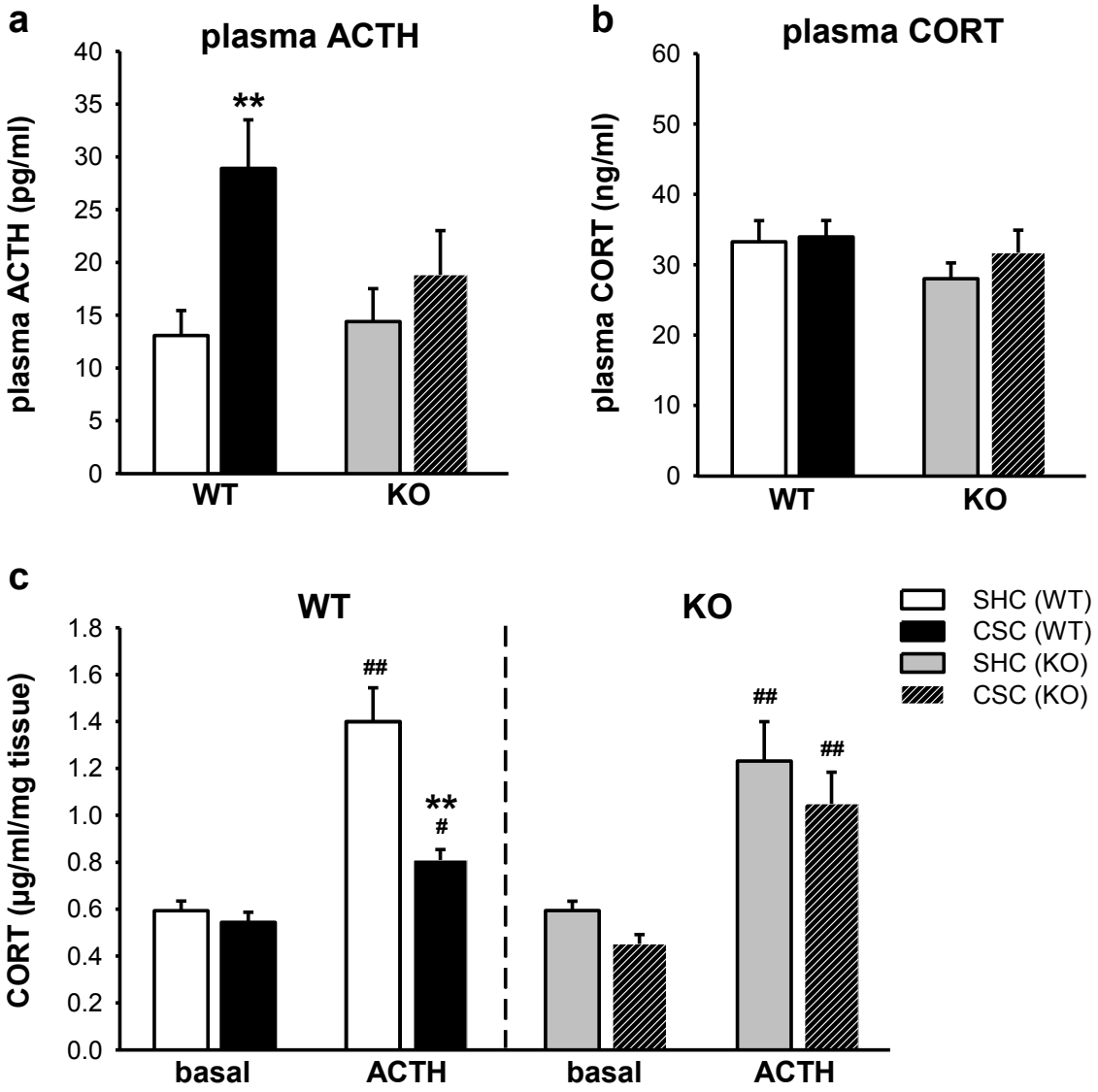


Figure 11. Stress-protective effects of mGlu7 deficiency on CSC-induced alterations in HPA axis functionality. (a) The CSC-induced increase in basal morning plasma ACTH levels in wildtype (WT) mice was absent in knockout (KO) mice. (b) There was neither an effect of housing nor genotype on basal morning plasma CORT levels. (c) 19 days of CSC resulted in an impaired adrenal *in vitro* ACTH responsiveness in WT mice (left panels), an effect absent in KO mice (right panels). White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). n = 15-23 (a, b) and n = 11-13 (c) per genotype and housing group. Data represent mean + S.E.M. $^{**}p \leq 0.01$ *vs.* respective SHC group; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ *vs.* respective basal stimulation (c); two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016c).

3.4 mGlu7 genetic ablation protects against CSC-induced immunological alterations

Reliable immunological alterations induced by CSC exposure include splenomegaly and the development of spontaneous colitis (Füchsl et al., 2014; Langgartner et al., 2015; Reber et al., 2007). In the present study, absolute spleen weight was dependent on both the factor housing ($F_{1,22}$ = 11.069, p = 0.003) as well as the factor genotype ($F_{1,22}$ = 12.917, p = 0.002). In line with the literature (Füchsl et al., 2014; Reber et al., 2007), Bonferroni *post hoc* analysis revealed a significant increase in spleen weight in CSC compared to SHC mice of the WT group (p = 0.001). This CSC effect was absent in the KO group. In addition, spleen weight was significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Figure 12a). Beneficial effects of mGlu7 genetic ablation were also found with respect to CSC-induced colonic inflammation. *In vitro* IFN-γ secretion from isolated and anti-CD3/anti-CD28-stimulated mesLNC were found to be dependent on the factors housing ($F_{1,19}$ = 5.476, p = 0.030) and genotype ($F_{1,19}$ = 12.358, p = 0.002), as well as a factor housing x genotype interaction ($F_{1,19}$ = 5.632, p = 0.028). Bonferroni *post hoc* analysis revealed significantly increased IFN-γ secretion in CSC compared to SHC mice of the WT group (p = 0.004). This CSC effect was again absent in mGlu7 KO mice. Moreover, IFN-γ secretion was significantly lower in the CSC KO group compared to CSC WT mice ($p \leq 0.001$; Figure 12b). In support of colonic inflammation, the histological damage score was found to be dependent on the factors housing ($F_{1,49}$ = 6.332, p = 0.015) and genotype ($F_{1,49}$ = 6.967, p = 0.011), as well as a factor housing x treatment interaction ($F_{1,49}$ = 6.967, p = 0.011). Bonferroni *post hoc* analysis revealed a significantly increased damage score in CSC compared to SHC mice of the WT group ($p \leq 0.001$; Figure 12c), reflected by an increased epithelial damage and a more severe inflammatory infiltration (Figure 12d). This CSC effect was again absent in the mGlu7 KO group. Furthermore, the damage score was significantly lower in CSC KO compared to CSC WT mice ($p \leq 0.001$; Figure 12c).

Together, these findings suggest that mGlu7 genetic ablation also protects against developing CSC-induced immunological alterations including colonic inflammation.

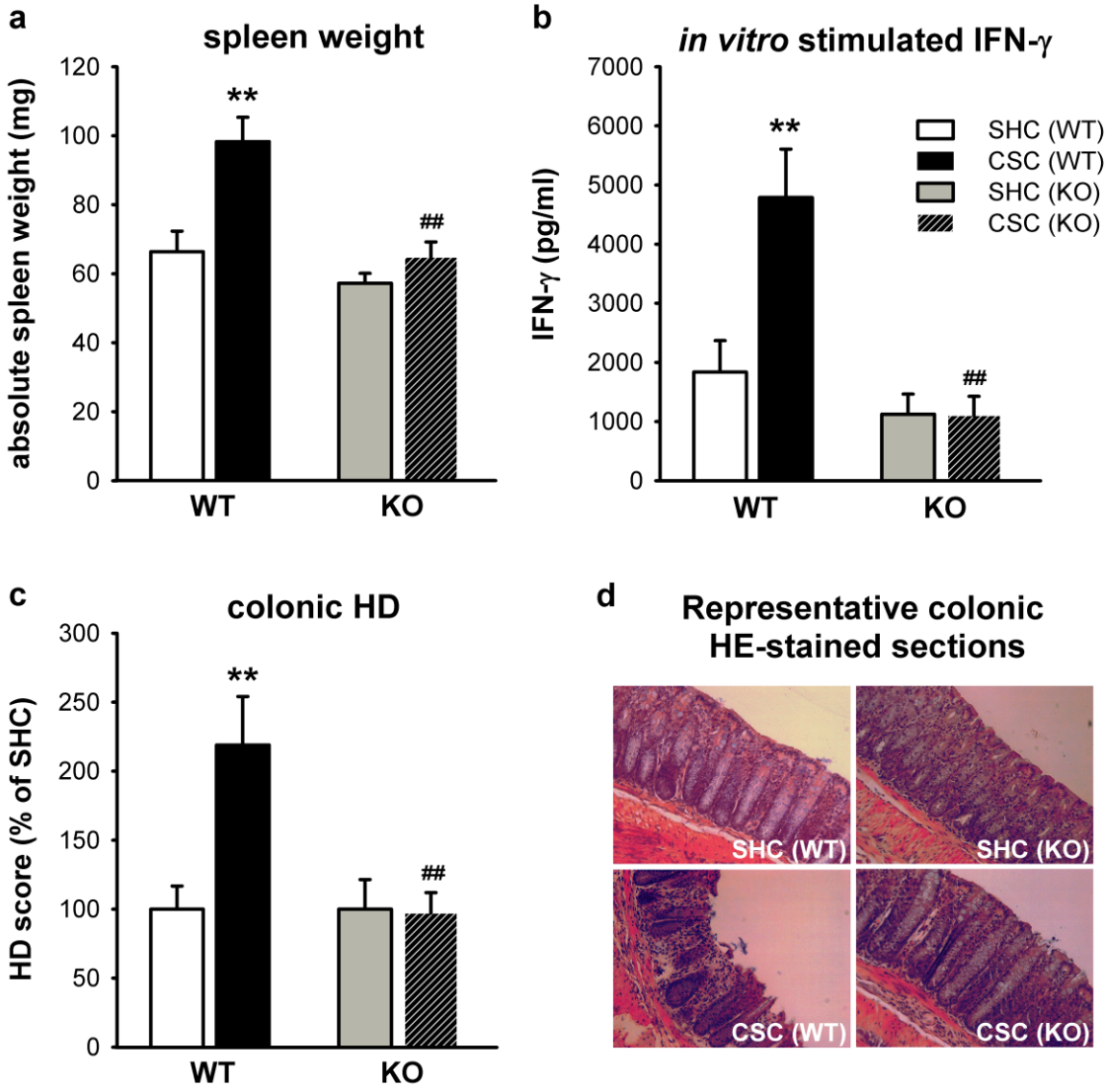


Figure 12. Stress-protective effects of mGlu7 deficiency on CSC-induced immunological alterations. (a) Absolute spleen weight was significantly increased in CSC compared to SHC mice of the wildtype (WT) but not of the knockout (KO) group. In addition, spleen weight was significantly lower in CSC KO compared to CSC WT mice. (b) 19 days of CSC resulted in a significantly increased secretion of IFN-γ from isolated and anti-CD3/anti-CD28 stimulated mesenteric lymph node cells (mesLNC). This effect was absent in KO mice. Hence, IFN-γ secretion was also significantly lower in CSC KO compared to CSC WT mice. (c) CSC exposure induced a significant increase in the histological damage (HD) score of colonic tissue in the WT group, whereas this damage was not present in KO mice. Furthermore, HD score was significantly lower in CSC KO compared to CSC WT mice. (d) Representative colonic sections stained with hematoxylin and eosin from SHC WT (left; normal colon histology) and CSC WT (right; goblet cell loss and crypt loss in locally restricted areas and infiltration of cells reaching the Lamina muscularis mucosae) mice. White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); dashed bar, CSC (KO). n = 5-8 (a), n = 4-8 (b) and n = 11-15 (c) per genotype and housing group. Data represent mean + S.E.M. $^{**}p \leq 0.01$ *vs.* respective SHC group; $^{\#\#}p \leq 0.01$ *vs.* respective WT group; two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016c).

4. mGlu5 functional blockade relieves maladaptive stress consequences induced by CSC

In order to investigate the functional role of the mGlu5 receptor in modulating somatic as well as affective consequences of CSC exposure, genetic and pharmacological approaches were designed. First, mGlu5 KO mice exposed to CSC were analyzed with respect to selected physiological, immunological and behavioral parameters. Second, the effects of chronic systemic pharmacological blockade of mGlu5 were assessed on a broader range of physiological, immunological and behavioral consequences of CSC. For this purpose, C57Bl/6N WT mice, separated into CSC and SHC groups, were chronically infused with the allosteric mGlu5 receptor antagonist CTEP at different doses; vehicle infusions were used as control.

4.1 Chronic stress-protective phenotype in mice lacking mGlu5

To evaluate a functional role of mGlu5 in chronic psychosocial stress-induced somatic and affective alterations, different selected physiological, immunological, and behavioral parameters were assessed in male mGlu5 KO mice and their WT littermates after CSC exposure. Absolute adrenal weight was found to be dependent on the factor housing ($F_{1,46}$ = 22.717, $p \leq 0.001$) and a factor housing x genotype interaction ($F_{1,46}$ = 5.516, p = 0.023), with a significant increase in weight in WT animals exposed to CSC compared to respective SHC mice ($p \leq 0.001$). This CSC effect was absent in mGlu5 KO animals. Moreover, adrenal weight was significantly lower in CSC mGlu5 KO compared to CSC WT mice (p = 0.010; Figure 13a). Next, a potential influence of chronic stressor exposure on the number of viable mesLNC was analyzed and it was found a main effect of the factor genotype ($F_{1,47}$ = 18.228, $p \leq 0.001$). Bonferroni *post hoc* analysis indicated a significantly lower number of viable mesLNC in CSC mGlu5 KO mice compared to their CSC WT littermates ($p \leq 0.001$). Moreover, an independent Student's *t*-test revealed an increase by trend in the number of viable mesLNC in CSC compared to SHC mice of the WT group (t_{28} = -1.868, p = 0.072; Figure 13b). In addition, stimulated IFN-γ secretion of mesLNC *in vitro* was dependent on a factor housing x genotype interaction ($F_{1,43}$ = 4.116, p = 0.049), with a significantly increased secretion in CSC compared to SHC mice of the WT group (p = 0.003). This effect was again absent in the mGlu5 KO group (Figure 13c). Moreover, there was a significantly lower IFN-γ secretion in CSC KO compared to CSC WT mice (p = 0.043; Figure 13c). To assess genotype-specific effects of CSC on anxiety-related

behavior, especially physiological anxiety, the SIH response on day 19 of CSC exposure was evaluated. A main effect was found of the factor housing ($F_{1,44} = 23.754$, $p \leq 0.001$) and of the factor housing x genotype interaction ($F_{1,44} = 5.002$, $p = 0.030$). Bonferroni *post hoc* analysis revealed a significantly increased SIH response in CSC compared to SHC mice of the WT group ($p \leq 0.001$); this CSC effect was again absent in the mGlu5 KO group (Figure 13d). Notably, it was shown for the first time that CSC exposure – in addition to the typical increase in innate anxiety – also increased physiological anxiety in WT mice. Unfortunately, innate anxiety on the EPM could not be evaluated due to technical limitations in experimental setups at the time the experiments was performed.
Together, the present findings indicate a stress-protective phenotype in mice lacking the mGlu5 receptor, at least with respect to the selected parameters assessed.

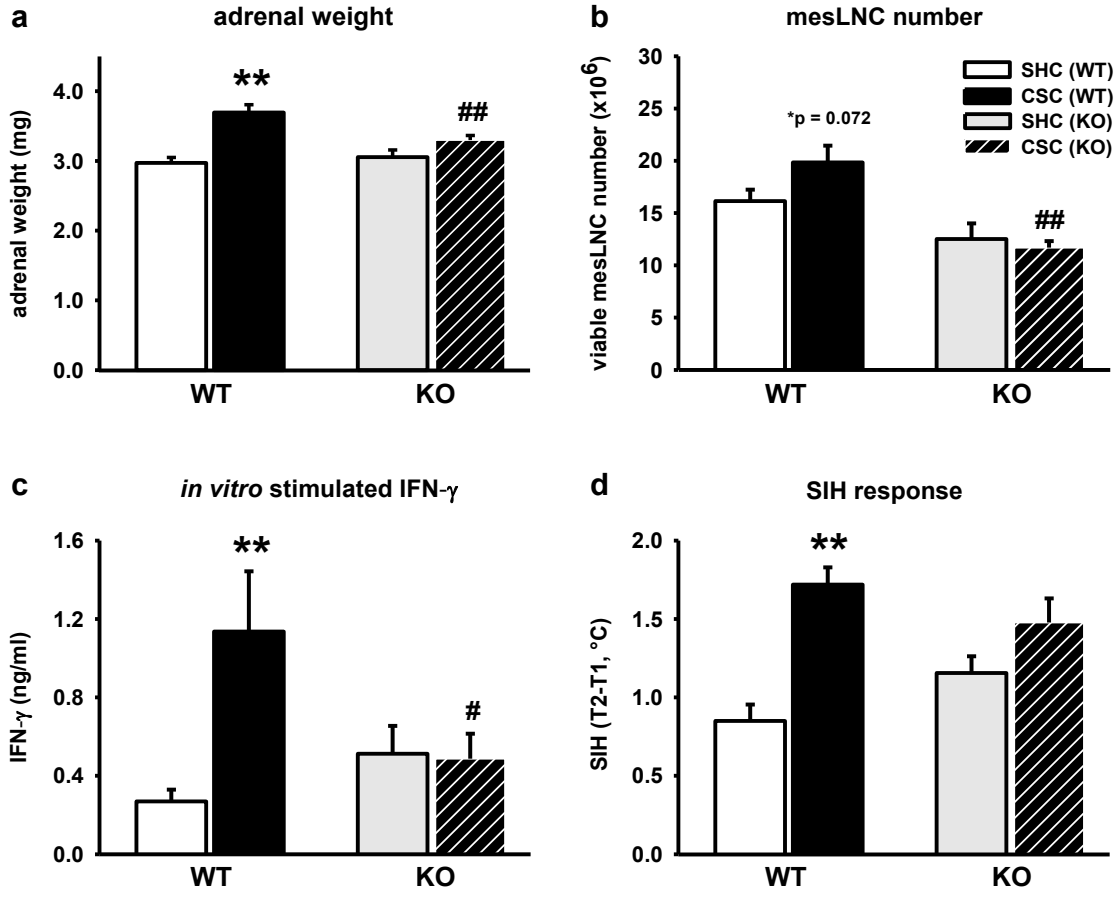


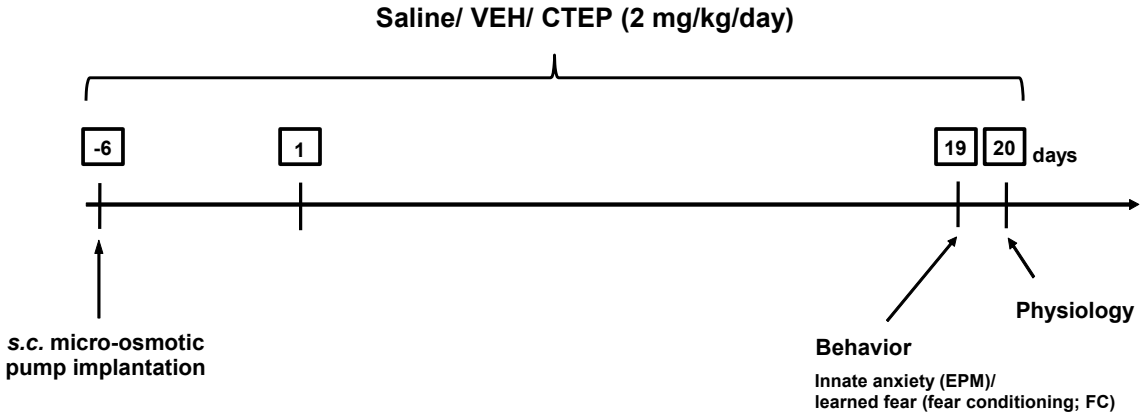
Figure 13. Physiological, immunological, and behavioral profile of WT mice and their mGlu5 KO littermates exposed to 19 days of CSC. (a) CSC exposure induced a significant increase in absolute adrenal weight in WT mice; this CSC effect was absent in KO mice. In addition, CSC KO mice showed a significantly lower adrenal weight compared to CSC WT mice. (b) In WT mice, CSC exposure resulted in a strong trend towards an increased number of viable mesenteric lymph node cells (mesLNC; $p = 0.072$),

whereas it remained unaffected in KO mice. In addition, the number of viable mesLNC was significantly lower in CSC KO compared to CSC WT mice. (c) The CSC-induced increased IFN-γ secretion of stimulated mesLNC *in vitro*, as observed in WT mice, was not present in KO mice. Furthermore, IFN-γ secretion *in vitro* was significantly lower in CSC KO compared to CSC WT mice. (d) CSC exposure significantly increased the hyperthermic response in the SIH test in WT mice. This CSC effect was again not present in KO mice. White bar, SHC (WT); black bar, CSC (WT); grey bar, SHC (KO); striped bar, CSC (KO). n = 9-16 per genotype and housing group. Data represent mean + S.E.M. $^{**}p \leq 0.01$ *vs.* respective SHC mice; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ *vs.* respective WT genotype; two-way ANOVA followed by Bonferroni *post hoc* analysis or independent Student's *t*-test. WT, wildtype; KO, knockout. Adapted from Peterlik et al. (2016b).

4.2 Pilot-study: Chronic application of vehicle (PEG) and CTEP in naïve mice precludes any undesirable effects on typical CSC-affected parameters

In preparation for the pharmacological approach of studying mGlu5's functional role in CSC-induced consequences, it was essential to provide experimental evidence for the compatibility of chronic vehicle (PEG) administration via micro-osmotic pumps with typical CSC-affected parameters. Therefore, it was tested whether chronic administration of the vehicle (PEG) and CTEP (2 mg/kg/day) *s.c.* via micro-osmotic pumps had any undesirable effects under non-stress conditions on parameters typically assessed after CSC exposure (Figure 14a). Importantly, there was no effect of either vehicle or CTEP treatment (2 mg/kg/day) in comparison to chronic saline administration (Figure 14b) in non-stressed mice after 26 days. This is in line with a previous study conducted by Peters et al. (2014), where they revealed no confounding influence of either the surgical procedure or the chronic *i.c.v.* administration of vehicle (in this case Ringer's solution) via micro-osmotic pumps.

a **Experimental design**



b

	Saline	VEH (PEG)	CTEP (2 mg/kg/day)
innate anxiety (EPM, time ratio OA/TA (%)), *n* = 4-6	49.88 ± 14.50	64.72 ± 7.60	69.51 ± 13.16
locomotion (EPM, CA entries), *n* = 4-6	7.83 ± 2.17	6.17 ± 1.35	4.5 ± 1.26
fear acquisition (cued FC, freezing response (%) after 5 CS-US pairings), *n* = 5-6	67.14 ± 9.94	47.62 ± 9.96	57.14 ± 7.93
fear expression (+ 24 h, freezing response (%) after 2 CS presentations), *n* = 5-6	65.33 ± 5.73	50.56 ± 5.67	54.44 ± 6.59
body weight gain (g), *n* = 11-12	3.3 ± 0.5	2.9 ± 0.2	2.9 ± 0.4
thymus weight (mg), *n* = 11-12	44.81 ± 2.59	41.91 ± 1.99	40.91 ± 2.19
pituitary weight (mg), *n* = 11-12	1.63 ± 0.10	1.55 ± 0.05	1.53 ± 0.07
adrenal weight (mg), *n* = 11-12	3.69 ± 0.15	3.64 ± 0.05	3.54 ± 0.10
plasma ACTH (pg/ml), *n* = 11-12	10.68 ± 0.73	12.66 ± 1.92	10.41 ± 0.40
plasma CORT (ng/ml), *n* = 8-12	37.43 ± 4.32	38.43 ± 3.15	31.29 ± 3.00

Figure 14. Chronic application of vehicle (PEG) and CTEP via micro-osmotic pumps in naïve mice precludes any undesirable effects on typical CSC-affected parameters. (a) Schematic illustration of the experimental design of chronic (*i.e.* 26 days) administration of saline, VEH (polyethylene glycol, PEG), and CTEP at a dose of 2 mg/kg/day via micro-osmotic pumps (*s.c.*) in unstressed mice to test for possible undesirable effects on the parameters typically assessed following CSC exposure. (b) Neither VEH nor CTEP treatment had any effects on the parameters assessed compared to mice treated with saline only. Adapted from Peterlik et al. (2016b).

4.3 Chronic pharmacological mGlu5 blockade reverses the CSC-induced decrease in body weight

To further address the functional involvement of the mGlu5 receptor in chronic psychosocial stress physiology, it was assessed whether chronic pharmacological blockade of the mGlu5 receptor interferes with the well-known CSC-induced somatic and affective changes. It was found a main effect of the factor treatment ($F_{3,125} = 3.352$; $p = 0.021$) and a

factor housing x treatment interaction ($F_{3,125} = 3.017$; $p = 0.032$) on body weight gain, with a decrease in CSC compared to SHC mice of the vehicle group ($p = 0.028$). This stress effect was abolished by CTEP at all three doses applied (Figure 15a). Moreover, body weight gain was significantly higher in CSC mice treated with CTEP at a dose of 0.05 mg/kg/day ($p = 0.011$) and 0.5 mg/kg/day ($p = 0.003$) compared to CSC mice of the vehicle group. A closer look on body weight development of mice treated with vehicle and 2 mg/kg/day of CTEP (exemplary for all CTEP doses) during CSC procedure revealed a main effect of the factors housing ($F_{3,75} = 3.373$, $p = 0.023$), time ($F_{6,450} = 41.282$, $p \leq 0.001$) and a factor housing x time interaction ($F_{18,450} = 1.658$, $p = 0.044$). Mice from all treatment/housing groups showed a positive body weight development over a period of 19 days. Moreover, an independent Student's *t*-test revealed a decreased body weight in CSC compared to SHC mice of the vehicle group on day 10 ($t_{42} = 2.062$, $p = 0.045$), day 15 ($t_{42} = 2.186$, $p = 0.034$) and day 17 ($t_{42} = 2.116$, $p = 0.040$). This CSC effect was blocked in mice treated with CTEP at a dose of 2 mg/kg/day (Figure 15b).

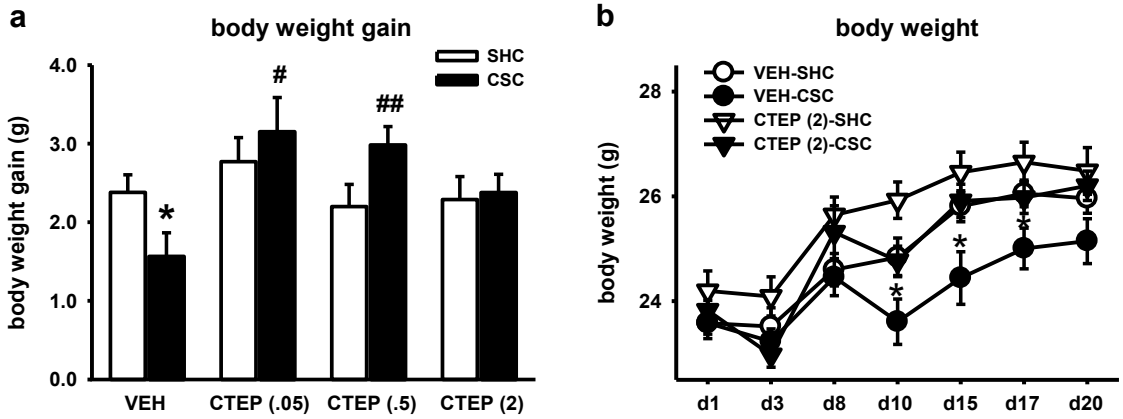


Figure 15. Stress-protective effects of chronic CTEP treatment on CSC-induced changes in body weight gain and body weight development. (a) CSC induced a significant decrease in body weight gain (day 1 to day 20) in VEH-treated mice. This stress effect was blocked by chronic CTEP treatment. Also, body weight gain was significantly higher in CSC mice treated with CTEP at the doses of 0.05 and 0.5 mg/kg/day compared to CSC VEH-treated mice. (b) In VEH-treated mice, CSC resulted in a significantly lower absolute body weight on days 10, 15, and 17; this effect was blocked with chronic CTEP treatment (2 mg/kg/day). White bar, SHC; black bar, CSC. n = 8-24 per treatment and housing group. Data represent mean ± S.E.M. $^{*}p \leq 0.05$ *vs.* respective SHC mice; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ *vs.* respective VEH group; two-way ANOVA followed by Bonferroni *post hoc* analysis (a) or independent Student's *t*-test (b). Adapted from Peterlik et al. (2016b).

4.4 Chronic pharmacological mGlu5 blockade corrects CSC-induced alterations in HPA axis functionality in a dose-dependent manner

As CSC exposure has been shown to result in profound physiological and neuroendocrine changes (Füchsl et al., 2014; Reber et al., 2007; Uschold-Schmidt et al., 2012; Veenema et al., 2008), further focused was placed on different HPA axis-related parameters. It was found a main effect of the factor housing ($F_{1,127}$ = 7.366, p = 0.008) on absolute pituitary weight with an increased weight in CSC compared to SHC mice treated with vehicle (p = 0.008) and CTEP at a dose of 0.05 mg/kg/day (by trend; p = 0.057). This increase could be reversed with the two higher doses of CTEP (Figure 16a). Basal morning plasma ACTH levels were found to be dependent on the factors housing ($F_{1,123}$ = 3.873, p = 0.051) and treatment ($F_{3,123}$ = 3.356, p = 0.021). Bonferroni *post hoc* analysis showed a significant stress-induced increase only in basal morning plasma ACTH in mice treated with CTEP at a dose of 0.05 mg/kg/day, but failed to show any differences between mice treated with CTEP in comparison to respective vehicle groups. In addition, an independent Student's *t*-test revealed a significant increase in plasma ACTH in CSC compared to SHC mice treated with vehicle (t_{42} = -2.297, p = 0.027; Figure 16b). Analysis of absolute adrenal weight revealed a main effect of the factor housing ($F_{1,128}$ = 96.834, $p \leq 0.001$) and a factor housing x treatment interaction ($F_{3,128}$ = 9.646, $p \leq 0.001$). CSC exposure induced an increase in adrenal weight in mice treated with vehicle and CTEP at a dose of 0.05 and 0.5 mg/kg/day ($p \leq 0.001$ for each). This effect was abolished by treatment with CTEP at a dose of 2 mg/kg/day, and consequently, adrenal weight was significantly lower in stressed mice treated with the high dose of CTEP compared to CSC mice of the vehicle group ($p \leq 0.001$; Figure 16c). Basal morning plasma CORT levels were neither influenced by the factor housing nor by the factor treatment (Figure 16d).

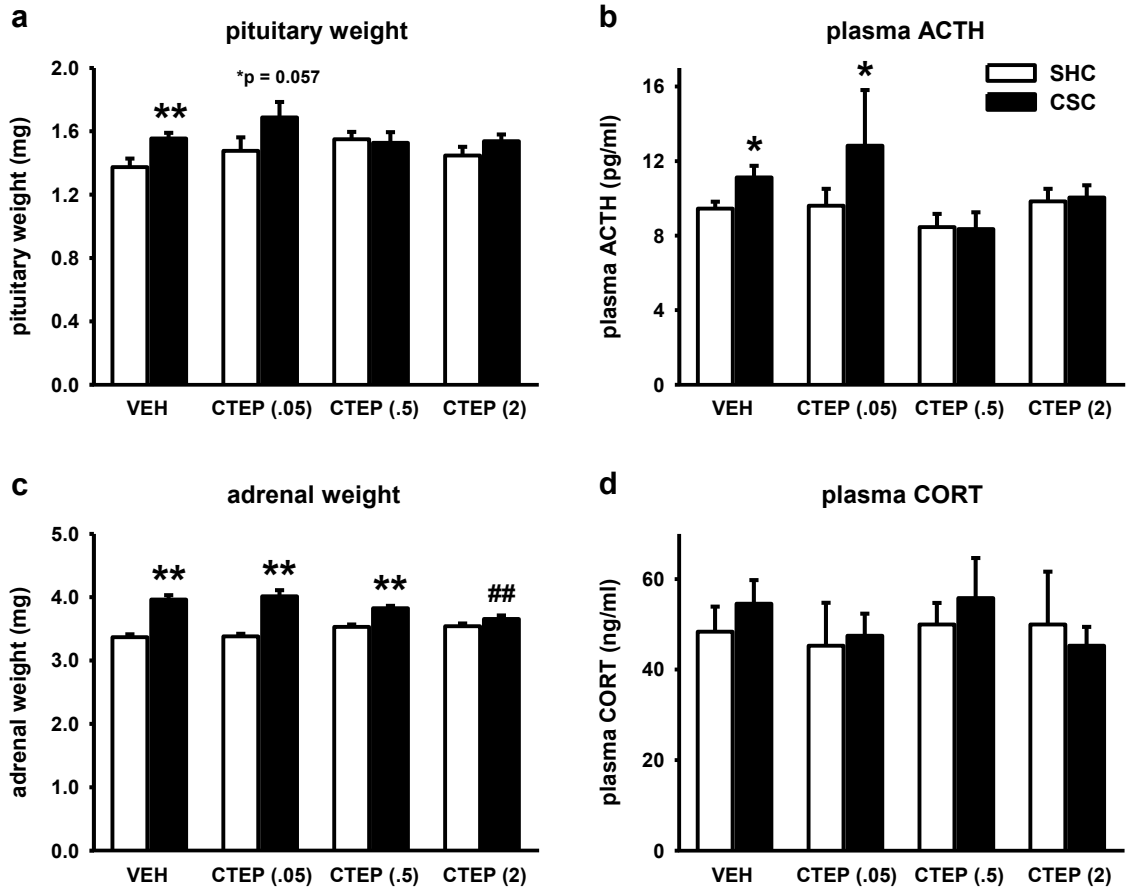


Figure 16. Stress-protective effects of chronic CTEP treatment on CSC-induced physiological and neuroendocrine alterations. (a) Absolute pituitary weight was significantly increased in CSC compared to SHC mice treated with VEH and increased by trend in mice treated with 0.05 mg/kg/day (p = 0.057). CTEP at doses of 0.5 and 2 mg/kg/day were able to abolish this increase. (b) The CSC-induced increase in basal morning plasma ACTH levels found in VEH-treated mice was still present in mice treated with CTEP at a dose of 0.05 mg/kg/day, but completely abolished in mice treated with 0.5 and 2 mg/kg/day of CTEP. (c) CSC resulted in an increased absolute adrenal weight in VEH-treated mice; this CSC effect was still present in mice treated with CTEP at doses of 0.05 and 0.5 mg/kg/day, but completely abolished with 2 mg/kg/day of CTEP. In addition, adrenal weight was significantly lower in CSC mice treated with 2 mg/kg/day of CTEP compared to CSC mice of the VEH group. (d) Neither CSC nor chronic CTEP treatment significantly affected basal morning plasma CORT levels. White bar, SHC; black bar, CSC. n = 8-24 per treatment and housing group. Data represent mean + S.E.M. *$p \leq 0.05$, **$p \leq 0.01$ *vs.* respective SHC mice; $^{\#\#}p \leq 0.01$ *vs.* respective VEH group; two-way ANOVA followed by Bonferroni *post hoc* analysis or independent Student's *t*-test (b). Adapted from Peterlik et al. (2016b).

Next, the effects of CSC on adrenal *in vitro* ACTH responsiveness and potential effects of CTEP treatment were analyzed. In mice treated with vehicle and 0.05 mg/kg/day of CTEP, there was a main effect of the factors housing (vehicle: $F_{1,86}$ = 8.660, p = 0.004; 0.05 CTEP: $F_{1,32}$ = 14.705, $p \leq 0.001$), stimulation (vehicle: $F_{1,86}$ = 42.362, $p \leq 0.001$; 0.05

CTEP: $F_{1,32} = 37.689$, $p \leq 0.001$) and a factor housing x stimulation interaction (vehicle: $F_{1,86} = 8.282$, $p = 0.005$; 0.05 CTEP: , $F_{1,32} = 5.313$, $p = 0.028$) on adrenal CORT secretion *in vitro* (Figure 17). In contrast, adrenal CORT secretion from explants of mice treated with 0.5 and 2 mg/kg/day of CTEP were only dependent on the factor stimulation (0.5 CTEP: $F_{1,64} = 108.316$, $p \leq 0.001$; 2 CTEP: $F_{1,58} = 60.518$, $p \leq 0.001$). In all groups, adrenal explants from both SHC ($p \leq 0.001$ for each) and CSC (vehicle: $p = 0.013$; 0.05 CTEP: $p = 0.015$; 0.5 and 2 CTEP: $p \leq 0.001$) mice showed an increased CORT secretion in response to ACTH compared to basal (saline) stimulation (Figure 17). However, in mice treated with vehicle and 0.05 mg/kg/day of CTEP, adrenal CORT secretion in response to ACTH was significantly lower in CSC compared to SHC mice ($p \leq 0.001$ for each). This CSC-induced attenuation of adrenal *in vitro* ACTH responsiveness was absent in mice treated with CTEP at doses of 0.5 and 2 mg/kg/day (Figure 17). Together, these findings indicate that HPA axis functionality is generally less vulnerable to chronic psychosocial stressor exposure in mice with pharmacological mGlu5 receptor inhibition.

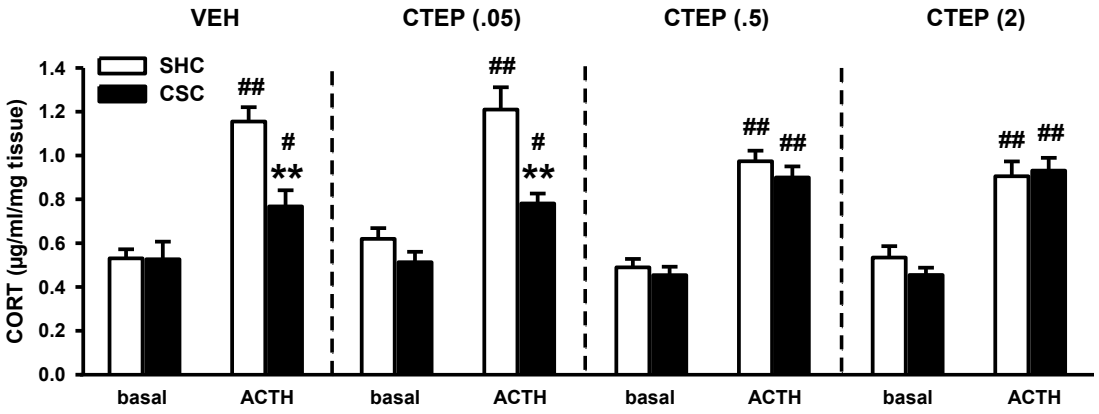


Figure 17. Stress-protective effects of chronic CTEP treatment on CSC-induced reduction of adrenal *in vitro* ACTH responsiveness. CSC resulted in an impaired adrenal *in vitro* ACTH responsiveness in VEH-treated mice as well as in mice treated with CTEP at a dose of 0.05 mg/kg/day. This CSC effect was absent in mice treated with 0.5 and 2 mg/kg/day of CTEP**.** White bar, SHC; black bar, CSC. n = 8-24 per stimulation and housing group. Data represent mean + S.E.M. $^{**}p \leq 0.01$ *vs.* respective SHC mice; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ *vs.* respective basal stimulation; two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016b).

4.5 Chronic pharmacological mGlu5 blockade dose-dependently prevents CSC-induced immunological alterations

CSC housing represents an established model to also assess immunological consequences of chronic psychosocial stress (Füchsl et al., 2014; Langgartner et al., 2015; Peters et al., 2012; Reber et al., 2007) and has been shown to result in thymic atrophy and splenomegaly (Füchsl et al., 2014; Reber et al., 2007) and to cause spontaneous colitis (Reber et al., 2007). In the present study, assessment of absolute thymus weight revealed a main effect of the factor housing ($F_{1,128}$ = 19.837, $p \leq 0.001$) and a factor housing x treatment interaction ($F_{3,128}$ = 3.296, p = 0.023), with a decreased thymus weight in CSC compared to SHC mice treated with vehicle ($p \leq 0.001$) and 0.05 mg/kg/day of CTEP (p = 0.021). This CSC-induced thymic atrophy was blocked by treatment with CTEP at the doses of 0.5 and 2 mg/kg/day. Furthermore, Bonferroni *post hoc* analysis also revealed a lower thymus weight in CSC mice treated with CTEP at a dose of 0.5 (p = 0.005) and 2 mg/kg/day (p = 0.047) compared to CSC mice of the vehicle group (Figure 18a). Absolute spleen weight was dependent on the factor housing ($F_{1,95}$ = 30.239, $p \leq 0.001$) and a factor housing x treatment interaction ($F_{3,95}$ = 7.527, $p \leq 0.001$). CSC exposure induced an increase in spleen weight in mice treated with vehicle ($p \leq 0.001$) and CTEP at the doses of 0.05 (p = 0.037) and 0.5 mg/kg/day (p = 0.002). This effect was blocked by treatment with 2 mg/kg/day of CTEP. In addition, spleen weight was lower in CSC mice of all CTEP groups compared to stressed vehicle-treated animals (0.05 CTEP: p = 0.017; 0.5 CTEP: p = 0.002; 2 CTEP: $p \leq 0.001$; Figure 18b).

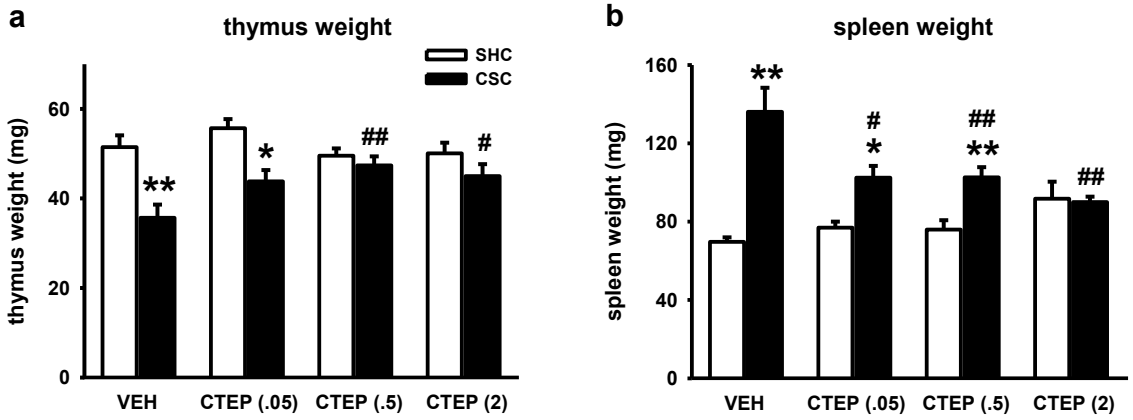


Figure 18. Stress-protective effects of chronic CTEP on CSC-induced weight changes in organs of the immune system. (a) CSC exposure induced a significant decrease in absolute thymus weight in VEH-treated mice as well as in mice treated with CTEP at a dose of 0.05 mg/kg/day. This stress effect was blocked in

mice treated with CTEP at the doses of 0.5 and 2 mg/kg/day. In addition, thymus weight was significantly higher in CSC mice treated with CTEP at the doses of 0.5 and 2 mg/kg/day compared to CSC mice of the VEH group. (b) The CSC-induced increase in absolute spleen weight found in VEH-treated mice was still present in mice treated with CTEP at doses of 0.05 and 0.5 mg/kg/day, but prevented by 2 mg/kg/day of CTEP. In addition, spleen weight was significantly lower in CSC mice of all CTEP groups compared to CSC mice of the VEH group. White bar, SHC; black bar, CSC. n = 8-24 per treatment and housing group. Data represent mean + S.E.M. *$p \leq 0.05$, **$p \leq 0.01$ *vs.* respective SHC mice; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ *vs.* respective VEH group; two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016b).

Beneficial effects of chronic mGlu5 receptor blockade were also found with respect to CSC-induced colonic inflammation. The number of viable mesLNC was dependent on the factor housing ($F_{1,93} = 36.841$, $p \leq 0.001$) and a factor housing x treatment interaction ($F_{3,93} = 3.424$, $p = 0.020$). Bonferroni *post hoc* analysis revealed an increased number in CSC compared to SHC mice treated with vehicle and CTEP at doses of 0.05 and 0.5 mg/kg/day ($p \leq 0.001$ for each). This increase was blocked by 2 mg/kg/day of CTEP. Moreover, the number of viable mesLNC was significantly lower in CSC mice treated with CTEP at a dose of 2 mg/kg/day compared to CSC mice of the vehicle group ($p = 0.015$; Figure 19a). Analysis of the IFN-γ secretion of mesLNC *in vitro* revealed a main effect of the factors housing ($F_{1,89} = 7.708$, $p = 0.007$), treatment ($F_{3,89} = 3.401$, $p = 0.021$) as well as a housing x treatment interaction ($F_{3,89} = 4.555$, $p = 0.005$). Bonferroni *post hoc* tests showed a significantly increased IFN-γ secretion in CSC compared to SHC mice treated with vehicle ($p \leq 0.001$). This CSC effect was blocked by CTEP-treatment independent of the dose applied. Furthermore, *in vitro* IFN-γ secretion was lower in CSC mice of all CTEP groups compared to CSC mice of the vehicle group (0.05 CTEP: $p = 0.006$; 0.5 CTEP: $p = 0.001$; 2 CTEP: $p = 0.003$; Figure 19b). In support of CSC-induced development of colonic inflammation, the histological damage score was dependent on the factor housing ($F_{1,82} = 34.776$, $p \leq 0.001$), the factor treatment ($F_{3,82} = 5.075$, $p = 0.003$), as well as a factor housing x treatment interaction ($F_{3,82} = 5.075$, $p = 0.003$). It was found an increased score in CSC compared to SHC mice treated with vehicle ($p \leq 0.001$; Figure 19d), indicated by an increased epithelial damage and more severe inflammatory infiltration (Figure 19c). This CSC effect was also present in mice treated with CTEP at the doses of 0.05 ($p = 0.035$) and 0.5 mg/kg/day ($p = 0.003$), but absent in mice treated with 2 mg/kg/day of CTEP (Figure 19d). Moreover, histological damage was significantly higher in CSC mice treated with vehicle compared to CSC mice of all CTEP groups (for each $p \leq 0.014$).

Together, these findings suggest a dose-dependent reduction of the vulnerability to chronic stressor exposure with respect to immunological consequences including colonic inflammation in CTEP-treated mice.

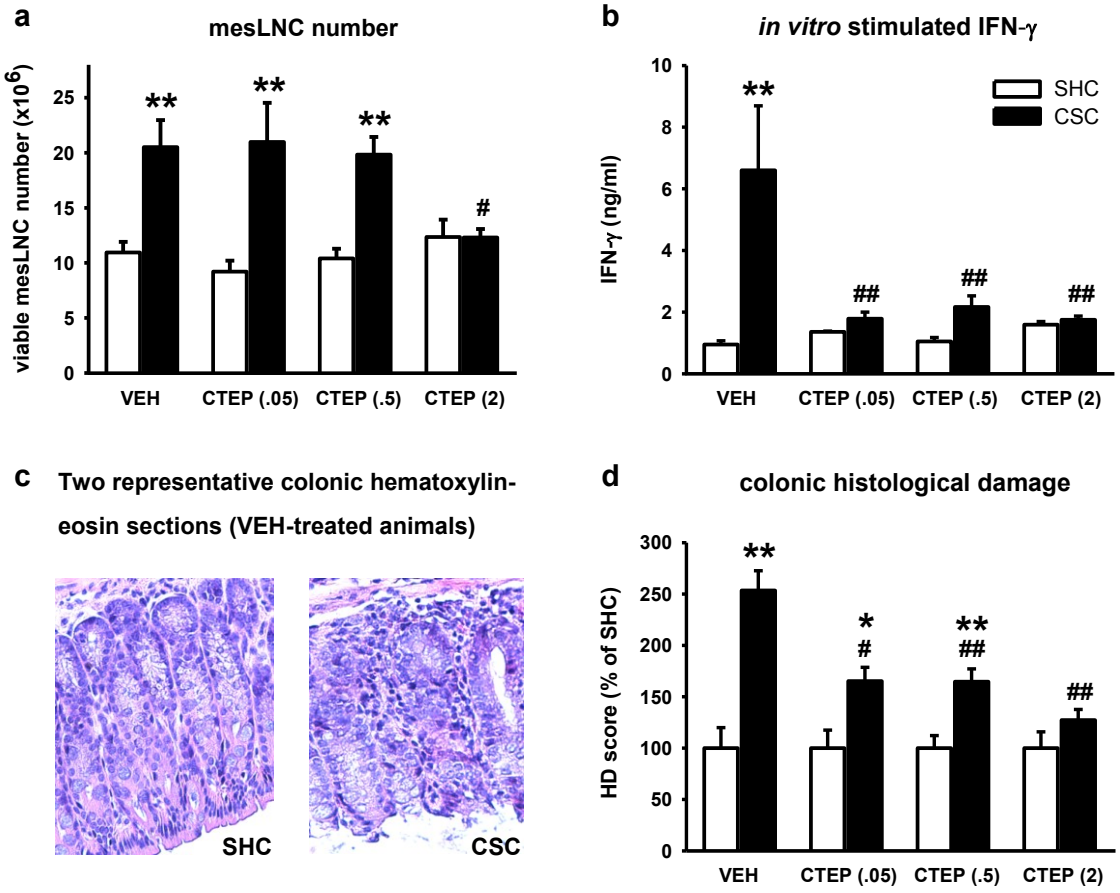


Figure 19. Prevention of CSC-induced colonic inflammation by chronic CTEP. (a) In a similar fashion, the CSC-induced increase in the number of viable mesLNC in the VEH group was also present in mice treated with CTEP at doses of 0.05 and 0.5 mg/kg/day, but abolished with 2 mg/kg/day of CTEP. In addition, CSC mice treated with 2 mg/kg/day of CTEP had a significant lower number of viable mesLNC compared to CSC mice of the VEH group. (b) Stimulated IFN-γ secretion *in vitro* was significantly increased in VEH-treated animals after CSC exposure. All CTEP doses applied were able to reverse this CSC effect. Also, IFN-γ secretion was significantly lower in CSC mice of all CTEP groups compared to CSC mice treated with VEH. (c) Representative colonic sections stained with hematoxylin and eosin from SHC (left; normal colon histology) and CSC (right; goblet cell loss and crypt loss in locally restricted areas and infiltration of cells reaching the Lamina muscularis mucosae) mice of the VEH group. (d) CSC exposure induced a significant increase in the histological damage (HD) score of colonic tissue in mice treated with VEH as well as CTEP at doses of 0.05 and 0.5 mg/kg/day. This damage was blocked by treatment with 2 mg/kg/day of CTEP. White bar, SHC; black bar, CSC. n = 8-24 per treatment and housing group. Data represent mean + S.E.M. *$p \leq 0.05$, **$p \leq 0.01$ *vs.* respective SHC mice; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ *vs.* respective VEH group; two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016b).

4.6 Chronic pharmacological mGlu5 blockade has no influence on the CSC-induced anxiety-prone phenotype

As CSC exposure reliably increases anxiety-related behavior in mice (Langgartner et al., 2015; Reber and Neumann, 2008), possible beneficial effects were evaluated of CTEP-treatment on CSC-induced physiological and innate anxiety with the SIH and EPM test, respectively. It was found a main effect of the factor housing ($F_{1,129}$ = 32.902, $p \leq 0.001$) on the stress-induced hyperthermic response. Bonferroni *post hoc* analysis revealed an increased SIH response in CSC compared to SHC mice treated with vehicle (p = 0.002) as well as CTEP at a dose of 0.5 and 2 mg/kg/day ($p \leq 0.001$ for each). In addition, an independent Student's *t*-test also revealed a CSC-induced increase in the SIH response in mice treated with 0.05 mg/kg/day of CTEP (t_{16} = -2.645, p = 0.018; Figure 20).

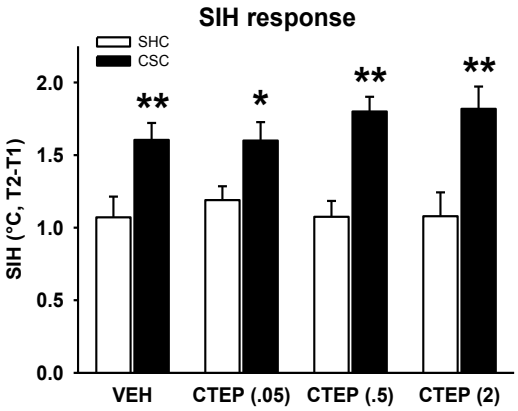


Figure 20. Chronic CTEP has no influence on CSC-induced increase in physiological anxiety. CSC exposure induced an increased hyperthermic response in the SIH test independent of treatment, indicative for an increase in physiological anxiety. White bar, SHC; black bar, CSC. Data represent mean + S.E.M. **$p \leq$ 0.01 *vs.* respective SHC mice. n = 8-24 per treatment and housing group; two-way ANOVA followed by Bonferroni *post hoc* analysis or independent Student's *t*-test (0.05 mg/kg/day). Adapted from Peterlik et al. (2016b).

The time the mice spent on the open arms of the EPM, indicative for their level of innate anxiety, was also dependent on the factor housing ($F_{1,92}$ = 31.780, $p \leq 0.001$), with a decreased percentage of time spent on the open arms in CSC compared to SHC mice of the vehicle group ($p \leq 0.001$). This CSC-induced decrease was still present in mice treated with CTEP independent of the dose applied (Bonferroni *post hoc* analysis, 0.05 CTEP: by trend p = 0.065; 0.5 CTEP: by trend p = 0.057; 2 CTEP: $p \leq 0.001$), suggesting also no beneficial effects of CTEP on CSC-induced increase in innate anxiety (Figure 21a). Although there was a main effect of the factor housing on the number of CA entries ($F_{1,92}$ = 4.501, p = 0.037), Bonferroni *post hoc* analysis did not show any significant differences of CA entries within and between all groups, indicating comparable locomotor activity

(Figure 21b). Together, these findings indicate that chronic CTEP treatment had no influence on the CSC-induced increase in physiological and innate anxiety.

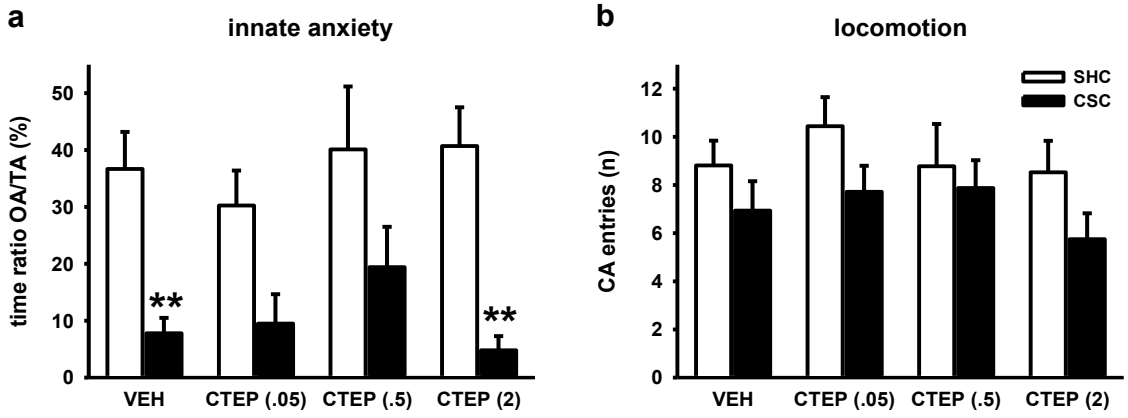


Figure 21. Chronic CTEP has no influence on CSC-induced increase in innate anxiety. (a) The percentage of time spent on the open arms (time ratio OA/TA in %) on the EPM was decreased in CSC mice of all treatment groups (in the groups of 0.05 and 0.5 mg/kg/day of CTEP by trend, $p \leq 0.057$; significance was reached in the 2 mg/kg/day group, $p \leq 0.01$), indicating CSC-induced increase in innate anxiety independent of treatment. (b) The number of closed arm (CA) entries were not different within and between all groups, indicating comparable locomotor activity. White bar, SHC; black bar, CSC. Data represent mean + S.E.M. $^{**}p \leq 0.01$ *vs.* respective SHC mice. n = 8-16 per treatment and housing group; two-way ANOVA followed by Bonferroni *post hoc* analysis. Adapted from Peterlik et al. (2016b).

4.7 Acute and sub-chronic pharmacological mGlu5 blockade induce anxiolytic-like effects in the SIH test

Pharmacological inhibition of the mGlu5 receptor by CTEP-treatment already demonstrated robust activity in the SIH test in mice, with a minimal effective oral dose of 0.1 mg/kg. In addition, unspecific effects of CTEP such as sedation or general changes in overall health and body temperature were absent in a dose range of up to 2 mg/kg (Lindemann et al., 2011). In the present study, these findings were extended towards a different administration route of CTEP (*i.p.*) and CTEP's *in vivo* activity was verified in the SIH test upon acute and sub-chronic treatment at the doses of 0.1, 0.5, and 2 mg/kg (see experimental design, Figure 22a).

The SIH response following acute CTEP-treatment was found to be dependent on the factor treatment (one-way ANOVA; $F_{3,23} = 8.464$, $p = 0.001$), with a reduced stress-induced hyperthermic response 1 h after a single *i.p.* injection in mice treated with 0.5 ($p = 0.008$) and 2 mg/kg ($p \leq 0.001$) of CTEP compared to vehicle treatment (Figure 22b).

Furthermore, the SIH response following sub-chronic CTEP-treatment (injected *i.p.* every 48 h) was dependent on the factors treatment (repeated measures ANOVA; $F_{3,70} = 2.858$, $p = 0.043$) and time (repeated measures ANOVA; $F_{1,70} = 4.631$, $p = 0.035$). LSD *post hoc* analysis revealed a reduced stress-induced hyperthermic response in mice treated with CTEP at a dose of 2 mg/kg compared to the respective vehicle group on day 16 ($p = 0.007$) and on day 24 ($p = 0.035$; Figure 22c). These findings indicate that acute and sub-chronic treatment with CTEP given *i.p.* every 48 h is able to induce anxiolytic-like effects in the SIH test.

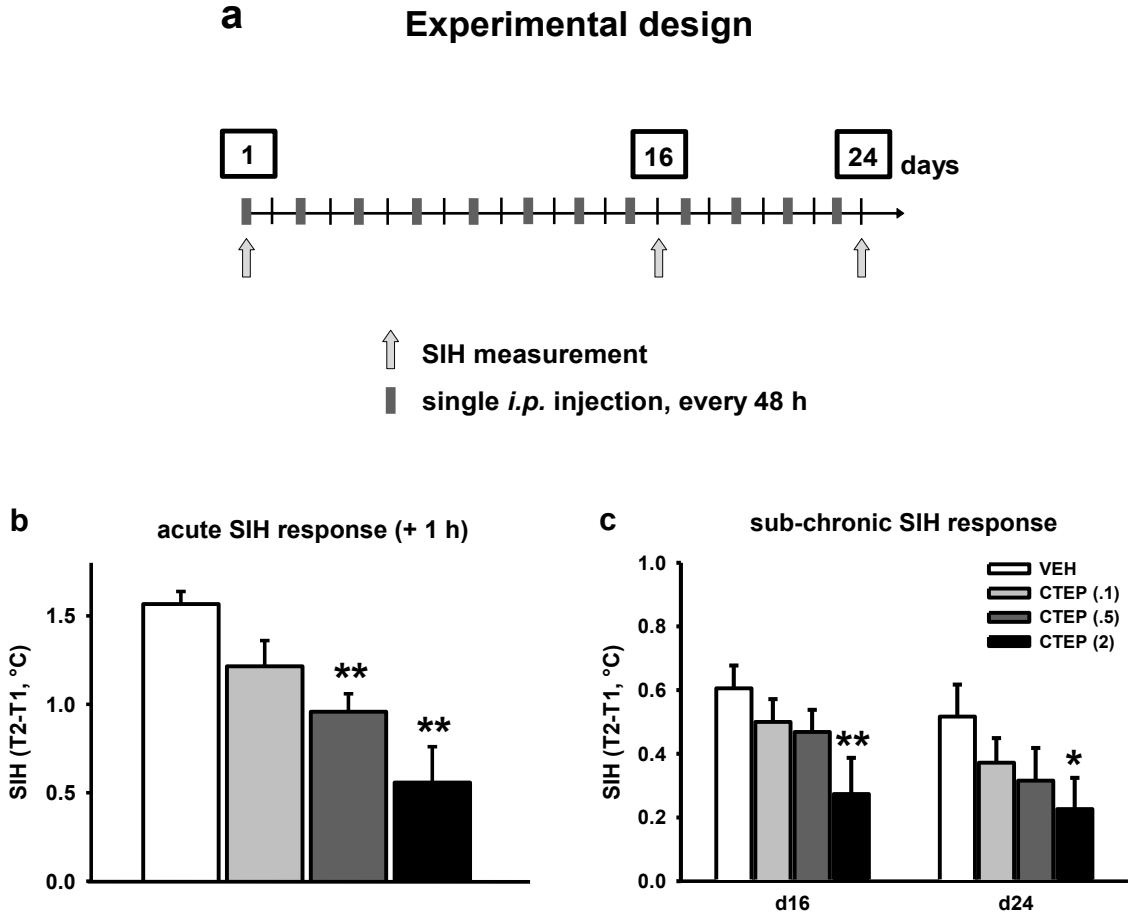


Figure 22. Acute and sub-chronic CTEP (*i.p.*) induces anxiolytic effects in the SIH test. (a) Schematic illustration of the experimental design of acute (1 h following single *i.p.* injection) and sub-chronic (day 16 and day 24 following 8x and 16x *i.p.* injections every 48 h, respectively) CTEP treatment assessed in the SIH test. (b) Acute CTEP (1 h after single *i.p.* injection) was dose-dependently active in reducing the SIH response compared to VEH. (c) Sub-chronic CTEP injected *i.p.* every 48 h was dose-dependently active in reducing the SIH response compared to VEH on day 16 and day 24. White bar, VEH; light grey bar, CTEP 0.1 mg/kg; dark grey bar, CTEP 0.5 mg/kg; black bar, CTEP 2 mg/kg. Data represent mean + S.E.M. * $p \leq 0.05$, ** $p \leq 0.01$ *vs.* respective VEH group. n = 6-7 for acute and n = 18-19 for sub-chronic treatment per group; one-way ANOVA followed by LSD *post hoc* analysis (b) or two-way ANOVA followed by Bonferroni *post hoc* analysis (c). Adapted from Peterlik et al. (2016b).

Discussion

The Discussion section includes chapters taken and adapted from different (joint) first author publications and submitted first author manuscripts:

1.) **Peterlik, D.**, Flor, P.J., Uschold-Schmidt, N., 2016a. The Emerging Role of Metabotropic Glutamate Receptors in the Pathophysiology of Chronic Stress-Related Disorders. *Curr. Neuropharmacol.,* 14(5), 514–39. (*Curr Neuropharmacol* permits the author to include published journal articles in full or in part in the author's dissertation)

2.) Gee, C.E.*, **Peterlik, D.***, Neuhäuser, C., Bouhelal, R., Kaupmann, K., Laue, G., Uschold-Schmidt, N., Feuerbach, D., Zimmermann, K., Ofner, S., Cryan, J.F., van der Putten, H., Fendt, M., Vranesic, I., Glatthar, R., Flor, P.J., 2014. Blocking metabotropic glutamate receptor subtype 7 (mGlu7) via the Venus flytrap domain (VFTD) inhibits amygdala plasticity, stress, and anxiety-related behavior. *J. Biol. Chem.* 289, 10975–87. doi:10.1074/jbc.M113.542654.* **Both authors contributed equally to this work.** (*J. Biol. Chem.* permits the author to include published journal articles in full or in part in the author's dissertation)

3.) **Peterlik, D.**, Stangl, C., Bauer, A., Bludau, A., Keller, J., Grabski, D., Killian, T., Schmidt, D., Zajicek, F., Jaeschke, G., Lindemann, L., Reber, S.O., Flor, P.J., Uschold-Schmidt, N., 2016b. Blocking Metabotropic Glutamate Receptor Subtype 5 Relieves Maladaptive Stress Consequences Induced by Chronic Male Subordination. *Brain. Behav. Immun.*, doi:10.1016/j.bbi.2016.08.007, *in press.* (*Brain. Behav. Immun.* permits the author to include published journal articles in full or in part in the author's dissertation)

4.) **Peterlik, D.**, Stangl, C., Bludau, A., Grabski, D., Strasser, R., Schmidt, D., Flor, P.J., Uschold-Schmidt, N., 2016c. Relief from detrimental consequences of chronic psychosocial stress in mice deficient for the metabotropic glutamate receptor subtype 7. *Neuropharmacology*, doi:10.1016/j.neuropharm.2016.04.036, *in press.* (*Neuropharmacology* permits the author to include published journal articles in full or in part in the author's dissertation)

Peterlik D. is responsible for design and performance of the experiments, data collection, analysis and interpretation and writing of the first drafts of the manuscripts.

1. Acute pharmacological blockade of mGlu7 inhibits depressive-, anxiety-like and fear-related behavior

Gee et al. (2014) recently identified and thoroughly characterized XAP044, the first mGlu7-selective full antagonist that blocks the receptor's signaling pathways via binding to the large extracellular VFTD region. Thus, XAP044 presumably acts via a novel site compared to known selective allosteric ligands that were often associated with off-target effects. XAP044 was furthermore shown to selectively block LTP in the lateral amygdala in an mGlu7-dependent manner, *i.e.* it blocked LTP only in WT but not mGlu7-KO mice. In support of the literature, this further substantiates a role for mGlu7 in the cellular physiology of the fear and emotion circuitry. In addition, XAP044 treatment reduced in a dose-dependent manner the stress-induced rise in rectal body temperature in the mouse SIH test without affecting basal body temperature, a finding that gave the first hint of XAP044's systemic *in vivo* activity.

It was now an essential part of the present PhD thesis to further characterize XAP044 *in vivo* in a battery of behavioral tests including innate anxiety, depression and conditioned fear tests in mice. These experiments should a.) add further knowledge to and support studies that have investigated mGlu7's role in emotional behavior suggesting receptor blockade as a promising pharmacotherapeutic approach and b.) proof and specify systemic *in vivo* activity of mGlu7 receptor blockade with a compound that binds via a new mechanism, *i.e.* to the receptor's VFTD.

Systemic application of XAP044 in the present study slightly reduced immobility in the TST and an increased ratio of open/total arm entries in the EPM test (Figures 4, 5), indicative of decreased depression- and anxiety-related behavior, respectively. XAP044 furthermore resulted in less freezing during the acquisition session of Pavlovian fear conditioning (Figures 6). All these findings are well consistent with published data on the behavior of mGlu7-deficient mice and with mGlu7 siRNA-mediated knockdown studies using several paradigms to assess anxiety-, depression- and fear-related behavior (Callaerts-Vegh et al., 2006; Cryan et al., 2003; Dobi et al., 2013; Fendt et al., 2013, 2008; Goddyn et al., 2008; Mitsukawa et al., 2006; O'Connor et al., 2013b; Stachowicz et al., 2008). Interestingly, the effects of XAP044 are also, at least in part, similar to those previously reported for the allosteric agonist AMN082 (Palucha et al., 2007; Stachowicz et al., 2008). However, as discussed above, this apparent discrepancy can potentially be explained by the rapid AMN082-induced mGlu7 internalization or effects of active

metabolites (Pelkey et al., 2007; Sukoff Rizzo et al., 2011). It is worth noting that despite widespread efficacy of XAP044 in tests of anxiety and stress, the magnitudes of such effects were less than those of either benzodiazepines, the mGlu5 antagonist MPEP, or mGlu7 deficient mice. This difference in efficacy might be explained by the low concentration of free compared to total XAP044 in brain and plasma (see pharmacokinetic studies reported in Gee et al. (2014)), a drawback of XAP044 that can likely be overcome with future chemical derivatives. Nonetheless, the present results with XAP044 further substantiate an important role for mGlu7 in the behavioral physiology of acute stress, fear, and anxiety.

In conclusion, Gee et al. (2014) have identified XAP044 as an mGlu7-selective antagonist that acts via binding to the VFTD of this class C GPCR. The present *in vivo* findings indicate that XAP044's novel molecular mode of pharmacological blockade carries significant application potential for the treatment of stress-related pathologies. Moreover, modeling the mechanism of binding of XAP044 within the VFTD of mGlu7, followed by computer-assisted drug design, could possibly facilitate future drug development.

2. Chronic psychosocial stress in mice induces changes of specific mGlu receptors in the mouse brain

Despite well-established links between acute stress, fear and depression-related behaviors and specific mGlu receptor subtypes (Kew and Kemp, 2005; Swanson et al., 2005), only little is known about their role(s) in chronic psychosocial stress in rodents and even much less in humans. Therefore, it was part of the present PhD thesis to investigate the molecular changes that occur within the brain mGlu receptor system with a focus on mRNA expression of selected mGlu receptor subtypes in response to chronic psychosocial stress. To this end, the CSC model was used, a valuable animal model whose diverse effects on behavioral, physiological and immunological parameters are well characterized (Füchsl et al., 2014; Peterlik et al., 2016a; Peters et al., 2012; Reber and Neumann, 2008; Reber et al., 2007; Schmidt et al., 2010; Uschold-Schmidt et al., 2013, 2012; Veenema et al., 2008). Given the crucial involvement of in particular mGlu5 and mGlu7 subtypes in the regulation of fear and acute stress and anxiety (see Introduction), robust CSC-induced alterations in the mRNA expression of these two receptor subtypes were expected to occur in brain regions relevant for the regulation of emotion and stress-related behaviors, namely the prefrontal cortex, the hypothalamus and the hippocampus.

A trend towards a CSC-induced increase of mGlu5 mRNA levels was detected specifically in the hypothalamus (Peterlik et al., 2016b). In order to assess whether these changes also reflect changes at the protein level, mGlu5 protein binding sites were measured using the [^{3}H]ABP688 radioligand binding assay (performed by F. Hoffmann-La Roche, Basel, Switzerland). Interestingly, mGlu5 protein levels were specifically increased in the hippocampus of CSC compared to SHC mice (Figure 23). Together, these findings suggest a prominent and region-specific activation of the mGlu5 receptor system during chronic psychosocial stress in the hippocampus.

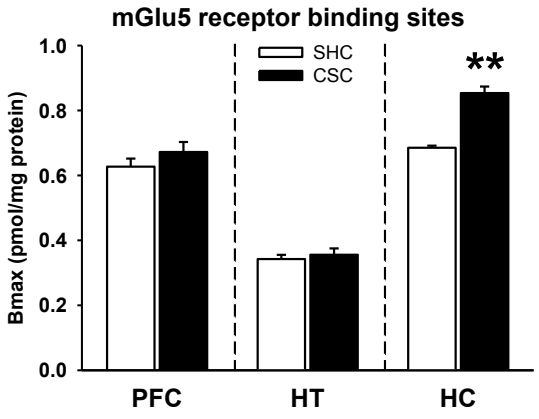


Figure 23. Selective changes in brain mGlu5 receptor binding sites in response to 19 days of CSC. Saturation analysis using the highly mGlu5-selective radioligand [^{3}H]-ABP688 (performed by F. Hoffmann-La Roche, Basel, Switzerland) revealed significantly increased mGlu5 receptor binding sites (B_{max}) in HC after 19 days of CSC exposure, whereas mGlu5 receptor binding sites in the PFC and HT remained unaffected. White bar, SHC (n = 3); black bar, CSC (n = 3). Data represent mean + S.E.M. **$p \leq$ 0.01 *vs.* respective SHC mice, Student's t-test; PFC, prefrontal cortex; HT, hypothalamus; HC, hippocampus. Adapted from Peterlik et al. (2016b).

This finding is in line with previous reports on alterations of mGlu5 protein expression following chronic stressor exposure. Employing the CMS model, Wierońska et al. (2001) showed changes in hippocampal mGlu5 receptor protein expression with an increase in the CA1 and a decrease in the CA3 region, paralleled by an increase in depressive-like behavior. In contrast, mice exposed to CSC typically show an increased anxiety-related behavior without changes of depressive-like behavior (Slattery et al., 2012). Thus, different qualities of chronic stressors (*i.e.* a continuous psychosocial stressor with only repeated physical interaction *vs.* continuous exposure to a combination of physical and psychological stress) may have different effects on brain mGlu5 expression and/or signaling. Further supporting the functional relevance of hippocampal mGlu5 in stress physiology, Yim et al. (2012) revealed in a recent study a link between hippocampal mGlu5 and behavioral coping strategy in response to inescapable and unpredictable footshock stress. They could demonstrate that mGlu5 protein expression was increased in rats that showed helpless behavior and a lack of adaptation in a novel environment, whereas it was decreased in those that did not. These results suggested that hippocampal mGlu5 has a pivotal role in the controllability-based coping strategy. Translated to the CSC-induced changes found in the present study, *i.e.* increase in hippocampal mGlu5 protein expression and increase in anxiety-related behavior, this would also suggest mGlu5 to be critically involved in the development of the chronic stress-related anxiety-prone phenotype found in CSC mice. At least, the present data suggest an increased mGlu5 activity in stress-sensitive brain regions involved in the regulation of behavior and HPA axis functionality following CSC exposure.

With respect to the mGlu7 receptor subtype, downregulated mGlu7 transcript levels were found specifically in the prefrontal cortex. Of course, these transcript data may not necessarily reflect functional relevance of mGlu7 in chronic psychosocial stress-induced pathology. However, due to the poor quality of antibodies raised against the mGlu7 protein, measuring selective mGlu7 protein levels is still a challenge to many different laboratories worldwide (O'Connor et al., 2013; O'Connor et al., 2013a). Presumed that also mGlu7 protein levels are reduced specifically in the prefrontal cortex, the following speculation can be made: The specific reduction of the primarily presynaptically located mGlu7 in the prefrontal cortex might lead to a reduction in the negative feedback at the synapses of glutamatergic neurons, which in turn could result in excessive L-glutamate release. The consequent hyperexcitability in the prefrontal cortex might, at least in part, contribute to the CSC-induced anxiety-prone phenotype (Bi et al., 2013; Bruening et al., 2006). In support, anxiety-inducing stimuli have been reported to be associated with increased neuronal activation in prefrontal cortical areas (Singewald et al., 2003). Furthermore, the fact that mGlu7 is also presynaptically located on inhibitory GABAergic neurons (Kinoshita et al., 1998; Somogyi et al., 2003), where the receptor negatively regulates inhibitory neurotransmitter release (Klar et al., 2015; Schrader and Tasker, 1997), adds further complexity. Given that anxiety disorders are often viewed as a result of an imbalance between L-glutamate and GABA (Wierońska and Pilc, 2009), mGlu7 is in a prime position to act as a key regulator of this imbalance in such disorders. In view of this, the L-glutamate/GABA balance might be disturbed due to the CSC-induced dysregulation of mGlu7 in the prefrontal cortex.

Interestingly, no changes of mGlu2 and mGlu3 mRNA levels were found in response to CSC in each of the three brain regions, possibly suggesting that group II mGlu receptors may play a less prominent role in CSC-induced pathophysiology. As it would have gone beyond the scope of the present thesis, the determination of a possible functional relevance of group II mGlu receptors in CSC-induced maladaptations may be part of future investigation.

Overall, the present findings represent very early evidence towards a role of specific mGlu receptor subtypes in chronic psychosocial stress-induced pathophysiology. At least, the data suggest that there are dysregulated activities and thus possibly controlling roles of the mGlu5 and mGlu7 receptor subtypes in the hippocampus and the prefrontal cortex, respectively, two brain regions involved in the regulation of behavior and HPA axis functionality. Together, these findings may indicate that these receptor subtypes should be

pursued as further research topics in chronic stress-induced conditions. The functional relevance of these receptor subtypes can be further addressed by the investigation of their associated intracellular proteins like Homer1, Calmodulin, Norbin, Tamalin and protein interacting with PKC alpha (PICK1), which have important regulatory functions (Enz, 2007; Niswender and Conn, 2010).

3. Chronic psychosocial stress-protective phenotype of mGlu7 KO mice

The present study provides first evidence for the involvement of mGlu7 in mediating behavioral, physiological and immunological consequences of chronic psychosocial stress in mice using CSC as a male subordination paradigm. Genetic ablation of mGlu7 relieved multiple chronic stress-induced maladaptations: in addition to protection against the CSC-induced anxiety-prone phenotype, mGlu7 deficient mice were also less vulnerable to CSC with respect to reliable physiological and immunological consequences such as HPA axis dysfunction and colonic inflammation. Together, these findings evidence the involvement of the mGlu7 subtype in a wide range of affective and somatic alterations that emerge upon chronic psychosocial stressor exposure and suggest that mGlu7 pharmacological blockers could be a relevant option for the treatment of chronic stress-related emotional and somatic dysfunctions in man.

Genetic ablation of mGlu7 has been demonstrated to be associated with several selective changes in molecular targets that participate in the acute stress response and in psychopathological states (Cryan et al., 2003; Mitsukawa et al., 2006). In particular, increased GR levels in the hippocampus combined with an increased GR-mediated feedback suppression of the HPA axis, as well as elevated hippocampal BDNF and 5-HT_{1A} receptor levels in mGlu7 KO mice suggest selective dysregulation of stress response integration opposite to that found in humans suffering from chronic stress-related pathologies (Holsboer and Barden, 1996; Webster et al., 2002). Moreover, these changes correlate well with the previously identified acute antistress, antidepressant- and anxiolytic-like phenotype in mice with genetic mGlu7 ablation (Callaerts-Vegh et al., 2006; Cryan et al., 2003), with pharmacological mGlu7 blockade (Gee et al., 2014; Kalinichev et al., 2013) as well as with siRNA-mediated knockdown of mGlu7 (Fendt et al., 2008; O'Connor et al., 2013b).

The present study demonstrated a stress-protective phenotype in mice with genetic ablation of mGlu7 in the context of chronic psychosocial stress. First of all, in support of previous findings (Füchsl et al., 2014; Peters et al., 2013; Reber and Neumann, 2008; Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012), CSC exposure resulted in increased anxiety-related behavior in mice of the WT group tested on the EPM. However, this CSC-induced anxiety-prone phenotype was not present in mGlu7 KO mice, suggesting a stress-protective effect of genetic mGlu7 ablation with respect to the chronic stress-induced increase in innate anxiety. Of note, this lack of CSC-induced anxiety observed in

mGlu7 KO mice was not due to changes in locomotor activity. This is an important consideration as mGlu7 genetic ablation has been associated with an increased susceptibility to sensory stimulus-induced seizures (Sansig et al., 2001). Interestingly, the previously described anxiolytic-like effect of genetic mGlu7 ablation could not be reproduced in the present study, as innate anxiety levels on the EPM were comparable between mGlu7 KO and WT mice in the SHC group. However, the findings are still in line with the study of Cryan et al. (2003) who showed a significant anxiolytic-like effect of genetic ablation of mGlu7 in mice using the light-dark box (LDB) test, but couldn't easily confirm this effect with the EPM test. Thus, it seems that the detection of the anxiolytic-like phenotype of mGlu7 KO mice depends on the exact behavioral test employed.

A stress-protective effect of genetic mGlu7 ablation was also found regarding HPA axis functionality. In mGlu7 KO mice, no CSC-induced increase in absolute pituitary weight and associated basal morning plasma ACTH levels were detected, which are reliable indicators for chronic psychosocial stress induced by CSC exposure (Füchsl et al., 2013; Langgartner et al., 2015) and could be confirmed in the WT group of the current study. Furthermore, mGlu7 KO, in contrast to WT mice, did not develop the previously shown CSC-induced increase in absolute adrenal weight accompanied by a reduction in adrenal ACTH responsiveness *in vitro* (Uschold-Schmidt *et al*, 2012). These findings suggest that the HPA axis of mGlu7-deficient mice is less vulnerable to chronic psychosocial stressor exposure.

Analysis of immunological parameters further supported a stress-protective phenotype in mGlu7 KO mice. First, the previously described CSC-induced splenomegaly (Füchsl et al., 2014), which was confirmed in the WT group of the present study, appears completely abolished in mGlu7 KO mice. Given the suggested role for mGlu7 in the regulation of gastrointestinal function (Julio-Pieper et al., 2010), it was also of great interest to assess whether genetic ablation of mGlu7 is protective against CSC-induced development of spontaneous colitis. Indeed, an increased IFN-γ secretion from isolated and anti-CD3/anti-CD28-stimulated mesLNC and an increased histological damage of colonic tissue, indicating development of mild colonic inflammation (Füchsl et al., 2014; Reber et al., 2007), were detected in CSC mice of the WT group but not in mGlu7 KO mice.

Together, the present findings indicate that mice with genetic ablation of mGlu7 are less vulnerable to various behavioral, physiological as well as immunological consequences of chronic psychosocial stressor exposure. The mechanisms underlying these stress-protective effects of genetic mGlu7 ablation might be explained by the role of mGlu7 in regulating

neurotransmitter release: The presynaptically located mGlu7 is thought to act as an auto- and heteroreceptor on glutamatergic and GABAergic neurons in key limbic brain regions involved in the regulation of the stress response. Thus, a complete lack of mGlu7 might induce region-specific changes in excitatory and inhibitory neurotransmitter release through altered negative feedback regulation eventually leading to a stress-resilient phenotype. Moreover, downstream effects on other neurotransmitter systems such as the dopamine and serotonin (5-HT) systems (Müller and Schwarz, 2007) that are known to alter e.g. anxiety-related behavior (Lucki, 1998) and/or altered neurodevelopmental mechanisms due to mGlu7 deficiency may also account for stress resilience observed in mGlu7 KO mice.

The finding of above demonstrated that 19 days of CSC lead to a decrease of mGlu7 mRNA levels in the PFC in WT mice is possibly well consistent with the physiological/behavioral observations: A downregulation of mGlu7 and hence an L-glutamate/GABA imbalance in a specific brain region, namely the PFC, may account for an increase in anxiety-related behavior following CSC. This would be in line with studies suggesting that hyperexcitability in the PFC contributes to an anxiety-prone phenotype (Bi et al., 2013; Bruening et al., 2006; Singewald et al., 2003). Conversely, altered inhibition and/or excitation in several brain regions at the same time, due to the complete lack of mGlu7, may compensate CSC-induced changes in neurotransmitter release via complex circuitry adaptations and thereby account for the stress-protective phenotype of mGlu7 KO mice. In addition, mGlu7 is known to be expressed in endocrine organs like adrenal glands and also in the gastrointestinal tract (Julio-Pieper et al., 2011). Therefore, peripheral mechanisms may also contribute to the stress-protective phenotype in mice lacking mGlu7, at least with respect to immunological/inflammatory parameters. In support of this, glutamatergic activation of colonic mGlu7 was recently associated with the pathophysiology of secretory disorders (Julio-Pieper et al., 2010).

In conclusion, the present results demonstrate a stress-protective phenotype of mice lacking mGlu7 in the context of chronic psychosocial stress, and thus clearly indicate a role for mGlu7 in mediating affective as well as somatic consequences induced by chronic psychosocial stressor exposure.

4. mGlu5 functional blockade relieves maladaptive stress consequences induced by CSC

The present thesis could reveal that also the mGlu5 receptor is crucially involved in mediating physiological, immunological, and behavioral consequences of chronic psychosocial stress in mice using CSC as a male subordination paradigm. First, it was shown that genetic ablation of mGlu5 decreases the vulnerability to selected CSC-induced alterations. Second, it was demonstrated that chronic treatment of WT mice with CTEP, a systemically active mGlu5 NAM, is in a dose-dependent manner protective against the physiological and immunological consequences of CSC. These findings suggest for mGlu5 to be a valuable and novel target for the treatment of chronic stress-induced pathologies in man.

4.1 Stress-protective phenotype in mice lacking mGlu5

In line with previous reports (Füchsl et al., 2014; Reber et al., 2007; Uschold-Schmidt et al., 2012) and the findings described above, WT littermates of mGlu5 KO mice showed a CSC-induced increase in adrenal weight. Moreover, CSC compared to SHC WT mice showed a trend towards an increased number of isolated viable mesLNC as well as an increased secretion of the pro-inflammatory cytokine IFN-γ from these cells following *in vitro* anti-CD3/anti-CD28 stimulation. Both findings are in agreement with previous reports and indicative for the development of spontaneous colonic inflammation (Langgartner et al., 2015; Reber et al., 2007). However, these reliable physiological and immunological CSC-induced alterations were not present in mice lacking mGlu5. CSC exposure also reliably results in an increased anxiety-related behavior, recorded e.g. in the EPM, in the elevated platform (EPF), in the LDB, and in the open-field tests (Reber and Neumann, 2008; Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012). In the present study, CSC versus SHC mice were exposed to the SIH test, an animal model addressing the physiological component of anxiety and sensitive to anxiolytic drugs (Adriaan Bouwknecht et al., 2007; Borsini et al., 1989). Interestingly, it could be shown for the first time that 19 days of CSC, in addition to innate anxiety, also increases physiological anxiety levels, indicated by a CSC-induced increased hyperthermic response in WT mice in the SIH test. Again, this chronic stress effect was absent in mGlu5 KO mice, suggesting a stress-protective effect of genetic mGlu5 ablation also with respect to physiological anxiety. Unfortunately, the previously reported anxiolytic-like effect of

mGlu5 receptor depletion in the SIH test (J Brodkin et al., 2002) was not reproduced as a lower SIH response was not found in KO compared to WT mice within the SHC group. This might be due to genetic differences between mouse strains (B6;129-*Gprc1e*^{tm1Rod} *vs.* C57/BL6 mice) and differences in the experimental setup (measurement of T2 30 min *vs.* 15 min after T1 in the SIH test).

Taken together, the present findings suggest that mice deficient for mGlu5 are protected from important CSC-induced physiological, immunological, and behavioral (at least with respect to physiological anxiety) alterations, and thus, seem to be resilient to a variety of maladaptive consequences of chronic psychosocial stressor exposure.

4.2 Stress-protective effects of chronic CTEP treatment

Given the promising stress-protective phenotype of mGlu5 KO mice, a further step was analyze the effects of pharmacological mGlu5 inhibition in mice exposed to CSC. The typical CSC-affected parameters were assessed in even more detail after chronic (during CSC) administration of the mGlu5 NAM CTEP. Different doses of CTEP (0.05, 0.5, and 2 mg/kg/day) were used to evaluate a possible dose-dependency. These doses were chosen according to previously published data showing sufficient plasma and brain exposure and sustained receptor occupancy as well as activity in animal models of anxiety (SIH, Vogel conflict test; Lindemann et al. (2011)), with minimal effective oral doses between 0.1 and 0.3 mg/kg. Of note, the *in vivo* experiments reported by Lindemann et al. (2011) did not reveal any unspecific effects of CTEP like sedation or general changes in overall health, body temperature, and body weight, in a dose range of up to 2 mg/kg.

In a pilot study, it was imperative to provide experimental evidence for the compatibility of chronic vehicle administration via micro-osmotic pumps with typical CSC-related parameters. Importantly, no effects of either vehicle or CTEP treatment (2 mg/kg/day) were found in comparison to chronic saline administration (Figure 14) in unstressed mice. This was in line with the study conducted by Peters et al. (2014), where they revealed no confounding influence of either the surgical procedure or the chronic *i.c.v.* administration of vehicle (in that case Ringer's solution) via micro-osmotic pumps.

In line with previous studies using untreated mice (Reber et al., 2007; Veenema et al., 2008), vehicle-treated CSC *vs.* SHC mice of the present study showed a reduced body weight gain. This effect was absent in chronically stressed mice treated with CTEP at all doses. A stress-protective effect of CTEP is further supported by findings assessing HPA

axis functionality. Absolute pituitary weight and associated basal morning plasma ACTH levels, typically increased in mice exposed to CSC (Füchsl et al., 2013; Langgartner et al., 2015), were also increased in CSC *vs.* SHC mice of the vehicle group, but not in mice treated with the two higher doses of CTEP. Furthermore, in vehicle-treated mice, CSC exposure resulted in enlarged adrenals accompanied by a reduced adrenal CORT response to ACTH *in vitro*, fully in accordance with previous reports (Uschold-Schmidt *et al*, 2012). CTEP dose-dependently prevented these CSC-induced changes, together with pituitary and plasma ACTH findings indicating that the HPA axis is generally less vulnerable to chronic psychosocial stressor exposure in mice with pharmacological mGlu5 inhibition. These findings seem to be in contrast to the study by Wagner et al. (2014), showing no beneficial effects of oral CTEP (2 mg/kg; every 48 h) on the dysregulated HPA axis activity in mice exposed to CSDS. These discrepancies might be due to differences in the chronic stress paradigms likely resulting in different changes in the mGlu5 system in the brain and/or periphery and, thus, different CTEP-mediated effects. Moreover, it cannot be excluded that the stress induced by repeated oral applications of CTEP interfered with possible beneficial effects of mGlu5 blockade on the CSDS-induced dysregulation of HPA axis activity.

The assessed immunological parameters suggest that pharmacological blockade of mGlu5 by CTEP attenuates or even abolishes the vulnerability also to CSC-induced immunological alterations. In accordance with previous studies (Füchsl et al., 2014; Reber et al., 2007), CSC mice of the vehicle group showed a decrease in absolute thymus weight and an increase in absolute spleen weight. These effects were dose-dependently reduced with CTEP. Anti-stress effects of mGlu5 blockade were also found with respect to colonic inflammation. Although an increased number of isolated viable mesLNC found in vehicle-treated CSC mice was still detectable in the groups with the two lower CTEP doses, it was absent in mice treated with 2 mg/kg/day of CTEP. Furthermore, CTEP in contrast to vehicle treatment abolished the CSC-induced increase in *in vitro* IFN-γ secretion from isolated viable mesLNC and, at least at a dose of 2 mg/kg/day, in the histological damage score of the colon. Thus, CTEP is protective against two reliable CSC consequences indicative for colonic inflammation (Reber et al., 2011, 2007).

In line with previous data (Reber and Neumann, 2008; Reber et al., 2007) and the above mentioned data from WT mice, CSC exposure of vehicle-treated mice in the present study increased innate and physiological anxiety measured on the EPM and in the SIH test, respectively. Unexpectedly, however, CTEP treatment did not normalize the CSC-induced anxiety-prone phenotype, despite the above reported attenuated SIH response of mGlu5

KO CSC mice and the recovery of behavioral alterations by chronic oral CTEP administration in CSDS mice reported in a recent study (Wagner et al., 2014). This raises the suspicion that in the present study the mGlu5 receptor saturation upon chronic CTEP administration via micro-osmotic pumps was not sufficient to induce any behavioral effects – at least on the days of behavioral testing (EPM and SIH tests on days 18 and 19, respectively). Indeed, subsequent drug exposure analysis (performed by F. Hoffmann-La Roche Ltd., Basel, Switzerland) revealed only low plasma CTEP concentrations within a range of approximately 1.0 ng/ml up to 1.5 ng/ml (Figure 24), far below the expected values.

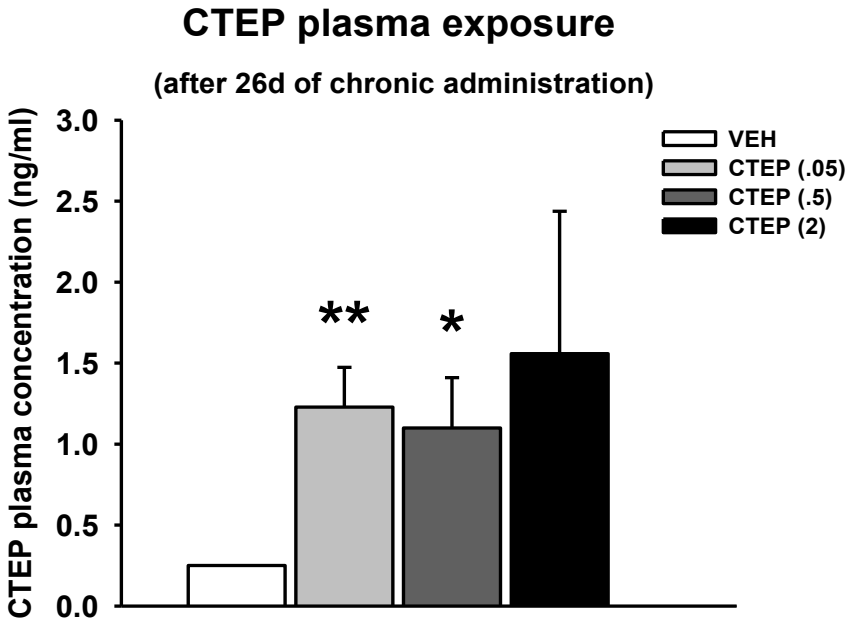


Figure 24. Plasma CTEP exposure after chronic (26 days) drug dosing via micro-osmotic pumps. Plasma samples were collected after 26 days of chronic CTEP (1 and 2 day(s) after SIH and EPM testing, respectively) at doses of 0.05, 0.5 and 2 mg/kg/d. Chronic CTEP administration at the two lower doses resulted in significantly increased CTEP plasma concentrations (ng/ml) compared to VEH (CTEP .05: p = 0.001 and CTEP .5: p = 0.016). Unexpectedly, chronic application of CTEP at a dose of 2 mg/kg/d did not result in significantly increased CTEP plasma concentrations compared to VEH (CTEP 2: p = 0.159)**.** White bar, VEH; light grey bar, CTEP 0.05 mg/kg; dark grey bar, CTEP 0.5 mg/kg; black bar, CTEP 2 mg/kg. As drug exposure levels were not different between the SHC and CSC group (raw data not shown), data of SHC and CSC mice was averaged per data set. CTEP plasma concentration values obtained from VEH-treated animals were ≤ 0.25 ng/ml and therefore averaged to 0.25 ng/ml. Data represent mean + S.E.M. * $p \leq 0.05$, ** $p \leq 0.01$ *vs.* respective VEH group. n = 8 per group (n = 4 SHC and n = 4 CSC mice); independent Student's t-test.

In a recent study, Michalon et al. (2012) reported a minimal CTEP plasma concentration of 98 ± 14 ng/ml following two weeks of chronic oral dosing at 2 mg/kg every 48 h, corresponding to an estimated mean receptor occupancy level of 81%.

Of course, by comparing the study conducted by Michalon et al. (2012) with the present study, possible differences in CTEP plasma exposure due to different route and duration of drug administration seem very likely. However, the low plasma CTEP levels on day 20 of CSC yielded in the present study strongly suggest only inadequate mGlu5 receptor saturation insufficient to induce any beneficial effects on behavior, but apparently sufficient to prevent CSC-induced physiological and immunological alterations. It could also be (which is even quite likely), that CTEP plasma exposure was actually higher at an earlier time point during CSC exposure when physiological (Uschold-Schmidt et al., 2012) and immunological changes (Reber et al., 2007) start to develop, but declined due to improper functioning of micro-osmotic pumps and eventually resulted in a lack of behavioral effects. In view of this, it will be part of future experiments to determine the actual levels of mGlu5 receptor saturation at various earlier time points during chronic administration of CTEP via micro-osmotic pumps. If the assessed CTEP plasma exposure rate will be found on a low level also at earlier time points, the present findings suggest that CSC-induced physiological and immunological alterations are more sensitive to mGlu5 inhibition compared to the behavioral consequences of CSC. Together with the stress-protective phenotype of mGlu5 KO CSC mice seen in the SIH test, this also suggests that a higher dose of CTEP and subsequently a higher receptor saturation in relevant brain regions might correct the CSC-induced anxiety-prone phenotype. Unfortunately, attempts to use a higher CTEP dose (6 mg/kg/day; data not shown) failed due to plugging of the pumps and resultant loss of pump functioning and, thus, one can only speculate about potential results of experiments using higher drug doses. However, if this hypothesis proves correct, this would make CTEP a powerful tool for the independent treatment of stress-induced somatic and affective diseases in man depending on the dose applied.

A further explanation for the missing behavioral effects in the present study could be that chronic CTEP administration caused an upregulation of mGlu5 receptor expression accompanied by functional changes in specific brain regions, which possibly compensated for any potential effects on behavior. Indeed, antagonist-induced upregulation and concomitant functional supersensitivity has been reported for 5-HT_{3A}-, opioid- and NMDA receptors (Follesa and Ticku, 1996; Morton et al., 2015; Yoburn, 1988; Yoburn et al., 1994). Therefore, and especially in view of the close physical and functional interaction

between NMDA and mGlu5 receptors, an antagonist-induced upregulation of mGlu5 upon chronic CTEP dosing may have occurred and confounded any behavioral effects in the present study. However, this is only part of speculation at the moment and has to be investigated in future projects.

Of note, the proposed anxiolytic-like efficacy of CTEP could be verified in a further experiment with naïve mice *i.p.*-administered with CTEP at doses of 0.5 and 2 mg/kg in an acute and of 2 mg/kg/day in a sub-chronic design. Therefore, the anti-stress/anxiolytic-like effect of CTEP reported in the literature (Lindemann et al., 2011) could be confirmed and extended (with respect to the route and the duration of administration). Future experiments will be needed to resolve the underpinnings of the discrepancies in the behavioral effects of CTEP obtained in the present study (chronic *s.c. vs.* sub-chronic *i.p.* treatment).

With respect to the molecular mechanisms underlying the beneficial anti-stress effects of mGlu5 receptor blockade, one so far can only speculate based on findings reported in the literature:

First of all, it's interesting to consider that the selective mGlu5 antagonist MPEP has been shown to dose-dependently induce c-Fos neuronal activation in stress-related brain areas including the PVN of the hypothalamus, CeA and BNST (Inta et al., 2012), similar to the activation pattern induced by imipramine (Sumner et al., 2004) and/or diazepam (Salminen et al., 1996) treatment. This would implicate that functional mGlu5 blockade might result in neuronal activation of stress-sensitive brain areas that are also stimulated by antidepressant and anxiolytic drugs. Whether CTEP does the same as MPEP remains to be tested.

Given the synergistic reciprocal interaction between mGlu5 and NMDA receptors, mGlu5 inhibition may reduce glutamatergic transmission at NMDA receptors (O'Leary et al., 2000), thereby shifting the balance towards inhibitory GABAergic neurotransmission in brain regions regulating behavioral and physiological responses to stress (Palucha and Pilc, 2007). On the other hand, similar to the proposed mechanisms underlying the effects of the clinically active CTEP analogue basimglurant (Fuxe and Borroto-Escuela, 2015), CTEP may preferentially target mGlu5 heteroreceptor complexes of cortical limbic GABA interneurons, especially those inhibiting the glutamate projection neurons of the circuits of stress-related mood and emotion pathways. These glutamate neurons may therefore be activated by being set free from GABA inhibition, which in turn restores activity in relevant brain circuits. Moreover, concurrent increased AMPA receptor activation might

involve increased glutamate release (Thomas et al., 2000) as well as increased BDNF levels and release associated with increased TrkB signaling (Chaki et al., 2013; Pilc et al., 2008). Subsequent activation of the PI3-kinase/Akt/mTOR pathway might improve the formation of synaptic proteins and/or the return of stress-induced loss of synaptic function (as briefly discussed by Fuxe and Borroto-Escuela (2015)). In support of this, activation of mTOR signaling has recently been shown to be associated with the antidepressant-like activity of the mGlu5 antagonist MTEP and the NMDA antagonist ketamine (Li et al., 2010; Pałucha-Poniewiera et al., 2014a; Tang et al., 2015). Interestingly, recent studies showed reduced phosphorylation levels of mTOR and its downstream signaling components in the amygdala in rats (Chandran et al., 2013) and the prefrontal cortex in mice (Tang et al., 2015) following CUS exposure. Whether also CSC exposure actually induces dysregulation of mTOR and whether this dysregulation can be corrected by CTEP treatment remains to be assessed in future studies.

Other reports suggest the involvement of the serotonergic system in the action of mGlu5 blockade. The existence of mGlu5-5-$HT_{2A/2C}$ heteroreceptor complexes implicates specific functional interaction between the glutamate and serotonin systems. Stachowicz et al. (2007) supposed that the anxiolytic effect of mGlu5 blockade via MTEP in the Vogel conflict drinking test observed in rats occurs due to an increased release of serotonin in the PFC with subsequent activation of 5-$HT_{2A/2C}$ receptors and, therefore, changes in the balance between the glutamatergic and serotonergic system in specific brain regions. Moreover, it was recently shown that MPEP-induced blockade of mGlu5 exerted anxiolytic-like effects in the novelty-suppressed feeding test in a 5-$HT_{2A/2C}$ receptor-dependent manner (Fukumoto and Chaki, 2015).

Others suggest an interaction with neuropeptides that are crucial for modulating stress responses, such as neuropeptide Y (NPY, Heilig, 2004), in the stress-protective action of mGlu5 blockade. Wierońska et al. (2004) showed that NPY neurons that are highly expressed in the amygdala are critically involved in MPEP's anxiolytic action. The concrete investigation of an involvement of NPY and its receptors in CTEP's stress-protective effects is part of future studies.

Furthermore, as the mGlu5 expression is also abundant in peripheral endocrine organs like the adrenal glands (Pokusa et al., 2014), in the gastrointestinal tract (Julio-Pieper et al., 2011) and in immune cells (Storto et al., 2000), also peripheral mechanism have to be considered to play a role in mGlu5's involvement in chronic psychosocial stress conditions. For example, although a clear functional role is not much explored, Nasser et

al. (2007) found that chemically induced colitis was associated with redistribution of mGlu5 and that a mouse model of spontaneous chronic intestinal inflammation showed dysregulated glial mGlu5 expression in colon myenteric plexus, suggesting involvement of mGlu5 in inflammatory processes.

In conclusion, the present studies employing genetic and pharmacological inhibition of mGlu5 activity suggest a stress-protective effect of mGlu5 functional blockade. Moreover, the results suggest that the stress-protective effects are due to acute mGlu5 inhibition as opposed to potential neurodevelopmental effects of mGlu5 ablation in the KO mice. Together, these findings strongly indicate a role for mGlu5 in mediating at least somatic consequences induced by chronic psychosocial stressor exposure and, thus, substantiate that the approach of mGlu5 receptor blockade represents a very promising strategy towards future treatment of chronic stress-induced pathologies in man.

Future Outlook

Given the promising stress-protective phenotype of mice lacking mGlu7, pharmacological blockade of mGlu7 might represent a relevant option for the treatment of chronic stress-induced affective and somatic maladaptations. Future studies will encompass chronic application of selective mGlu7 antagonists, such as XAP044 (see characterization above) and/or related compounds, at different doses, aiming to reverse CSC-induced emotional and physiological dysfunctions, and thus, extend the findings obtained from mGlu7 deficient mice to pharmacological approaches. Moreover, given that functional expression of mGlu7 in specific brain regions, such as PFC and HT, is expected to be necessary and possibly sufficient to exert regulatory effects on certain consequences of CSC, viral vectors for selective and regions-specific knockdown (or overexpression) of mGlu7 will help to reveal region-specific functional relevance of mGlu7 in mediating CSC-induced alterations. Furthermore, detailed behavioral analysis will be performed to assess whether the animal's stress coping style (active *vs.* passive; which is regulated by the PFC) during CSC exposure is altered in mGlu7 KO compared to WT mice and also in mGlu7 antagonist-treated compared to vehicle-treated mice. In view of the present data, mGlu7 blockade is expected to be associated with rather active stress coping behavior which might contribute to and explain the overall stress-protective phenotype observed in mGlu7 KO mice. The potentially beneficial influence of mGlu7 blockade on CSC-induced basal evening hypocorticism (and subsequent impaired GC signaling), a phenomenon that is also seen in humans suffering from chronic stress (see Introduction), will also be addressed. In addition, future studies will aim at analyzing mGlu7's impact on the development of CSC-induced somatic alterations (HPA axis dysfunction, colonic inflammation and damage, etc.); CSC time-course experiments (10 h, 48 h, 7 d, 19 d) will be conducted to find the earliest point-in-time when stress protection in mGlu7 KO mice emerges. Furthermore, detailed immunohistochemistry for mGlu7 protein distribution needs to be conducted in sections of organs such as the pituitary, adrenal glands and colonic tissue, in order to reveal whether alterations in peripheral mGlu7 might contribute to the development of CSC-induced somatic dysfunctions, or if they are solely centrally mediated.

Genetic and pharmacological mGlu5 inhibition was shown to exert beneficial effects on a broad range of CSC-induced maladaptations. Thus, mice lacking mGlu5 were protected against a variety of CSC-induced alterations, including the newly established CSC-induced increase in SIH response. Future studies will address also behavioral analysis of the stress coping styles of mGlu5 KO mice compared to their WT littermates.

Interestingly, chronic application of CTEP relieved only somatic, but not the typical behavioral alterations induced by CSC exposure. This lack of effect on behavioral aspects might be due to relatively low plasma CTEP levels assessed on day 20 of CSC, strongly suggesting only insufficient mGlu5 receptor saturation. It is quite likely that CTEP plasma exposure was actually higher at earlier time points of CSC exposure. Future experiments will be needed to determine the actual levels of mGlu5 receptor saturation at various earlier time points during chronic administration of CTEP via micro-osmotic pumps and to study its possibly stress-protective effects on CSC-induced increased physiological and innate anxiety (e.g. SIH and EPM test, respectively). Thus, these experiments will reveal whether CTEP (or close analogues such as basimglurant) can be used for the independent treatment of stress-induced somatic and affective pathologies depending on the dose applied.

Given that CSC exposure increased mGlu5 protein expression specifically in the HC, the application of CTEP (and/or related compounds) locally into the HC (or into other stress-sensitive brain regions) in combination with CSC exposure and its functional analysis will help to find out whether mGlu5 in the HC (or in other brain regions) might have functional relevance for any CSC-induced behavioral and/or somatic alterations.

Summary

Etiology and pharmacotherapy of stress-related psychiatric conditions and comorbid somatic pathologies are nowadays areas of high unmet medical need and intense research. It is widely accepted that stressors holding a chronic and psychosocial component represent the most acknowledged risk factor. The L-glutamatergic system represents the primary excitatory neurotransmitter system of the mammalian brain and mGlu receptors acting as important pre- and postsynaptic regulators of neurotransmission provide a mechanism by which fast synaptic responses through iGlu receptors can be fine-tuned. During the last decades, research on mGlu receptors advanced remarkably and much attention was given to the mGlu5 and mGlu7 subtypes in acute stress, fear and depression-related behavior and physiology (see Introduction). As the most widely distributed throughout the mammalian brain, the presynaptic mGlu7 receptor is an important regulator of glutamatergic function and postulated to be critical for both normal CNS functioning and a range of stress-related disorders. Although genetic and pharmacological approaches have helped to understand mGlu7's function in a host of behavioral and physiological processes, available allosteric ligands have often yielded disparate results despite displaying similar pharmacological properties *in vitro*. The recent discovery of XAP044 raised great hope to resolve this discrepancy. XAP044 was characterized as the first mGlu7-selective full antagonist that blocks the receptor's signaling pathways by binding to its large VFTD, but not to allosteric sites within the transmembrane domain. Thus, XAP044 presumably binds via a novel mechanism compared to that from known selective ligands. It was part of the present PhD thesis to characterize this novel compound XAP044 *in vivo* in a broad battery of acute stress tests for depression, fear and anxiety in mice. It was shown that XAP044 is systemically active and demonstrates a wide spectrum of anti-stress, antidepressant and anxiolytic-like efficacy, strongly supporting pharmacological blockade of mGlu7 as a promising mode of action for future treatment of stress-related disorders of emotion in man.

Also the mGlu5 subtype has become a recent focus for drug discovery efforts (see Introduction). Due to its physical and functional association with the postsynaptic NMDA receptor the mGlu5 subtype is considered as a good target to modulate NMDA receptor function. This is an important consideration as, for instance, the NMDA receptor antagonist ketamine – despite showing rapid and sustained efficacy in clinical depression trials – is also associated with severe cognition-altering and dissociative effects. To date, several mGlu5 NAMs have been reported to have therapeutic potential for numerous conditions including clinical depression and anxiety disorders. A recent study revealed the

mGlu5 NAM basimglurant as a promising antidepressant drug with the potential to also alleviate comorbidities such as anxiety and pain.

Despite the overall well-established link between mGlu5 and mGlu7 and acute stress-related behavior and physiology, the roles these receptors play in chronic stress-related conditions is only little explored. The CSC paradigm represents a powerful animal model as it displays harmful behavioral, physiological and immunological changes induced by chronic psychosocial stress. Those consequences are relevant for the development of psychiatric, somatic and/or gastrointestinal disorders in humans and the question whether mGlu5 and mGlu7 have the potential to exert control on these pathological consequences is of great interest, and it may suggest future therapeutic strategies for the treatment of chronic stress-related disorders in humans, *i.e.* a wide clinical application spectrum. In a first step of the present PhD thesis, the molecular changes were assessed that occur within the mGlu receptor system in response to CSC exposure. Here, an increase by trend of mGlu5 mRNA was found in the hypothalamus. Additional saturation binding analysis revealed increased mGlu5 protein binding specifically in the hippocampus. Furthermore, robust downregulation of mGlu7 mRNA was found specifically in the PFC. In contrast, mGlu2 and mGlu3 were not dysregulated upon CSC exposure. Taken together, the present results indicate specific CSC-induced alterations of mGlu5 and mGlu7 expression in stress-sensitive brain regions involved in the regulation of behavior and HPA axis functionality, and thus provide early evidence towards a role of specific mGlu receptor subtypes in chronic psychosocial stress-induced pathophysiology.

In a next step, the influence of genetic ablation of mGlu7 on behavioral, physiological and immunological consequences of CSC was analyzed to reveal the potential role of the endogenous mGlu7 receptor during chronic psychosocial stress. Indeed, genetic ablation of mGlu7 relieved multiple CSC induced alterations; mGlu7 deficient mice were protected against the CSC-induced anxiety-prone phenotype as well as against several CSC-induced physiological and immunological consequences such as HPA axis dysfunction and colonic inflammation, respectively. These findings point to a distinct role of mGlu7 in modulating a wide range of affective and somatic alteration that occur upon CSC exposure. Moreover, the stress-protective phenotype of genetic mGlu7 ablation suggests mGlu7 pharmacological blockade to be a possible treatment strategy for chronic stress-related emotional and somatic conditions in man.

In the last part of the present thesis, the potentially beneficial role of genetic and pharmacological mGlu5 inhibition on CSC-induced alterations (the same broad range as

presented above) was analyzed. Interestingly, also mGlu5 deficient mice were protected against a variety of CSC-induced physiological and behavioral changes, including the newly established CSC-induced increase in SIH response. Moreover, the effects of the mGlu5 NAM CTEP, a close analogue to the clinically active drug basimglurant with long half-life in rodents, were studied on a wider range of CSC-affected parameters. Here, CTEP relieved in a dose-dependent manner various CSC-induced consequences such as HPA axis dysfunction, immunological alterations and colonic inflammation, suggesting that mGlu5 is a relevant mediator for a wide range of alterations induced by chronic psychosocial stress and a potentially valuable drug target for the treatment of stress-related somatic pathologies.

In conclusion, the present PhD thesis provides clear evidence for the importance of especially the mGlu5 and mGlu7 subtypes in the regulation of acute and chronic stress-related behavior and physiology, lending further support towards future development of mGlu5- and mGlu7-selective antagonists and their administration as therapy for stress-related psychiatric and somatic disorders in humans.

German Summary

Die Ursachenforschung und Therapiemöglichkeiten stressbedingter psychischer und somatischer Erkrankungen sind heutzutage von großer medizinischer Bedeutung. Es ist allgemein anerkannt, dass Stressoren mit chronischer und psychosozialer Komponente dabei den stärksten Risikofaktor darstellen. Das L-Glutamatsystem mit seinen ionotropen und metabotropen Rezeptoren ist das primäre exzitatorische Neurotransmittersystem im Säugergehirn, wobei die prä- und postsynaptischen metabotropen Glutamatrezeptoren die Feinmodulation der schnellen synaptischen Signalweiterleitung der ionotropen Glutamatrezeptoren ermöglicht. In den letzten Jahrzehnten waren erstaunliche Fortschritte in der Forschung an metabotropen Glutamatrezeptoren festzustellen. Ein besonderes Augenmerk wurde hier auf die Rolle der Subtypen mGlu5 und mGlu7 in akutem stress-, furcht- und depressionsbezogenem Verhalten gesetzt (siehe Einleitung). Im Säugergehirn ist der mGlu7 Subtyp der am weitesten verbreitete Glutamatrezeptor und spielt somit vermutlich eine bedeutende Rolle im zentralen Nervensystem, unter normalen sowie pathologischen Zuständen. Obwohl genetische und pharmakologische Ansätze hilfreich dafür waren, die Funktionsweise des mGlu7 in vielen verhaltensbedingten und physiologischen Prozessen zu verstehen, sind die Ergebnisse von Studien mit verfügbaren allosterischen Liganden trotz ähnlicher pharmakologischer Eigenschaften *in vitro* nicht immer eindeutig. Vor kurzem hat die Entdeckung von XAP044 große Hoffnung geweckt, um dieses Dilemma zu lösen. Charakteristisch für XAP044 ist, dass diese Substanz die Signalwege des mGlu7 durch Bindung an dessen Venusfliegenfalle-Domäne blockiert, und nicht wie die bisher bekannten selektiven Liganden durch Bindung an allosterische Transmembranstellen. XAP044 verwendet also vermutlich einen neuartigen Regulationsmechanismus. Ein Abschnitt der vorliegenden Doktorarbeit hat nun darauf abgezielt, diese neue Substanz XAP044 in verschiedenen Tests für Depression, Furcht und Angst in der Maus *in vivo* zu charakterisieren. Mir gelang es nachzuweisen, dass XAP044 systemisch aktiv ist und eine antistress, antidepressive und anxiolytische Wirkung aufweist. Diese Ergebnisse unterstützen die pharmakologische Blockade von mGlu7 als vielversprechenden therapeutischen Ansatz für die Behandlung von stressbedingten emotionalen Erkrankungen des Menschen.

Ebenso steht der mGlu5 Subtyp im Fokus der aktuellen Arzneimittelforschung (siehe Einleitung). Aufgrund seiner physikalischen und funktionellen Wechselwirkung mit dem postsynaptischen NMDA Rezeptor bietet der mGlu5 eine vielversprechende Möglichkeit, die Funktion des NMDA Rezeptors zu modulieren. Dies ist insofern von besonderer Bedeutung, da z.B. der NMDA Rezeptor Antagonist Ketamin – trotz schneller und

langanhaltender Wirkung in klinischen Depressionsstudien – sich ernsthaft auf die Wahrnehmung und Psyche auswirkt. Bis heute sind einige mGlu5 negative allosterische Modulatoren (NAM) bekannt, die z.B. bei Depressions- oder Angsterkrankungen therapeutisches Potential besitzen. Eine Studie hat vor kurzem gezeigt, dass der mGlu5-selektive NAM Basimglurant eine vielversprechende Substanz mit antidepressiver Wirkung ist, die zugleich Angst-und Schmerzzustände lindert.

Trotz der gut etablierten Rolle von mGlu5 und mGlu7 in akutem stressbezogenen Verhalten und Physiologie, ist deren Beteiligung bei chronischem Stress nur sehr gering erforscht. Das CSC-Modell stellt ein geeignetes Tiermodell dar, um genau dies näher zu untersuchen. Durch chronisch psychosoziale Stressor-Exposition werden hier verhaltensbedingte, physiologische und immunologische Veränderungen bei Mäusen induziert, welche vergleichbar sind mit den psychischen, somatischen und/oder gastrointestinalen Erkrankungen bei chronisch gestressten Menschen. Dabei stellte sich die Frage, ob die Glutamatrezeptorsubtypen mGlu5 und mGlu7 das Potential haben, diese pathologischen Veränderungen zu kontrollieren und womöglich therapeutische Ansätze für die Behandlung verschiedener chronisch stressbedingter Erkrankungen im Menschen zu liefern. Im Zuge dieser Doktorarbeit wurden zuerst mögliche Effekte von CSC auf das metabotrope Glutamatrezeptorsystem untersucht. Dabei wurde herausgefunden, dass im Hypothalamus die mGlu5 mRNA tendenziell hochreguliert ist. Eine zusätzliche Saturationsanalyse hat gezeigt, dass die Proteinbindung des mGlu5 spezifisch im Hippocampus erhöht ist. Des Weiteren ergab sich eine deutliche Abnahme der mGlu7 mRNA spezifisch im präfrontalen Cortex. Im Gegensatz dazu waren weder mGlu2 noch mGlu3 nach CSC-Exposition beeinflusst. Zusammenfassen waren nun als Folge von CSC-Exposition spezifische Expressionsänderungen der Subtypen mGlu5 und mGlu7 in stresssensitiven Gehirnregionen festzustellen, welche für die Regulation von Verhalten und der HPA-Achsen Funktion bedeutend sind. Somit konnten erste Hinweise auf eine mögliche wichtige Rolle dieser Rezeptoren in chronisch psychosozialem Stress geliefert werden.

Im nächsten Schritt wurden die Effekte der genetischen Blockade des mGlu7 auf verhaltensbiologische, physiologische und immunologische Konsequenzen der CSC-Exposition untersucht. Dabei wurde herausgefunden, dass das genetische Entfernen des mGlu7 viele CSC-induzierte Veränderungen abschwächt oder sogar verhindert; mGlu7-defiziente Mäuse entwickelten keine Angst und waren auch vor den physiologischen und immunologischen Folgen von CSC geschützt. Diese Ergebnisse deuten auf eine wichtige

Rolle des mGlu7 in der Modulation einer großen Bandbreite der affektiven und somatischen Veränderungen nach CSC-Exposition hin. Sie suggerieren darüber hinaus, dass die pharmakologische Blockade des mGlu7 eine mögliche Behandlungsstrategie bei chronisch gestressten Menschen darstellt.

Im letzten Teil der vorliegenden Doktorarbeit wurden mögliche positive Effekte der genetischen sowie pharmakologischen Blockade des mGlu5 Subtyps auf CSC-induzierte Veränderungen (dieselbe Bandbreite an Parametern wie oben beschrieben) analysiert. Interessanterweise waren mGlu5-defiziente Mäuse vor einigen Folgen von CSC, u.a. vor dem von mir neu etablierten CSC-induzierten Anstieg stressbedingter Hyperthermie, geschützt. Des Weiteren wurden die Effekte des mGlu5 NAM CTEP, welcher chemisch gesehen der klinisch aktiven Substanz Basimglurant sehr ähnlich ist und eine vergleichsweise hohe Halbwertszeit bei Nagern hat, auf eine größere Bandbreite CSC-induzierter Veränderungen untersucht. CTEP hat dosisabhängig tatsächlich viele Konsequenzen von CSC gelindert, wie z.B. die Dysfunktion der HPA-Achse und die Darmentzündung. Dies deutet darauf hin, dass auch der mGlu5 Subtyp einen relevanten Mediator für viele stress-induzierten Veränderungen darstellt und ein mögliches Zielprotein für die Behandlung stressbedingter somatischer Pathologien ist.

Somit konnte die vorliegende Doktorarbeit einen deutlichen Beweis dafür liefern, dass besonders die mGlu5- und mGlu7-Subtypen eine wichtige Rolle in der Regulation akut- und chronisch-stressbedingter Veränderungen im Verhalten und der Physiologie spielen. Womöglich kann dadurch auch die Entwicklung mGlu5- und mGlu7-gerichteter Therapieansätze für stress-bedingte psychische und somatische Erkrankungen beim Menschen unterstützt werden.

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Inhibition in Human Glioma U87 Cells: Involvement of PI3K/Akt/mTOR Pathway. Cell. Physiol. Biochem. 35, 419–32. doi:10.1159/000369707

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
ACPT-I	(1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid
ACTH	adrenocorticotropic hormone
ADX71743	6-(2,4-dimethylphenyl)-2-ethyl-4,5,6,7-tetrahydro-1,3-benzoxazol-4-one
ADX88178	4-methyl-N-[5-methyl-4-(1H-pyrazol-4-yl)-1,3-thiazol-2-yl]pyrimidin-2-amine
AFQ056, mavoglurant	methyl(3aR,4S,7aR)-4-hydroxy-4-[2-(3methylphenyl)ethynyl]-3,3a,5,6,7,7a-hexahydro-2H-indole-1-carboxylate
AMN082	N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride
AMPA	2-amino-3-(5-methyl-3-oxo-2,3-dihydro-1,2-oxazol-4-yl)propanoic acid
ANOVA	analysis of variance
ANS	autonomic nervous system
AVP	arginine vasopressin
AZ12216052	2-{[(4-bromophenyl)methyl]sulfanyl}-N-[4-(butan-2-yl)phenyl]acetamide
basimglurant	2-chloro-4-{[1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl]ethynyl}pyridine
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
Ca	calcium
CA	closed arm
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CMS	chronic mild stress
CNS	central nervous system
CO_2	carbon dioxide
CORT	corticosterone
CPPG	2-amino-2-cyclopropyl-2-(4-

	phosphonophenyl)acetic acid
CRC	colorectal cancer
CRH	corticotropin releasing hormone
CS	conditioned stimulus
CSC	chronic subordinate colony housing
CSDS	chronic social defeat stress
CSF	cerebrospinal fluid
CTEP	4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy) phenyl]imidazol-4-yl]ethynyl]pyridine
CUS	chronic unpredictable stress
DAG	diacylglycerol
DAG	diacylglycerol
db	decibel
DCPG	4-[(1S)-1-amino-2 hydroxy-2-oxoethyl]phthalic acid
DMEM	Dulbecco`s Modified Eagle Medium
DOPA	dehydroxyphenylalanine
DZP	diazepam
EDTA	ethylendiamintetraacetic Acid
ELISA	enzyme linked immunosorbent assay
EPF	elevated platform
EPM	elevated plus-maze
ERK	extracellular receptor kinase
EtOH	ethanol
fenobam	3-(3-chlorophenyl)-1-(1-methyl-4-oxo-5H-imidazol-2-yl)urea
FST	forced swim test
FXS	Fragile X syndrome
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid

Glu	glutamate
GPCR	G-protein-coupled receptor
GR	glucocorticoid receptor
GRM7	gene coding for mGlu7 receptors
GRN-529	2-{2-[2-(difluoromethoxy)-5-({5H,6H,7H-pyrrolo[3,4-b]pyridin-6-yl}carbonyl)phenyl]ethynyl}pyridine
h	hour
HC	hippocampus
HDRS	Hamilton depression rating scale
HPA	hypothalamic-pituitary-adrenocortical
HPLC/MS	high-performance liquid chromatography/mass spectrometry
HT	hypothalamus
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IBD	inflammatory bowel disease
IFN-γ	interferon-γ
iGlu	ionotropic glutamate receptor
IMI	imipramine
IP_3	inositol 1,4,5-trisphosphate
IP_3	{[(1R,2S,3R,4R,5S,6R)-2,3,5-trihydroxy-4,6-bis(phosphonooxy)cyclohexyl]oxy}phosphonic acid
kainate, KA	(2S,3S,4S)-3-(carboxylatomethyl)-4-(prop-1-en-2-yl)pyrrolidine-2-carboxylate
kg	kilogram
kHz	kilohertz
KO	knockout
LDB	light-dark box
LH	learned helplessness
LPS	lipopolysaccharide

LTP	long-term potentiation
Lu AF21934	(1S,2R)-2-[(aminooxy)methyl]-N-(3,4-dichlorophenyl)cyclohexane-1-carboxamide
lx	lux
mA	milliampere
MADRS	Montgomery-Asberg depression rating scale
MAPK	mitogen-activated protein kinase
MDD	major depressive disorder
mesLNC	mesenteric lymph node cells
Met-1	N-benzhydrylethane-1,2-diamine
mg	milligram
mGlu	metabotropic glutamate receptor
min	Minute
mM	millimolar
MMPIP	6-(4-methoxyphenyl)-5-methyl-3-(pyridin-4-yl)-4H,5H-[1,2]oxazolo[4,5-c]pyridin-4-one
MPEP	2-methyl-6-(2-phenylethynyl)pyridine
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MTEP	3-[2-(2-methyl-1,3-thiazol-4 yl)ethynyl] pyridine
mTOR	mammalian target of rapamycin
NAM	negative allosteric modulator
nM	nanomolar
NMDA	(2R)-2-(methylamino)butanedioic acid
NPY	neuropeptide Y
OA	open arm
OB	olfactory bulbectomy
OF	open field
PAM	positive allosteric modulator
PBS	phosphate-buffered Saline

PD	Parkinson's disease
PEG	Polyethyleneglycol
PFC	prefrontal cortex
PFC	prefrontal cortex
PHCCC	7-hydroxyimino-N-phenyl-1,7a dihydrocyclopropa[b]chromene-1a-carboxamide
PI3-kinase	phosphatidyl inositol 3-kinase
PICK1	protein interacting with PKC alpha
PKA	proteinkinase A
PKC	proteinkinase C
PLC	phospholipase C
PSD-95	postsynaptic density-95
PVN	paraventricular nucleus
qPCR	quantitative polymerase chain reaction
RPMI	Roswell Park Memorial Institute
RS-PPG	2-amino-2-(4-phosphonophenyl)acetic acid
s	seconds
s.c.	sub-cutaneous
S.E.M	standard error of mean
SHC	single-housed control
SIH	stress-induced hyperthermia
siRNA	small interfering RNA
SNS	sympathetic nervous system
SPAT	social preference/avoidance test
T1, T2	temperature 1, temperature 2
TCA	tricyclic antidepressant
Th2	T helper 2
TRD	treatment-resistant depression
US	unconditioned stimulus
VEH	vehicle

VFTD	Venus flytrap domain
vs.	versus
VU0155041	(1R,2S)-2-[(3,5-dichlorophenyl)carbamoyl] cyclohexane-1-carboxylic acid
WT	Wildtype
XAP044	7-hydroxy-3-(4-iodophenoxy)-4Hchromen-4-one

First pages of first/joint-first author publications

Original research articles

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Blocking metabotropic glutamate receptor subtype 5 relieves maladaptive chronic stress consequences.

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Abstract

Etiology and pharmacotherapy of stress-related psychiatric conditions and somatoform disorders are areas of high unmet medical need. Stressors holding chronic plus psychosocial components thereby bear the highest health risk. Although the metabotropic glutamate receptor subtype 5 (mGlu5) is well studied in the context of acute stress-induced behaviors and physiology, virtually nothing is known about its potential involvement in chronic psychosocial stress. Using the mGlu5 negative allosteric modulator CTEP (2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4yl]ethynyl]pyridine), a close analogue of the clinically active drug basimglurant - but optimized for rodent studies, as well as mGlu5-deficient mice in combination with a mouse model of male subordination (termed CSC, chronic subordinate colony housing), we demonstrate that mGlu5 mediates multiple physiological, immunological, and behavioral consequences of chronic psychosocial stressor exposure. For instance, CTEP dose-dependently relieved hypothalamo-pituitary-adrenal axis dysfunctions, colonic inflammation as well as the CSC-induced increase in innate anxiety; genetic ablation of mGlu5 in mice largely reproduced the stress-protective effects of CTEP and additionally ameliorated CSC-induced physiological anxiety. Interestingly, CSC also induced an upregulation of mGlu5 in the hippocampus, a stress-regulating brain area. Taken together, our findings provide evidence that mGlu5 is an important mediator for a wide range of chronic psychosocial stress-induced alterations and a potentially valuable drug target for the treatment of chronic stress-related pathologies in man.

KEYWORDS: CTEP; Chronic psychosocial stress; Chronic subordinate colony housing; Knockout; Stress-protective phenotype; mGlu5

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Relief from detrimental consequences of chronic psychosocial stress in mice deficient for the metabotropic glutamate receptor subtype 7.

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Abstract

Chronic stress-related psychiatric conditions and comorbid somatic pathologies are an enormous public health concern in modern society. The etiology of these disorders is complex, with stressors holding a chronic and psychosocial component representing the most acknowledged risk factor. During the last decades, research on the metabotropic glutamate receptor (mGlu) system advanced dramatically and much attention was given to the role of the metabotropic glutamate receptor subtype 7 (mGlu7) in acute stress-related behavior and physiology. However, virtually nothing is known about the potential involvement of mGlu7 in chronic psychosocial stress-related conditions. Using the chronic subordinate colony housing (CSC, 19 days) in male mice, we addressed whether central mGlu7 is altered upon chronic psychosocial stressor exposure and whether genetic ablation of mGlu7 interferes with the multitude of chronic stress-induced alterations. CSC exposure resulted in a downregulation of mGlu7 mRNA transcript levels in the prefrontal cortex, a brain region relevant for stress-related behaviors and physiology. Interestingly, mGlu7 deficiency relieved multiple chronic stress-induced alterations including the CSC-induced anxiety-prone phenotype; mGlu7 ablation also ameliorated CSC-induced physiological and immunological consequences such as hypothalamo-pituitary-adrenal (HPA) axis dysfunctions and colonic inflammation, respectively. Together, our findings provide first evidence for the involvement of mGlu7 in a wide range of behavioral and physiological alterations in response to chronic psychosocial stressor exposure. Moreover, the stress-protective phenotype of genetic mGlu7 ablation suggests mGlu7 pharmacological blockade to be a relevant option for the treatment of chronic stress-related emotional and somatic dysfunctions.

KEYWORDS: Affective and somatic pathologies; Chronic psychosocial stress; Chronic subordinate colony housing; Knockout; Metabotropic glutamate receptor subtype 7

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Blocking metabotropic glutamate receptor subtype 7 (mGlu7) via the Venus flytrap domain (VFTD) inhibits amygdala plasticity, stress, and anxiety-related behavior.

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Abstract

The metabotropic glutamate receptor subtype 7 (mGlu7) is an important presynaptic regulator of neurotransmission in the mammalian CNS. mGlu7 function has been linked to autism, drug abuse, anxiety, and depression. Despite this, it has been difficult to develop specific blockers of native mGlu7 signaling in relevant brain areas such as amygdala and limbic cortex. Here, we present the mGlu7-selective antagonist 7-hydroxy-3-(4-iodophenoxy)-4H-chromen-4-one (XAP044), which inhibits lateral amygdala long term potentiation (LTP) in brain slices from wild type mice with a half-maximal blockade at 88 nm. There was no effect of XAP044 on LTP of mGlu7-deficient mice, indicating that this pharmacological effect is mGlu7-dependent. Unexpectedly and in contrast to all previous mGlu7-selective drugs, XAP044 does not act via the seven-transmembrane region but rather via a binding pocket localized in mGlu7's extracellular Venus flytrap domain, a region generally known for orthosteric agonist binding. This was shown by chimeric receptor studies in recombinant cell line assays. XAP044 demonstrates good brain exposure and wide spectrum anti-stress and antidepressant- and anxiolytic-like efficacy in rodent behavioral paradigms. XAP044 reduces freezing during acquisition of Pavlovian fear and reduces innate anxiety, which is consistent with the phenotypes of mGlu7-deficient mice, the results of mGlu7 siRNA knockdown studies, and the inhibition of amygdala LTP by XAP044. Thus, we present an mGlu7 antagonist with a novel molecular mode of pharmacological action, providing significant application potential in psychiatry. Modeling the selective interaction between XAP044 and mGlu7's Venus flytrap domain, whose three-dimensional structure is already known, will facilitate future drug development supported by computer-assisted drug design.

KEYWORDS: Elevated Plus-Maze; Fear Conditioning; Glutamate Receptors Metabotropic; Neurotransmitter Release; Pharmacology; Stress; Synaptic Plasticity; XAP044

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Review article

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The Emerging Role of Metabotropic Glutamate Receptors in the Pathophysiology of Chronic Stress-Related Disorders.

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Abstract

Chronic stress-related psychiatric conditions such as anxiety, depression, and alcohol abuse are an enormous public health concern. The etiology of these pathologies is complex, with psychosocial stressors being among the most frequently discussed risk factors. The brain glutamatergic neurotransmitter system has often been found involved in behaviors and pathophysiologies resulting from acute stress and fear. Despite this, relatively little is known about the role of glutamatergic system components in chronic psychosocial stress, neither in rodents nor in humans. Recently, drug discovery efforts at the metabotropic receptor subtypes of the glutamatergic system (mGlu1-8 receptors) led to the identification of pharmacological tools with emerging potential in psychiatric conditions. But again, the contribution of individual mGlu subtypes to the manifestation of physiological, molecular, and behavioral consequences of chronic psychosocial stress remains still largely unaddressed. The current review will describe animal models typically used to analyze acute and particularly chronic stress conditions, including models of psychosocial stress, and there we will discuss the emerging roles for mGlu receptor subtypes. Indeed, accumulating evidence indicates relevance and potential therapeutic usefulness of mGlu2/3 ligands and mGlu5 receptor antagonists in chronic stress-related disorders. In addition, a role for further mechanisms, e.g. mGlu7-selective compounds, is beginning to emerge. These mechanisms are important to be analyzed in chronic psychosocial stress paradigms, e.g. in the chronic subordinate colony housing (CSC) model. We summarize the early results and discuss necessary future investigations, especially for mGlu5 and mGlu7 receptor blockers, which might serve to suggest improved therapeutic strategies to treat stress-related disorders.

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Curriculum vitae, publications and awards

Curriculum vitae

PERSONAL INFORMATION

Name:	Daniel Peterlik, MSc
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Nationality:	German
Martial status:	married, one child

EDUCATION

07/2012 – present **PhD degree with "summa cum laude"** (awaiting receipt of PhD certificate), Regensburg International School of Life Sciences (RIGeL) PhD program, Laboratory of Molecular and Cellular Neurobiology, University of Regensburg, Germany

- Scientific focus on the functional involvement of metabotropic glutamate receptors in the regulation of acute and chronic stress-induced affective and somatic (i.e. physiological and immunological) consequences in mammals
- PhD Thesis: "Metabotropic Glutamate Receptors – Regulation of Acute and Chronic Stress-Related Behavior and Physiology", Supervisor: Prof. Dr. Peter J. Flor, Laboratory of Molecular and Cellular Neurobiology

10/2009 – 06/2012 **Master of Science (MSc)** in Biology, University of Regensburg, Germany

- Majors: Neurobiology, Immunology and Molecular Human Biology
- Overall grade: **1,5**
- Master Thesis: "Involvement of brain metabotropic glutamate receptor subtype 7 in chronic psychosocial stress and fear conditioning" (grade: 1.1), Supervisor: Prof. Dr. Peter J. Flor, Laboratory of Molecular and Cellular Neurobiology

10/2006 – 09/2009 **Bachelor of Science (BSc)** in Biology, University of Regensburg, Germany

- Bachelor Thesis: "Charakterisierung myeloider Zellen aus der Milz im CSC-Modell" (grade: 1.1), Supervisor: Prof. Dr. Inga Neumann, Department of Behavioural and Molecular Neurobiology

07/2006 **Abitur**, Johann-Michael-Fischer Gymnasium Burglengenfeld, Germany

Publications/submitted manuscripts

Peterlik, D., Stangl, C., Bludau, A., Grabski, D., Strasser, R., Schmidt, D., Flor, P.J., Uschold-Schmidt, N., 2016c. Relief from detrimental consequences of chronic psychosocial stress in mice deficient for the metabotropic glutamate receptor subtype 7. **Neuropharmacology (IF: 5.106)**, doi:10.1016/j.neuropharm.2016.04.036, *in press*

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Acknowledgements - Danksagung

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Author’s declaration – Eidesstaatliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als die der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Ort, Datum Daniel Peterlik