Studying prepulse inhibition in a stressed system

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Studying prepulse inhibition in a stressed system

Prepulsinhibitie in een gestrest systeem nader bekeken

(met een samenvatting in het Nederlands)

Proefschrift

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door

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Chapter 1

General introduction

1. Background and rational for this thesis

1.1. Stress and psychosis

Although genetic factors undoubtedly play an important role in the etiology of psychotic disorders such as schizophrenia, onset and course of these disorders are thought to be influenced by environmental factors, particularly stress. According to the original vulnerability-stress model developed by Zubin and Spring (1977), the experience of stress was even suggested to be essential to the onset of acute psychosis: "It is assumed that exogenous and/or endogenous challengers elicit a crisis in all humans, but depending on the intensity of the elicited stress and the threshold for tolerating it, that is, one's vulnerability, the crisis will either be contained homeostatically or lead to an episode of disorder". Indeed, after more than 30 years of research, researchers are still looking for epigenetic mechanisms that, driven by stress exposure, result in aberrant regulation of vulnerability genes to increase the risk for psychotic disorders, with some success [1, 2].

To date there is however no consistent evidence from observational studies that experience of stressful life events triggers disease onset in healthy individuals. Though, longitudinal studies link episodes of relapse in schizophrenia patients to elevated levels of experienced stress [3]. In order to test the theory linking stress and psychosis, animal models are indispensable. Although human lifelong stress and psychotic symptoms are difficult to mimic in laboratory animals, a particularly useful behavioral readout in this respect may be prepulse inhibition (PPI) of startle, which is a stress-sensitive translational readout (see below).

1.2. Prepulse inhibition of startle

Throughout this thesis, PPI was used as a tool to model psychotic-like behavior in rodents. In chapter 1, the concept of PPI is described in somewhat more detail. Briefly, PPI represents the degree to which a weak *sensory* event (the prepulse) inhibits the reflexive *motor* response to the subsequent intense startling stimulus, also referred to as sensorimotor gating. Typically, but not exclusively, PPI is deficient in schizophrenia. Other neuropsychiatric disorders that are associated with reduced PPI include bipolar [4] and panic disorder [5], obsessive-compulsive disorder [6], Huntington's disease [7] and Tourette's syndrome [8], generally reflecting impaired gating in the sensory, motor or cognitive domains. At the neural level, overlapping brain substrates and pharmacological mechanisms between PPI in humans and rodents have been broadly suggested, and consequently, PPI is considered a "cross-species" homologue behavioral measure. When comparing rodents and humans, however, it should be noted that rodent and human brains are not similar, so the PPI regulatory neural circuitry in rodents cannot be translated directly to the human situation. Species differences are expected to be most pronounced in phylogenetically newest regions, such as the frontal cortex, having much relevance to schizophrenia [9]. Also, the pharmacology of PPI appears to be different at several levels between humans and rodents, and even within rodent species and strains [10]. Within animal strains however, both PPI and drug sensitivity are considered robust and highly reliable phenotypes [9]. Another major strength of

the PPI test lies in its predictive validity in drug discovery and development. Although there is no evidence supporting either desirable or functionally enhancing effects of increased PPI per se, nor that increase of PPI by a drug should be necessary or sufficient for clinical benefit in schizophrenia, normalization of experimentally impaired PPI in rodents strongly predicts clinical utility and even potency of antipsychotic agents [11, 12].

1.3. Corticotropin-releasing factor systems

In 1981, corticotropin-releasing factor (CRF) was discovered as the hypothalamic neuropeptide that mediates the effects of stress on the hypothalamic-pituitary-adrenal (HPA) axis [13]. Since then, a growing family of related compounds has been characterized, including CRF and urocortin 1-3, and the CRF-binding protein [14, 15]. Together, these compounds mediate behavioral, autonomic and endocrine responses to stress [16]. However, importantly, in addition to hypothalamic sites, CRF is released from CRF-containing neurons that are broadly expressed in the brain [17]. CRF released from these neurons is also active as a neurotransmitter during stress, which can dramatically change the motivational state of an animal [18-21]. The biological actions of CRF and related peptides are mediated via two types of G-protein-coupled receptors, CRF_1 and CRF_2, which differ in their pharmacology and tissue distribution patterns. CRF is relatively selective for CRF_1 over CRF_2 receptors [16]. The distribution of CRF_2 receptors is restricted to discrete limbic and brainstem areas, while CRF_1 receptors are broadly distributed over the brain, including areas such as nucleus accumbens, amygdala, prefrontal cortex, hippocampus and the brainstem pedunculopontine tegmental nucleus and inferior colliculus [17]. These latter areas are part of the neural circuitries implicated in the modulation of startle and regulation of PPI [22], which brings CRF in a position to alter sensorimotor gating processes. Previously, it has been proposed that HPA axis over-activity may induce psychotic symptoms, via cortisol-induced enhancement of mesolimbic dopaminergic pathways relevant to psychosis [23, 24], but this hypothesis was questioned and refuted by others [25, 26]. Currently it is thought that CRF may be involved in psychotic processes via HPA-independent, central mechanisms. Indeed, in mice and rats, long-term overexpression or exogenous administration of CRF are found to reduce PPI, by a CRF_1-dependent mechanism [27-29]. Exogenous CRF also robustly increases startle, but this latter effect is thought to be independent of the compound's effect on PPI [27, 30]. As CRF_1 receptors are thought to be implicated in activating the stress response, a range of pharmacological agents has been developed which block the CRF_1 receptor, hoping that these drugs may have therapeutic effects in the treatment of stress-related mental disorders. To date, CRF_1 receptor antagonists have proven to be efficacious in animal models of human stress disorders, in which the CRF system is over-activated (reviewed by Kehne, 2007). However, due to the drugs' adverse side effects, further development has been laborious till thus far [31].

1.4. Aim and objectives

The aim of this thesis was to investigate the role of central CRF signaling in sensorimotor gating processes. Therefore, the experiments here described were dedicated to two objectives:

1. To determine which mechanisms are involved in CRF-induced PPI deficits
2. To assess if CRF1 receptor antagonists could have antipsychotic-like actions

1.5. Structure of the thesis

First, studies addressing the effects of stress on PPI are reviewed (**chapter 2**). Interestingly, results were found to be in line with neurodevelopmental theories of schizophrenia. For the rest of this thesis it is important to realize that schizophrenia is the most well-known psychotic illness, so most research on psychotic processes has focused on this illness. Throughout this thesis therefore the term schizophrenia is used, but it should be noted that similar neurochemical deficits are thought to underlie other forms of psychosis, and that schizophrenia is obviously more than psychosis. In this thesis the term 'schizophrenia' is used in context of this annotation. In **chapter 3 and 4,** mood stabilizers were central in our aim to unravel pathways through which CRF may alter PPI. Mood stabilizers are drugs that are used in the treatment of bipolar disorder. This is a neuropsychiatric illness characterized by manic and depressive episodes, the former being associated with psychosis and deficient PPI. In **chapter 4,** we investigated whether the potentiation of amphetamine-induced hyperlocomotion by chlordiazepoxide, putatively caused by mesolimbic dopamine-GABA interactions, could be extended to PPI. A well-known effect of valproate is facilitation of GABAergic neurotransmission, which could be related to its antimanic effects. As $GABA_B$ signaling is involved in the regulation of PPI, in **chapter 5** we investigated the interactions between CRF and $GABA_B$ signaling. Since schizophrenia is associated with impaired PPI, and CRFtg mice display PPI deficits, we investigated the actions of CRF_1 receptor antagonists in pharmacological models of PPI-disruption responsive to antipsychotics in **chapter 6**. Last, in **chapter 7** we summarize and discuss the findings of all experiments in the context of the formulated aims.

Chapter 2

Linking stress and schizophrenia: a focus on prepulse inhibition

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1. Introduction

Schizophrenia affects about 0.5-1.0% of people worldwide, occurring roughly equally in both men and women. The exact causes of schizophrenia are not fully understood, although the consensus of current research is that schizophrenia is a developmental disorder, caused by a genetic liability interacting with environmental and psychosocial stress. However, the possible neurobiological mechanisms underlying this gene-stress interaction are largely unknown.

To study the role of stress in the development of schizophrenia, it is useful to dissect this complex disease into specific symptoms. In this respect, a well-accepted model for psychotic-like behavior is prepulse inhibition (PPI) of the startle response. In an attempt to clarify the link between stress and schizophrenia, this chapter reviews experimental studies that determined the effect of acute and chronic stressors on PPI in humans and rodents. In section 2, studies that have focused on stress in adulthood will be discussed, and in section 3, studies that have addressed effects of early-life stress on PPI will be outlined. Together, the findings of these PPI studies support the neurodevelopmental theories of schizophrenia, which state that insults, including stress, experienced during early brain development could particularly increase risk of developing schizophrenia.

1.1. Symptom dimensions in schizophrenia

Schizophrenia is a mental disorder, characterized by a mixture of symptoms that are generally divided in three major clusters: positive, negative, and cognitive symptoms [32]. The first category signifies symptoms that reflect excess in normal function, which comprises psychotic symptoms. These mental phenomena are often dramatic; the patient appears to have lost contact with reality. Hallucinations are one type of positive symptom; they are perceptions disconnected from external stimuli, which may occur in any sensory modality, however, auditory hallucinations (i.e. hearing voices) are the most common hallucinations in schizophrenia. Delusions are another type of positive symptom, which are fixed, false beliefs that are not shared by other people in the patient's neighborhood. The most common form is the paranoid delusion, such as the false belief that one is spied on or persecuted. However, a variety of other themes is also possible, for instance the belief that the fillings in one's teeth are radio transmitters receiving extraterrestrial messages, or the belief that some outside agency has added or removed thoughts in one's brain.

Negative symptoms, on the contrary, describe loss or significant impairment of normal psychological functions, such as blunted affect, emotional withdrawal, poor rapport, passivity and apathy, and anhedonia [32]. Although this reduction in normal functioning may seem less dramatic as positive symptoms, particularly negative symptoms are associated with long periods of hospitalization and poor social functioning. Indeed, a patient's degree of negative symptoms appears to determine whether a patient is still able to function in society. Last, cognitive symptoms of schizophrenia comprise 'executive dysfunctions', including problems in maintaining goals, allocating attentional resources, evaluating and monitoring performance, and utilizing these capacities to solve problems.

1.2. Diathesis-stress hypothesis of schizophrenia

Schizophrenia is a complex illness, and its possible causes are still subject of debate. However, according to a widely adopted view, both a biological predisposition, and exposure to environmental stress are necessary ingredients for schizophrenia to become manifest – the so-called diathesis stress model [33]. A classical example of the association between environmental stress and the risk of developing schizophrenia, comes from studies of the 1944-1945 Dutch Hunger Winter [34-36]. In this discrete period during the Second World War, there was a serious decline in food intake in six cities of western Netherlands. Among people born in these cities between 1944 and 1946, the most exposed birth cohort (i.e., conceived at the height of the famine) showed a twofold and statistically significant increase in the risk for developing schizophrenia [36]. Since this particular famine, many others occurred worldwide, but these were less suitable for epidemiological investigations due to more disorganized conditions [37]. However, several years after the Dutch Hunger Winter, a similar occasion occurred in China. Specifically, from 1959-1961, people in affected provinces were starving and died in large numbers due to bad weather (for refs see [37]). Among births that occurred during the famine years, the risk of developing schizophrenia in later life was significantly increased when compared to those born before or afterwards [37].

These 'natural experiments' suggest that prenatal stress, i.e., exposure to maternal nutritional deficiency, could increase the risk of schizophrenia in later life. However, evidence from many observational studies that have investigated the association between experience of stress in adulthood and acute onset of psychotic illness, has not been confirmative [3]. In particular, there is no consistent evidence that experience of stressful life events is able to trigger onset of psychosis. Findings from longitudinal studies on the other hand, are stronger in linking episodes of relapse in schizophrenia patients to an elevated rate of stressful life events [52-53], thus providing additional evidence for a role of stress exposure in (the course of) schizophrenia.

1.3. Neurodevelopmental hypothesis of schizophrenia

As mentioned earlier, schizophrenia is generally not diagnosed before the third decade of life, suggesting that it is the end point of some pathological process acting on the immature brain. This observation has led to the formulation of the neurodevelopmental hypothesis of schizophrenia, which states that environmental disturbances during early brain development influence risk of developing schizophrenia [38-40]. Some candidates for these early disturbances are, for instance, prenatal influenza exposure, obstetric complications, prenatal maternal psychological stress, maternal and fetal nutritional deficiency, season of birth (for refs, see [37]). However, obviously, not every individual will get ill if they experience stress. Consequently, it is thought that manifestation of disease originates through an interaction with the genetic make-up of an individual [38-40]. Several lines of evidence support the neurodevelopmental hypothesis, including epidemiological studies, premorbid history and neuropathological postmortem studies [38]. With respect to genetics, many 'susceptibility' genes, found to be associated with a heightened risk for developing schizophrenia, have been linked to neurodevelopmental processes, such as synaptic connectivity, synaptogenesis, and growth factors [41].

However, a remaining question is, whether one single intervention early in development is enough to explain occurrence of schizophrenia much later in life. An alternative hypothesis, which works within the framework of the neurodevelopmental theory, is referred to as the "double-hit" model [42-44]. According to this model, maldevelopment within 2 critical 'windows of vulnerability' combines to lead to clinical manifestations of schizophrenia. First, early developmental risk factors (i.e. genetic predisposition, environmental stressor) will cause a heightened vulnerability to the illness through anomalous neural development and subtle changes in behavior. However, for schizophrenia to become manifest, an additional second 'hit' (i.e. an environmental factor such as drug abuse or social stress) is considered necessary. Thus, in this view, early and late risk factors are not simply additive, but instead, the first hit will increase an individual's vulnerability for effects of a subsequent hit [42-44].

1.4. Prepulse inhibition of the startle response

In order to test the above-mentioned theories, animal models could be used. However, due to the nature of the symptoms and the pathological complexity of schizophrenia, it is impossible to reproduce the disease in its entirety in an animal model. As a possible solution, one could model specific aspects or symptoms of the disorder. A highly validated model in this respect is the behavioral paradigm of prepulse inhibition (PPI) of the acoustic startle response, which is typically, but not exclusively, diminished in schizophrenic patients [9, 10]. As disrupted PPI is a trait marker of schizophrenia, which is also displayed by patients' unaffected relatives, as well as schizotypal (i.e., non-psychotic, unmedicated) patients, impaired sensorimotor gating is considered an endophenotype of schizophrenia [45].

PPI refers to the normal suppression of a startle response to a strong stimulus when it is preceded by a weaker stimulus (the prepulse). In rodents, PPI is commonly measured as whole-body startle responses, whereas in human experiments, generally eye-blink responses are used. In theory, deficient PPI in schizophrenic patients reflects a dysfunction in the gating of sensory and cognitive information, clinically manifesting as a patient's inability to filter irrelevant thoughts and sensory stimuli from intruding into awareness [45, 46]. Some cross-sectional and longitudinal studies demonstrate that in patients, deficits in sensorimotor gating are improved by atypical antipsychotics [9, 47]. Neurophysiologically, PPI is mediated via the brainstem, whereas it is regulated by an extensive set of interrelated projections from the forebrain [22]. Pharmacological interventions that diminish PPI are well characterized in animal models [10] and also increasingly applied in healthy human subjects [9, 48, 49]. In particular, PPI is disrupted by dopamine receptor agonists (e.g., apomorphine), serotonin receptor agonists (e.g., 8-OHDPAT), and NMDA receptor antagonists (e.g., PCP). Accordingly, the different models of disrupted PPI have been used in the search of novel antipsychotic treatments, and each of the models has proven to be sensitive to at least some antipsychotic medications [10]. Notably, concerning interventions in the dopaminergic system, application of receptor agonists into the (subcortically located) nucleus accumbens and receptor antagonists in the medial prefrontal cortex diminish PPI [50, 51], providing considerable construct validity to PPI as a model for deficient sensorimotor gating in schizophrenic patients. Thus, PPI is considered a robust,

predictable and neurobiologically informative experimental measure, broadly used in translational models for schizophrenia research. While PPI is largely determined by anatomical and genetic traits [9], it may also be sensitive to effects of stress – which may be even more relevant, considering the leading theories on the development of schizophrenia. Therefore, the aim of this chapter is to explore the studies that applied experimental stressors to investigate their influence on PPI.

2. Adult stress and gating mechanisms: evidence from animal and human studies

2.1. Effects of acute stress on sensorimotor gating: animal studies

In this section, we will review the existing literature on the effects of acute and chronic stress on PPI in adult rodents. For this purpose, some studies have pharmacologically interfered with a neural system that is fundamental to the biological stress response in mammals, the hypothalamic-pituitary-adrenal (HPA) axis, as discussed in section 2.1.1. Alternatively, external stressors have been artificially applied to rodents in the laboratory. In general, these stressors can be classified as either physical (i.e., nociceptive) or psychological, which will be outlined in sections 2.1.2. and 2.1.3., respectively.

2.1.1. HPA modulators

In a straightforward approach to address the link between stress in adulthood and PPI, some studies have pharmacologically interfered with the HPA-axis, a major mammalian stress system. Physiologically, the function of the HPA-axis is to transduce neural signals that arise in response to any physical or psychological stressor, into an endocrine response; starting at the level of the brain's major integrating center: the hypothalamus. In this structure, the neuropeptide corticotrophin-releasing factor (CRF) is produced. When CRF is released into the hypophyseal portal system, it travels to the pituitary gland, where it binds to CRF_1 receptors. This, in turn, triggers the secretion of adrenocorticotropic hormone (ACTH). Subsequently, ACTH is transported via the systemic circulatory system to the cortex of the adrenals, where it triggers the release of glucocorticoids. Through a negative feedback at the level of the hypothalamus and pituitary, glucocorticoids (cortisol in primates and corticosterone in rodents) ultimately inhibit their own release. For decades, the HPA-axis has been linked to schizophrenia [3, 52-54]. Notable findings in patients are elevated cortisol levels, especially shortly before onset of psychosis (reviewed by [23, 52], and altered stress responsiveness, with cortisol responses being both enhanced [52] and blunted [55, 56].

Some experimental animal studies have investigated the influence of HPA-axis manipulations on PPI. For instance, Van den Buuse and co-workers investigated the effects of a dopaminergic D2 receptor antagonist (i.e. haloperidol) on the PPI response in mice following adrenalectomy and corticosterone replacement (2, 10 or 50 mg) [57]. Subsequently, the animals were tested for PPI after injection of haloperidol. In adrenal-intact mice and in mice implanted with 10 mg corticosterone, haloperidol treatment increased PPI, while in both the 2 and 50 mg corticosterone-adrenalectomy groups, PPI

was unchanged. The authors explained their results by postulating a corticosterone-dopamine interaction; moderate levels of corticosterone would be needed for a normal dopaminergic tone, while both low and high concentrations of corticosterone would induce reductions in dopaminergic activity [57]. Indeed, an interaction between corticosteroids and central mesolimbic dopaminergic activity is suggested by several studies [58-60].

At the level of CRF, intraventricular brain injections with the neuropeptide lead to reliable alterations in PPI. This is not surprising, given the putative involvement of CRF in stress disorders and psychosis [61-64] and the fact that CRF receptors are expressed in areas that modulate startle and PPI, including brainstem, limbic and cortical nuclei [17]. In rodents, both acute central administration of CRF [27, 29, 30, 65] and chronic CRF overexpression [66] diminish PPI. However, unlike central CRF, peripherally injected CRF at doses that are known to cause the release of ACTH and corticosterone did not reduce PPI in rats [29]. Consequently, it was suggested that the effect of central CRF on PPI might be independent of its effects on the HPA axis. This finding is in agreement with a study of Groenink et al. (2008), which showed that neither glucocorticoid receptor antagonists nor adrenalectomy did improve perturbation of PPI in mice overexpressing CRF (CRF-OE mice). In addition, elevation of corticosterone levels by pellet implantation did not affect PPI in wild-type mice. In contrast, two different CRF_1 receptor antagonists significantly restored PPI in CRF-OE mice, based on which the authors concluded that chronic overactivation of CRF_1 receptors rather than excessive glucocorticoid receptor stimulation underlies PPI deficits in CRF-OE mice. Also in rats, neither acute, nor repeated administration of corticosterone decreased PPI [67]. In the brain, CRF acts via CRF_1 and CRF_2 receptors. Risbrough et al. [27] investigated the respective roles of these two receptor subtypes in the startle response and sensorimotor gating in mice. Regarding the magnitude of startle, they found that CRF_1 receptors are required for the effects of CRF, and CRF_2 receptors appear to have an auxiliary role. Furthermore, CRF_1 receptor blockade reversed CRF-induced deficits in PPI, whereas CRF_2 receptor blockade potentiated the latter effect. In addition, CRF_2 receptor activation increased PPI. Together, as was argued, these findings support the idea that CRF_1 and CRF_2 receptors exert opposing roles in inhibition of startle, with CRF_1 decreasing PPI and CRF_2 increasing it. Thus, the effect of central CRF on PPI is probably not mediated by corticosterone. However, as was mentioned before, corticosteroids could play a role in regulating PPI via an interaction with mesolimbic dopaminergic activity.

2.1.2. Physical stressors

From animal models, it has long been known that intermittent and inescapable foot-shock can induce a state of analgesia (stress-induced analgesia), which is reversed by the opiate receptor antagonist naloxone [68]. Functionally, this anticipation response to upcoming aversive stimuli reduces their impact and is thought to help the organism cope with the stressor [69]. To examine whether exposure to a severe stressor induces changes in sensory functioning that accompany stress-induced analgesia, Leitner and co-workers measured PPI in rats shortly (i.e., 20 min) after exposure of cold swim stress [70]. Next to a reliable analgesia, the stressed animals exhibited decreased prepulse inhibition. In a subsequent study, this stress-induced PPI-deficit appeared to

be of a multisensory nature, as reductions in PPI were found in reaction to both visual and acoustic prepulse stimuli [71]. The author interpreted these results as a general decrease in sensory sensitivity, which extends beyond the noxious stimulus (i.e., cold water). The finding supports a possible role for opiates in the PPI-disruptive effect of analgesia, in that opioid receptor agonists, which produce perceptual distortions in animals and humans, disrupt PPI in a dose-dependent fashion [72]. However, another nociceptive stressor, i.e., repeated inescapable foot-shocks, slightly increases PPI [73], or has no effect [65, 74]. Possibly, these conflicting findings can be partly explained by the longer stress-test intervals applied in the latter studies (see table 1). Lastly, the physical stressor referred to as 'restraint stress', comprises physically restraining a rodent in a narrow cylinder, usually for 15-20 minutes, sometimes on several subsequent days. Restraint stress has been shown to increase plasma ACTH, beta-endorphin, and corticosterone levels, and also brain levels of serotonin and norepinephrine (for refs, see [75]. However, the studies that examined the effects of restraint on PPI, have mostly reported inconsistent, or no effects of restraint [74-76], and one study showed that repeated, but not acute, restraint stress decreased PPI [77].

Thus, studies that have applied physical stressors in adult rodents have yielded inconsistent or no effects on PPI (table 1.a.). However, although some of these artificial stressors have been shown to be capable of producing elevated levels of stress hormones, the ethological validity of these stressors is not very high. In the next section, studies that examined the influence of ethologically more relevant psychological stressors will be discussed.

2.1.3. Psychological stressors

As opposed to physical stress, psychological stress does not involve a nociceptive component, as physical contact with the stressogenic stimulus is absent. This can be accomplished in several ways. One particularly simple method to induce emotional stress involves forcing rats to witness another rat being exposed to physical stress, such as repeated foot-shocks, or restraint. This observational stressor has been found to activate mesocortical dopamine systems [78]. However, it is also proposed to represent a milder form of stress, as plasma corticosterone levels were found to be less elevated, compared to the concomitant physical stress condition [75]. Studies that examined sensorimotor gating following witness stress, have reported no effects on PPI [73, 75].

Another psychological stressor, that is considered more potent, is referred to as social defeat. In this paradigm, an animal is made an intruder, by placing it into the residential cage of an aggressive conspecific, where it is attacked, though, generally, the experimenter will protect it from suffering too much physical harm. After a few minutes, the intruder is placed in a small cage within the resident's cage. As a consequence, it is not exposed to further injuries and direct attacks, but still remains in an unfamiliar environment, with olfactory, visual and to some extent physical (only via vibrissae) contact with the resident. Social defeat may have some face validity with respect to schizophrenia, as high levels of social competition and migration are proposed as risk factors for developing schizophrenia [79]. In rodents, social defeat has been associated with dopaminergic hyperactivity and to behavioral sensitization, whereby the animal displays an enhanced response to dopamine receptor agonists

[79]. With respect to PPI, significant impairments in the response were found following 3 weeks of daily social defeat in adult mice, which could be normalized by acute treatment with the cannabinoid receptor agonist WIN55212.2 [80].

Another ethologically valid psychological stressor involves exposure to a predator. Next to foot-shock stress (see section 2.1.1), Bakshi and co-workers also exposed their rats to ferrets, one of their natural predators [65]. To make sure that the stressor would be entirely psychogenic, the rats were protected from injury by a protective cage. This kind of predator exposure has been shown to elicit acute hypothalamic-pituitary-adrenal (HPA) axis (discussed in section 2.2.) activation, freezing behavior and ultrasonic vocalizations (for refs, see [65]). When compared to foot-shock stress, predator exposure was found to be equipotent in terms of the amplitude of acute corticosterone release. However, foot shock stress had no effects on PPI at any measured time-point, while predator exposure significantly disrupted PPI at 24 hours after the stress; but not acutely, or 48 hours, or 9 days later [65].

In our laboratory, we examined the influence of psychological stress, i.e., the potential threat of bright light, on PPI in Wistar rats. In rodents, high illumination potentiates startle (light-enhanced-startle, LES), an anxiety response sensitive to clinically effective anxiolytics [81]. As shown in figure 1, exposure to bright light significantly reduced PPI, whereas subsequent return to the safe condition enhanced PPI (for details see figure legend). These changes were most marked at lower prepulse intensities (interaction effect of prepulse intensity*phase $F_{4, 80}=4.57$, $p<0.005$).

Schmajuk and co-workers also reported diminished PPI induced by dark-to-light transitions, an effect that was blocked by haloperidol [82]. However, in addition to PPI, the animals also showed attenuated startle, which indicates that the (lower) illumination conditions used in the Schmajuk study did not induce anxiety. Accordingly, the authors explained their findings by stating that sudden changes in environment illumination (i.e. novelty) may evoke dynamic changes in dopaminergic circuits that modulate the startle response and prepulse inhibition [82].

In conclusion, the currently available studies, although limited, demonstrate that psychological stress can indeed affect PPI (table 1.b.). However, results are not unequivocal. Due to considerable variation in duration and time course of effect, it is unclear how long the PPI-disruptions will last, once the stressors are terminated, and further studies are warranted to assess time-course and robustness and underlying mechanism involved in the observed alterations in gating mechanisms.

2.2. Effects of acute stress on sensorimotor gating: human studies

In section 2.1. we have explored studies that investigated the influence of transient stress on the rodent PPI response. In the next sections, studies on human subjects are discussed, where stress has also been found to affect PPI. For instance, in a recent study, intravenous infusions of cortisol, in a dose resembling the physiological cortisol secretion in response to a moderate stressor, were shown to disrupt PPI in healthy participants [83]. The disruptions reached a maximum at 20 minutes after administration, and returned to baseline another 20 minutes later. In an older study, Grillon and Davis investigated effects of stress and shock anticipation on PPI and startle in healthy subjects [84]. Basically, they measured PPI both when the subjects

anticipated shocks (i.e. the threat condition) and when no shocks were anticipated (i.e. the safe condition). The authors argued that the fear induced by the threat condition would be superimposed on a generally stressful experience of the experiment. To distinguish effects of general alertness, they added a nonadversive control condition with a different group of participants. In this control experiment, participants were asked – and being paid for – to keep attention to auditory, visual and tactile stimuli in the test environment. As a result, both shock anticipation (i.e. fear) and attention to external stimuli significantly increased PPI. Regarding the increased PPI in the fear condition (later replicated by [85], the authors suggested that threat of shock may have increased the general level of alertness, which facilitates the processing of stimuli, thereby enhancing the effectiveness of the prepulse in inhibiting the startle response.

Repeated periods of shock anticipation however, were found to diminish PPI, an effect that was hypothesized to represent a progressive deficit in sensory functioning due to the prolonged stress of repeated shock anticipation. Apparently, this finding is not in agreement with animal work, where foot-shock stress had no effect on PPI (section 2.1.1). However, an important difference between human shock anticipation and the animal foot-shock paradigm is the physical component of the stress: during the entire experiment, human subjects only received a single shock, while animals were repeatedly exposed to shocks. In other words, the human threat-of-shock paradigm generally represented the psychological stress of potential threat, while the animal foot-shock stress had a large physical component of actual shocks, which might be less powerful. In this view, the human findings are consistent with the animal studies of (potential) threat, where diminished PPI was induced by the potential threat of bright light, and by the threat of predation (section 2.1.2.).

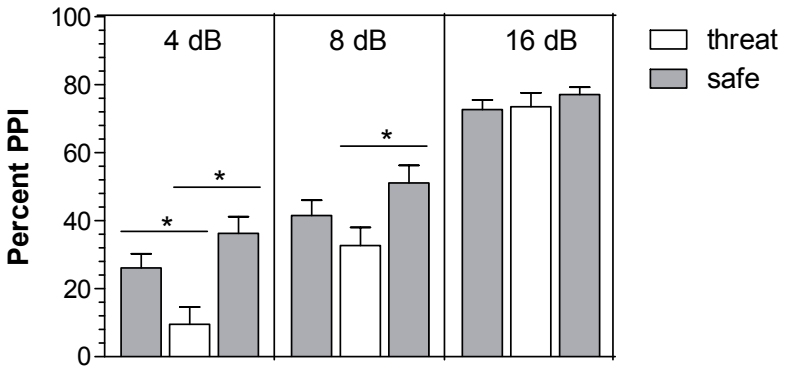

Fig. 1 PPI was measured under light-enhanced-startle conditions, with subsequent return to the dark (n=22). The test procedure was modified from [81], and consisted of three phases. During phase 1 and 3 rats were tested in the dark, whereas during phase 2 the test cubicle was brightly lit (900 lux). Following a 5-min acclimatization period, each phase started and ended with five pulse alone trials. In between, five different trials were presented 10 times each: pulse alone (115 dB, 50 ms duration), pulse preceded by a 20 msec prepulse of either 74, 78 or 86 dB and no-stimulus trials, average inter trial interval 15 s. Shown is percent PPI (mean + S.E.M.) in safe (black bars) and threat (open bars) conditions. Potential threat (i.e. bright light) significantly reduced PPI, and subsequent return to the safe (i.e. dark) condition significantly enhanced PPI. * $P < 0.05$.

2.3. Effects of acute stress on sensory gating: human studies

In addition to PPI, the brain's pre-attentive inhibitory functions are evaluated by an electrophysiological response-reduction paradigm, which is a paired-click test, wherein the P50 (or P1) wave of the vertex auditory event-related potential (i.e. computerized averages of the brain's electrical response to sound) is recorded. In healthy subjects, the P50 response to the second stimulus is generally attenuated (i.e. sensory gating), whereas in patients with schizophrenia and other psychiatric illnesses it is not suppressed [86, 87], indexing deficits in filtering out irrelevant sensory stimuli [88]. Like PPI, auditory sensory gating is influenced by stress. Note, that although the two experimental measures of brain inhibitory function are related, they are not identical in every respect, and partly regulated by different brain structures [89]. However, the fact that PPI and P50 gating are both measures of sensory gating that are influenced by stress, may point to possible common sources of functioning within the brain.

A few studies have reported decreased sensory gating following a stressful intervention. Effects of cold stress have been evaluated by the cold-pressor test, in which subjects have to submerge a hand to the wrist in cold water for a fixed period. To control for successful stress induction, subjective distress and arterial blood pressure were measured. With respect to PPI, the studies reported transient but significant deficits in response to cold stress, in addition to increased distress ratings and higher systolic arterial tension [90, 91]. Brief psychological stress has been found to exert similar effects. In a study by White and Yee [88], subjects were administered an oral arithmetic task; this mentally stressful task resulted in reduced P50 suppression in the participants. Possible effects of attention were ruled out by adding an equally difficult, but non-stressful arithmetic task, which did not affect P50 suppression.

2.4. Conclusion section 2

In table 1.a. and 1.b., an overview is given of studies reporting on effects of acute stressors in adult life on measures of sensory gating. Available evidence, from both animal and human subjects, indicates that acute stress can modify sensory gating. However, underlying neurobiological mechanisms remain poorly understood. Possibly, in first instance, moderate threat or attention to the environment could facilitate processing of sensory stimuli, for instance, via increased cortical arousal. Indeed, substantial evidence confirms that directing attention to the prepulse signal enhances PPI in humans and, likewise, emotional learning has been shown to enhance PPI in rats (reviewed by [92]). Severe or prolonged stressors on the contrary, may cause (progressive) loss of sensory perception that functions to reduce the impact of impending aversive events [84]. Particularly, these perceptual changes may be mediated by cortisol and CRF-induced activation of the mesolimbic dopamine system, a neural circuitry implicated in both stress responsivity and PPI.

However, some studies do not find an effect at all. From the animal work under review, this appeared to be most often the case when physical stressors were used, as opposed to psychological stressors (compare, table 1.a. and 1.b.). Due to the small number of available studies, it is difficult to draw definite conclusion from this finding. Clearly, the rodent PPI response is strain, age and gender dependent [93-95], and stress appears to differentially influence PPI across strains and sexes [96, 97]. Despite

these limitations, it could be speculated that psychological stressors may have a higher ethological validity, which could produce more pronounced effects on PPI. In this respect, it is interesting to note that the stress that is associated with episodes of relapse in schizophrenia patients is often of a psychological nature [23]. However, more studies on the effects of different types of stress on PPI are needed to reach a conclusion at this point.

Based on the available studies, it is suggested that in healthy organisms, alterations in PPI induced by acute stress in adulthood are probably reversible, not causing permanent break down of gating mechanisms. Considering the neurodevelopmental theory of schizophrenia (section 1.3), it may be more etiologically relevant to apply the experimental stressors in early life. Therefore, in the next section, the effects of neurodevelopmental interventions on the rodent PPI response will be discussed.

3. Early-life stress and prepulse inhibition: neurodevelopmental animal models

According to neurodevelopmental theories (section 1.3), schizophrenia is considered a developmental disorder, influenced by both genes and the (early) environment. To get more insight into the possible role of early risk factors in the development of schizophrenia, early-stress paradigms are applied to laboratory rodents. In the following section, some common approaches for introducing developmental stress are reviewed, with their subsequent impact on PPI in later life. Note that this section does not attempt to exhaustively cover all available types of early stress in relevant animal models. The interested reader is referred to more in-depth articles on this topic [98, 99].

3.1. Isolation rearing

One developmental manipulation that has received particular attention with respect to PPI is isolation-rearing. In this procedure, rats or mice are housed in single cages from the time of weaning (about 21 days after birth) until adulthood [100-103], and thereby deprived of social contact with their peers during (neuro)development [104]. In comparison to group-housed controls, postweaning-isolated rodents exhibit a range of brain and behavioral changes, reminiscent to schizophrenia [99, 105, 106]. Several studies have reported PPI deficits in isolation-reared rats [10] and mice [102, 103], which could be reversed by pretreatment with typical and atypical antipsychotics [107]. However, subsequent studies have indicated that the effect of isolation rearing is strain dependent [108], sensitive to housing conditions [109], developmental timing [110], and could be prevented by handling the isolated rats [111]. Moreover, in order to effectively disrupt PPI, isolation rearing has to be maintained until the moment of testing [100, 101]. Based on these drawbacks, the validity of isolation rearing as possible animal model for early life effects on schizophrenia-related behaviors is considered questionable [10, 97].

3.2. Maternal separation

Since rats and mice are born at a more immature stage of development than humans, neonatal interventions in these animals are comparable with adverse events in mid-late gestation in humans [99]. In this respect, maternal separation, being a neonatal neurodevelopmental model, differs fundamentally from isolation rearing (see previous section), which is a post weaning model. Maternal deprivation, or the temporary separation of rodent pups from their mother early in life, leads to various neurochemical changes, some with relevance to schizophrenia [99].

At the behavioral level, a single 24-hours period of maternal deprivation (at postnatal days 6 or 9) has been shown to induce deficits in PPI in a delayed fashion (i.e. arising after puberty), suggesting that certain long-term processes are set in motion by the early deprivation [50]. These deficits could be reversed by pretreatment with typical and atypical antipsychotics [50]. The fact that changes in PPI do not appear before adulthood, led Ellenbroek and co-workers to investigate whether the effect is unavoidable, or rather dependent on manipulations after the deprivation period [112]. First, they combined maternal deprivation with the post weaning isolation rearing procedure (see section 3.1.). Surprisingly, whereas both procedures were found to reliably disrupt PPI when applied separately, together they had no effect. Furthermore, they investigated the role of the mother, as the deprivation procedure obviously affects the dam, as well as the pups. To do this, either half of the litters were maternally deprived (in this way, the mother had pups to nurse during the deprivation period), or maternally deprived mothers were cross fostered to non-deprived pups and vice versa. In all cases, the pups displayed small deficits in PPI, compared to fully deprived controls, suggesting that the behavior of the mother, and possibly, her milk

Table 1.a. Overview of animal and human studies on effects of physical stressors in adult life on measures of sensory gating. PPI, prepulse inhibition; P50; P50 wave of event-related potential; n.d., not determined; ↑, ↓, =, response is improved, diminished, unchanged, respectively, when compared to control conditions.

Physical stress						
Subject	**Intervention**	**Stress-test interval**	**PPI**	**Startle**	**P50**	**Reference**
Human	Cold stress	0	n.d.	n.d.	↓	[90, 91]
Rat	Cold stress	20 min	↓	=	n.d.	[70]
Rat	Foot-shock	5 days	↑	↑	n.d.	[73]
Rat	Foot-shock	0, 24 and 48 hr, 9 d	=	=	n.d.	[65]
Rat	Foot-shock	2 weeks	=	=	n.d.	[74]
Rat	Restraint	0	=	=	n.d.	[75]
Rat	Restraint	5 min	↓, =	=	n.d.	[76]
Rat	Restraint	30 min	↓	=	n.d.	[77]
Rat	Restraint	3 weeks	=	=	n.d.	[74]

Table 1.b. Overview of animal and human studies on effects of emotional stressors in adult life on measures of sensory gating. PPI, prepulse inhibition; P50; P50 wave of event-related potential; n.d., not determined; ↑, ↓, =, response is improved, diminished, unchanged, respectively, when compared to control conditions.

Subject	Intervention	Stress-test interval	PPI	Startle	P50	Reference
Human	Mental stress	0	n.d.	n.d.		[88]
Human	Attention Threat - brief prolonged	0	↑ ↑ ↓	= ↑ n.d.	n.d.	[84]
Rat	Threat (intense light)	0	↓	↑	n.d.	Current chapter
Rat	Predator	24 hrs	↓	=	n.d.	[65]
Rat	Predator	0, 46 hrs, 9 d	=	=	n.d.	[65]
Rat	Novelty (light)	0	↓	↓	n.d.	[82]
Mouse	Social defeat	24 hrs	↓	=	n.d.	[80]
Rat	Witness stress	0	=	=	n.d.	[75]
Rat	Witness stress	5 days	=	=	n.d.	[73]

production, is also affected by the deprivation period. Thus, the authors concluded that the post-deprivation period is of crucial importance for the development of prepulse inhibition deficits in maternally deprived rats. Also, methodological factors such as timing, duration, and number of deprivation episodes, could possibly explain a lack of effect of maternal deprivation [112]. Thereby, the ability of maternal separation to produce PPI-disrupting effects appears to be dependent on genetic strain [113], and species under study (rat vs. mouse) [114-116].

Thus, maternal separation seems to induce PPI-deficits in a delayed fashion; however, the effect could be influenced by various protecting or facilitating post-deprivational factors, which might be similar to the influence of early-life stressors in humans.

3.3. Prenatal maternal immune activation

Epidemiological, clinical and preclinical studies have provided evidence that gestational exposure to certain infections, such as influenza, contributes to the etiology of schizophrenia (see Introduction). Similarly, animal models of maternal immune activation have yielded behavioral, neurochemical and neurophysiological findings that are consistent with observations in schizophrenia patients [117]. Currently, specific candidate infections have been identified that appear to be associated with an increased risk of schizophrenia, including rubella, influenza, herpes simplex, toxoplasma gondii, measles, polio, and genital and/or reproductive infections [118]. A mechanism common to the immune response accompanying these infections, is the release of inflammatory cytokines. Consequently, elevation of maternal cytokine levels during pregnancy is thought to alter the trajectory of brain development, resulting in the induction of

pathophysiological processes associated with mental illness [98]. Animal models of maternal immune challenge have used different immunogenic agents, all inducing a cytokine-associated inflammatory response in the mothers. However, other factors could be of relevance as well, for instance, immune activation is associated with fever, weight loss and elevated corticosteroids, which might compromise the offspring's *in utero* metabolic needs, thereby possibly affecting fetal brain development [98]. The effects of maternal infection on PPI have been investigated in several studies.

Systemic administration of bacterial endotoxin lipopolysaccharide (LPS) is capable of inducing a powerful immune response in the exposed animal, as well as fever and weight loss [98]. While LPS can be detected in both maternal and placental tissues, it is not found in the fetus [119], indicating that LPS itself is not responsible for the effects of maternal infection on the fetal brain. Several studies reported PPI disruptions in adult rat offspring of LPS infected mothers [120-123]. These PPI deficits were associated with changes in dopaminergic transmission, and could be reversed by adult treatment with antipsychotics [120, 122]. Interestingly, next to LPS, also turpentine, i.e. an inducer of local inflammation, at doses known to produce fever, significantly decreased PPI in adult offspring [121]. In analogy to the human situation, the influence of prenatal human influenza virus exposure on later brain development and behavior has been studied. Respiratory infection of pregnant BALB/c and C57BL/6 mice with the human influenza virus resulted in various behavioral abnormalities in the adult offspring, among which deficits in PPI, which were sensitive for antipsychotics [124]. In other studies, offspring of similarly infected mice displayed morphological and neurochemical changes reminiscent to schizophrenia, although the importance of these effects for the PPI deficits is unclear [99]. Of note, however, is the large reduction in expression of the brain protein reelin in cortex en hippocampus [125], which is associated with both schizophrenia [126] and impaired PPI [127]. Lastly, viral infection is simulated in rats and mice by polyriboinosinic-polyribocytidilic acid (poly I:C), an agent structurally similar to double-stranded RNA, which forms the genetic material of some viruses. While administration of poly I:C to pregnant rodents can result in increased levels of cytokines in fetal brain, it only generates a non-specific immune response, without particular anti-viral antibodies (for references, see [98]). With respect to PPI, treatment of pregnant mice or rats with poly I:C generates offspring that shows an impaired PPI response from post pubertal age [128-131], which is presumably mediated by dopaminergic maldevelopment [128, 131].

Thus, animal models of prenatal maternal infection show altered fetal brain development and disrupted PPI in adult offspring, probably mediated by the maternal immune response. These findings are in line with epidemiologic and clinical investigations on infection as a risk factor of schizophrenia [117].

3.4. Multiple stressors

Based on the two-hit hypothesis of schizophrenia (see Introduction), several experimental animal studies have applied multiple interventions at different stages of development, to investigate their combined influence on schizophrenia-like behaviors. Possibly, this method may give additional mechanistic insights compared

to single interventions alone. In the remaining part of this section, studies addressing the combined effects of multiple interventions on PPI will be discussed.

In one study, the interaction between stress and dopaminergic regulation of PPI was investigated [132, 133]. After combining two subsequent stressors in neonatal and young-adult life (i.e., maternal deprivation and prolonged corticosterone treatment, respectively), PPI was tested in rats following acute injections with apomorphine. In controls and in rats that had undergone either one of the two stressors, the apomorphine treatment was found to disrupt PPI, while in the group that had experienced the multiple stress, no PPI-disruptions were observed in response to apomorphine [132]. According to the authors, their findings implicate an inhibitory interaction of early and late developmental stress, on dopaminergic regulation of PPI [132]. In a follow-up study, the authors suggested that this 'inhibitory interaction' may be caused by receptor desensitization, because no changes were found in levels of dopamine D1 and D2 receptors [133]. Notably, the finding that a subsequent stressor could reverse PPI-disruptive effects of a particular stressor, has been reported elsewhere (i.e., maternal separation and isolation rearing, [112]). The above-mentioned experiments are in line with the idea that glucocorticoids could affect PPI by modulation of dopaminergic systems (see section 2.2.).

In most studies addressing the two-hit hypothesis, the 'first hit' comprises a genetic predisposition. Consequently, it is investigated how genes interact with early stress to produce schizophrenia-like neurochemical and behavioral alterations. As the focus of this chapter lies on the effects of stress on PPI, we will not discuss the neurochemical findings from genetic animal models of schizophrenia. Genotypes that have been found to interact with early stress to influence PPI include, nuclear receptor Nurr1 heterozygosity (i.e., 12 weeks of isolation rearing – Eells et al., 2006), Snap-25 mouse mutant *blind-drunk* (i.e., variable prenatal stress – Oliver & Davies, 2009), and NMDA receptor hypofunction mouse mutant (predation stress – Duncan et al., 2004). Notably, the latter study made use of predator olfactory cues (i.e., rat odor), which normalized the reduced PPI that was observed under control conditions in male mutants only. This result is in contrast to the study of Bakshi (section 2.1.3.), where predator exposure was found to decrease PPI in rats. However, an important methodological difference should be noted. Bakshi and co-workers actually introduced a predator in their experiment, while Duncan et al. only exposed the animals to predator odor. This difference in approach might have implications for the level of threat perceived by the subjects. In particular, when solely olfactory cues signal predation risk, perceiving animals may become more vigilant, which is thought to facilitate processing of sensory stimuli, possibly increasing PPI (section 2.5.). Proximal presence of the predator, on the other hand, is likely to represent a severe stressor, which may induce disruptions in PPI due to progressive loss of sensory perception (see section 2.5.). Possibly, the delayed stress effect observed in the Bakshi study could be accounted for by recruitment of central mediators of the stress response, such as CRF.

In conclusion, although limited, the studies mentioned in this section suggest that combining multiple stressors, or a genetic vulnerability and stress, may induce stronger alterations in sensorimotor gating, when compared to one single intervention. Further relevant studies are warranted to test this hypothesis.

4. General conclusions

In this chapter, we have discussed studies that investigated the association between experience of stress and PPI, the latter being a highly validated model for schizophrenia; a mental disorder that is thought to be caused by an interaction of a constitutional vulnerability with environmental stress (see Introduction). From both animal and human studies, it is found that – by an unknown mechanism – application of acute stressors in adulthood is able to affect PPI, at least, transiently (table 1). Possibly, on the short term, moderate threat or attention to the environment could facilitate processing of sensory stimuli via elevated cortical arousal, leading to increased information processing; while severe or prolonged stressors may cause (progressive) loss of sensory perception that functions to reduce the impact of impending aversive events (section 2.4.). These perceptual deteriorations may be mediated by, for instance, cortisol- and CRF-induced activation of the mesolimbic dopamine system, a neural system that is implicated in both stress responsivity and PPI. Also, it is suggested that alterations in PPI induced by the acute stressors under study are probably reversible, not causing permanent changes to brain structures important in the regulation of PPI. These results are in line with clinical and epidemiological findings, which suggest that experience of stressful life events does not trigger onset of psychotic illness in healthy individuals (see Introduction). However, clearly, the acute and chronic stressors applied in the laboratory do not mimic 'naturally occurring' transient stress in the real world. This type of stress may come and go, and, depending on how long it stays, it could ultimately cause permanent alterations in brain systems associated with psychosis in vulnerable individuals, as was observed in longitudinal studies (reviewed by [3, 52]).

The clinical observation that schizophrenia generally does not manifest until the third decade of life, suggests that it is the final outcome of pathological processes acting on the immature brain, and accordingly, it has led to formulation of the neurodevelopmental theory for the etiology of schizophrenia (see Introduction). In order to get more insight into the possible role of early environmental risk factors in illness development, early-stress paradigms are applied to rodents. Some developmental manipulations that have been shown to affect adult PPI include isolation rearing, maternal separation, and prenatal maternal immune activation (section 3). An alternative hypothesis within the framework of the neurodevelopmental theory of schizophrenia is referred to as the two-hit model. According to this model, early and late risk factors are not simply additive, but instead, the first hit will increase an individual's vulnerability for effects of a subsequent hit (see Introduction). Based on this theory, a few animal studies have applied multiple interventions at subsequent stages of development, to study their combined impact on schizophrenia-like behaviors, including PPI. Interestingly, although limited, results so far do not support the theory. Rather, instead of an augmentation, an inhibitory interaction of early and later developmental stress has been reported by two independent research groups. On the other hand, several studies have successfully identified candidate genes that could contribute to the induction of schizophrenia-like phenotypes in interaction with stress (section 3.3.). Possibly, investigating the interaction of these candidate genes

with experimental stressors in animal studies, could represent a fruitful approach to model the link between stress and schizophrenia.

In humans, it is known that a wide variation exists in response to adversity, with some individuals being more stress-sensitive than others, and some individuals being more prone to developing an illness in response to environmental adversity than others [134]. Attempts have been made to explain the source of this variation; several studies have investigated the link between genetic polymorphisms and environmental stress in the etiology of schizophrenia. Some progress has been made, for instance, a recent genome-wide association study – contrasting large numbers of genetic variants in patients and controls – revealed significant associations between schizophrenia and polymorphisms in major histocompatibility complex (MHC), a region implicated in the bodily reactions to stress and infection [135]. Also, although not yet replicated, the novel schizophrenia risk polymorphism ZNF804A was found to be associated with increased prefrontal-hippocampal and prefrontal-amygdala connectivity, possibly linking to increased sensitivity to stressful environments [136]. In another study, the association between a serotonin transporter gene polymorphism (i.e. 5-HTTLPR), stress and disease characteristics was investigated in individuals diagnosed with psychotic disease [137]. Therefore, symptoms occurring in the four-week period preceding hospitalization were evaluated in first-onset patients. As a result, stress (i.e. negative life-events preceding hospitalization) was found to be a predictor of depressive symptoms, but it did not interact with psychotic (or negative) symptoms. Together, the above-mentioned studies have genetically linked schizophrenia with several systems/brain structures important in stress regulation (e.g., MHC, amygdala, PFC). However, so far, no experimental studies have linked this gene-environment interaction with schizophrenia, which is probably caused by the fact that schizophrenia is a complex disorder, putatively determined by the sum of numerous small effects of individual genes; hence the importance of using endophenotypes such as PPI in association studies.

In conclusion, the link between stress and PPI appears to be in line with neurodevelopmental theories of schizophrenia; a single stressor in adult life does not seem to cause lasting alterations in PPI, however, when applied during a critical stage of (early) neurodevelopment or in genetically vulnerable organisms, stress could be more powerful in robustly affecting PPI. It is suggested, that future animal studies aimed at investigating the role of stress in the development of information processing dysfunctions in schizophrenia, should implement human risk gene polymorphisms that are associated with stress (e.g. by making use of inducible transgenic mouse models).

Chapter 3

Valproate improves prepulse inhibition deficits induced by corticotropin-releasing factor independent of $GABA_A$ and $GABA_B$ receptor activation

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Abstract

Corticotropin-releasing factor (CRF) is implicated in the pathogenesis of bipolar disorder, an illness associated with deficits in prepulse inhibition (PPI) of the acoustic startle response. Valproate is used in the treatment of bipolar disorder and may alter CRF activity via a $GABA_A$-ergic mechanism. This study determined the effect of valproate on CRF-disrupted PPI and examined the role of the hypothalamic-pituitary-adrenal axis and GABA-ergic signaling in the effect of valproate. Valproate (60-240 mg/kg) dose-dependently reversed PPI deficits displayed by transgenic mice overexpressing CRF (CRFtg), and normalized PPI deficits induced by CRF i.c.v. infusion in 129Sv mice. Valproate enhanced corticosterone secretion more effectively in CRFtg than in wild-type mice. The effect of valproate on PPI was not blocked by the $GABA_A$ receptor antagonist bicuculline, the $GABA_B$ receptor antagonists phaclofen and SCH-50911 or combined administration of a $GABA_A$ and $GABA_B$ receptor antagonist. The beneficial effect of valproate on PPI was not mimicked by the $GABA_A$ receptor agonist muscimol, the GABA transaminase inhibitor vigabatrin, the histone deacetylase (HDAC) inhibitor sodium butyrate or by the mood stabilizers lithium, carbamazepine, lamotrigine or topiramate.

Thus, we showed that valproate improves CRF-induced PPI deficits, albeit via a so far unknown mechanism. These marked beneficial effects of valproate on CRF-induced sensorimotor gating deficits suggest that valproate may be of particular value in specific subgroups of bipolar patients that are characterized by alterations in the CRF system.

1. Introduction

Cumulative evidence indicates that stress is an important factor in the pathogenesis of schizophrenia and bipolar disorder [138]. The corticotropin-releasing factor (CRF) system takes a cardinal position in the regulation of stress responses [139]. Decreased levels of CRF-binding protein mRNA were found in amygdala post-mortem tissue from male schizophrenic subjects [64], and positive treatment response to the antipsychotic quetiapine was associated with a decrease in CRF levels in CSF of schizophrenia patients [140]. Furthermore, genetic variations in CRF-related genes have been associated with schizophrenia, schizoaffective disorder and bipolar disorder (reviewed by [141].

These disorders are associated with deficits in prepulse inhibition (PPI) of the acoustic startle response [46]; that is, the reduction in the startle reflex produced by a weak prepulse stimulus. In schizophrenia patients, deficient PPI is thought to reflect a loss of sensorimotor gating that may lead to sensory flooding and the inability to filter irrelevant thoughts from intruding into awareness [45, 46]. In rodents, both intraventricular (i.c.v.) infusion of CRF and long-term central CRF overexpression disrupt PPI [28, 29, 66, 142]. Interestingly, transgenic mice overexpressing central CRF (CRFtg) show neuroendocrine changes [143], that are reminiscent of bipolar disorder [144] and schizophrenia [52]. Moreover, the disrupted PPI response of CRFtg mice is improved by antipsychotics such as haloperidol, risperidone, and clozapine [28].

Valproate is a mood stabilizer that has been used for the treatment of bipolar disorder [145] and, to a lesser extent, schizophrenia [146]. Definitive mechanisms mediating its clinical efficacy are unclear, but enhancement of GABAergic neurotransmission through inhibition of the metabolic enzyme GABA transaminase [147], reduction of neuronal excitability by affecting intracellular signaling pathways [148] and inhibition of histone deacetylase (HDAC) [149] may play a role. In rats, valproate has been shown to alter the expression of CRF and its receptors [150, 151]. In addition, valproate inhibited CRF secretion and synthesis in rat hypothalamic neurons *in vitro*. These effects could be blocked by the $GABA_A$ receptor antagonist bicuculline and be mimicked by the $GABA_A$ receptor agonist muscimol, suggesting involvement of increased $GABA_A$ receptor activation [152]. These are interesting findings, given the putative role of CRF in the pathophysiology of bipolar disorder and schizophrenia, and the proposed alterations in the GABA-ergic system upon prolonged exposure to elevated CRF levels [153-157]. The effects of valproate on CRF-induced behavioral alterations however, have not been studied so far. Such studies could contribute to our knowledge of mechanisms relevant for the etiology and treatment of these stress-related disorders.

Here we show that compared to other commonly used mood stabilizers, valproate is particularly effective in improving CRF-induced PPI deficits. Next we studied several potential mechanisms underlying the beneficial effect of valproate on PPI in CRFtg mice, including GABAergic signaling, the HPA axis and inhibition of histone deacetylase (HDAC).

2. Materials and Methods

2.1. Animals

All experiments were performed according to the Guide for Care and Use of Laboratory Animals and were approved by the Ethical Committee for Animal Research of Utrecht University.

CRFtg mice (line 2122, eighteenth generation) were generated as previously described [66]. Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF cDNA (600 bp fragment), which was inserted into a 8.2-kb genomic DNA-fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence. Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons. Subsequent breeding at the local breeding facilities (Utrecht, the Netherlands) consisted of matings between heterozygous transgenic males (C57BL/6J backgound) and C57BL/6JIco females (Charles River, the Netherlands). Genotyping was done by PCR. For PPI and corticosterone experiments, male CRFtg mice, 9-16 weeks old, were used, with their wild-type (WT) littermates serving as control mice. In the ultrasonic vocalization (USV) experiment, both male and female CRFtg and WT pups were used, 7-10 days of age.

The CRF infusion PPI experiment was performed on male 129SvEvTac mice, 10-16 weeks old (Taconic, Denmark). This strain was chosen, because it is more sensitive to the effects of CRF on PPI [142].

Animals were group-housed in bedded plastic cages (type X), enriched with a piece of PVC-tubing and paper tissue, at constant room temperature (21 ± 2°C) and relative humidity (50-60%). Mice were maintained on a 12:12 light/dark cycle (lights on: 06:00-18:00 h). Standard rodent food pellets (Special Diet Services, Witham, Essex, United Kingdom) and water were freely available.

2.2. CRF infusion: surgery

One week after arrival, the animals were systemically anesthetized using isoflurane gas anesthetic (2-3%, Isoflo, Abbott) mixed with oxygen and nitrous oxide. In the wound space, an additional local anesthetic was applied (Lidocaïne 5%, Alfacaine, Alfasan). Each mouse was prepared with a 23 gauge 2.5-mm-length unilateral guide cannula (Plastics One) into the lateral ventricle (flat skull; anteroposterior, -0.2 mm; mediolateral, + 1 mm; dorsoventral, -2.5 mm from bregma), which was secured with dental cement. To make sure the cement would be held in place, shallow lines were carved into the skull. Plastic dummies were used to close the cannulae. Before onset of testing, the animals were allowed to recover for one week. At the end of the experiment, cannula placements were assessed by infusion of methylene blue dye and verification of dye in the ventricular system (no mice excluded).

2.3. Corticosterone determination

Mice were decapitated 30 minutes after valproate treatment (0, 120, 240 mg/kg IP). Blood samples were collected between 9:00 AM and 12:30 PM. Trunk blood (500-700 µl) was collected in ice-cooled Eppendorf cups containing 25 µl (0.21 M) ethylenediamine

tetra-acetate. Plasma was separated by centrifugation (3000 rpm for 10 min at 4°C), and aliquots were stored at -70°C until assayed. Plasma corticosterone concentrations were measured in duplicate using a double antibody radioimmunoassay for rat corticosterone (ICN Biochemicals, Zoetermeer, the Netherlands).

2.4. Drugs

Valproic acid sodium salt (a.k.a. valproate) (60, 120, 240 mg/kg), bicuculline methiodide (2, 5 mg/kg), phaclofen (3-Amino-2-(4-chlorophenyl)propanephosphonic acid) (7.5, 15 mg/kg), SCH-50911 ((2*S*)-(+)-5,5-Dimethyl-2-morpholineacetic acid) (15, 30 and 60 mg/kg), muscimol (0.33, 1.0, 3.0 mg/kg) (3-Hydroxy-5-aminomethyl-isoxazole, 5-Aminomethyl-3-hydroxy-isoxazole, 5-Aminomethyl-3-isoxazolol), vigabatrin ((±)-γ-Vinyl-GABA) (200, 400, 800 mg/kg), sodium butyrate (0.3, 0.6, 1.2 mg/kg), and lithium chloride (10, 20, 40 mg/kg), were dissolved in saline. Mifepristone (45 mg/kg) (11**β**-(4-Dimethylamino)phenyl-17**β**-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), carbamazepine (5*H*-Dibenz[*b,f*]azepine-5-carboxamide) (20, 40, 80 mg/kg), lamotrigine (6-(2,3-Dichlorophenyl)-1,2,4-triazine-3,5-diamine, GI 267119X) (3, 9, 27 mg/kg) and topiramate (2,3:4,5-*Bis*-*O*-(1-methylethylidene)-β-D-fructopyranose sulfamate) (10, 100, 300 mg/kg) were suspended in a vehicle containing saline and Tween 80 (3%). Solutions were freshly prepared daily. Valproate, bicuculline, phaclofen, mifepristone, vigabatrin, lamotrigine and sodium butyrate were obtained from Sigma-Aldrich, Steinheim Germany; the other drugs were gifts from Servier, Croissy/Seine France. Compounds were administered intraperitoneally (IP) in a volume of 10 ml/kg, 30 minutes before the start of the test, except for mifepristone and vigabatrin which were injected 1 and 5 hours before the test, respectively. Drug doses were chosen based on literature, or dose-response pilots (bicuculline and phaclofen, not shown). Each drug was tested in a separate cohort of animals. In experiments *1.1, 3.1.c* and *3.1.d*, mice were tested in a within-subject, balanced Latin-square design, receiving each dose and vehicle once. In the other experiments, drugs were tested in a between-subjects design (CRF-infusion experiment, valproate with phaclofen and bicuculline, valproate with SCH-50911, muscimol, and vigabatrin). On any occasion, time between tests was at least one week.

2.5. CRF infusion

Human/rat corticotrophin-releasing factor (h/r-CRF, Bachem, Weil am Rein, Germany) was dissolved in 0.05M acetic acid and stored at -80°C until use. Infusion samples, freshly prepared on each testing day, were diluted 1:10 in sterile artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$ *$2H_2O$, 25 mM $NaHCO_3$, 25 mM glucose and 0.5% albumin). Vehicle consisted of 10% 0.05 M acetic acid in aCSF. 1 µg h/rCRF (based on pilot studies, not shown) or aCSF was infused 60 minutes before the test by means of an internal cannula projecting 0.5 mm beyond the tip of the guide cannula, in a volume of 2 µl (1 µl/min). The infusion cannulae were connected to Hamilton syringes, placed in a syringe pump (KdScientific 220 sersies, USA) via polyethylene tubing. During the infusion procedure, mice were lightly anesthetized using a cocktail of isoflurane gas anesthetic (2-3%), oxygen and nitrous oxide.

2.6. Prepulse inhibition of the acoustic startle reflex

Startle reflexes were measured in eight identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA). Startle stimuli (110 dB, 50 msec) were presented alone, or preceded by noise prepulses (20 msec) of 2, 4, 8, or 16 dB above background (70 dB), with 100 ms between onsets of the prepulse and startle stimuli. The test session started with a 5-min acclimation period followed by three consecutive blocks of test trials (block 1 and 3, startle-stimulus alone trials; block 2, startle-stimulus alone, startle + prepulse, and no-stimulus trials). Intertrial intervals ranged from 25 to 35 sec, and total test duration was 45 min. One week before drug testing, a baseline PPI measurement was performed, in order to familiarize the mice to the test procedure and to create treatment groups with equal mean percent PPI.

2.7. Data analysis

Percent PPI was calculated as the mean startle response to startle pulse-alone, minus the mean startle response to startle + prepulse stimuli, all divided by the mean startle response to startle pulse-alone stimuli, and multiplied by 100. For calculation of the means, only data from block 2 were used, as previously described [28].

In within-subjects PPI experiments in CRFtg mice, data were analyzed using repeated-measures analysis of variance (ANOVA) with prepulse intensity (four levels) and dose (four levels) as within-subject factors and genotype (two levels) as the between-subjects factor. Vigabatrin was tested in a between subjects design, because of its irreversible character. In the CRF infusion experiment, CRF and valproate treatment were tested as between-subjects and prepulse intensity as within subject facors. In the antagonist studies, PPI-data were collapsed across prepulse intensity, as there were no intensity x valproate drug interactions. Two repeated-measures ANOVAs were performed; one to determine the effect of valproate on PPI in CRFtg mice per se, the other to determine the effect of combined treatment with different doses of antagonists and valproate on PPI. In two antagonist experiments (valproate with SCH 50911, valproate with bicuculline and phaclofen), treatment effects were analyzed using a between subjects design, because of drug-unrelated death of two mice. Corticosterone data were analyzed using ANOVA with genotype and treatment as between-subject factors. *Post hoc* analyses followed significant interaction or main effects, using separate ANOVAs or Dunnett's and Bonferroni corrected *t* tests. All differences were considered to be significant if $p < 0.05$.

3. Results

In every PPI experiment reported here, percent PPI increased significantly with increasing prepulse intensity, and this effect will not be further described. Data on baseline startle are summarized in table 1.

Study 1: effect of valproate on CRF-induced PPI deficits

Experiment 1.1: effect of valproate on PPI deficits in CRFtg mice

The effect of valproate on PPI was dependent on genotype ($F[3,63]=3.5$, $p=0.02$) and on prepulse intensity ($F[9,189]=2.2$, $p=0.023$). Analysis per genotype showed that valproate improved PPI in CRFtg mice ($F[3,33]=8.9$, $p<0.001$) independent of prepulse intensity. At the highest dose tested (240 mg/kg), valproate completely reversed the PPI deficits of CRFtg mice. In WT mice, the effect of valproate was dependent on prepulse intensity ($F[9,99]=3.5$, $p=0.001$). Further analysis per prepulse intensity did not show significant effects of particular doses (figure 1*a*). Effects of valproate on baseline startle were dependent on genotype (see table 1, $F[3,63]=4.7$, $p=0.005$). Post hoc analysis revealed that valproate significantly reduced the startle response in WT mice at 240 mg/kg, whereas it tended to increase startle response in CRFtg mice (N.S.).

Experiment 1.2: effect of valproate on h/r-CRF (i.c.v.)-induced PPI disruption

CRF-induced PPI deficits were dependent on prepulse intensity ($F[2,111]=5.0$; $p=0.007$), and significant at 2, 4 and 8 dB. The effect of valproate on PPI was dependent on CRF treatment and prepulse intensity (3-way interaction; $F[2,111]=3.3$; $p=0.022$). Further analysis showed that valproate completely reversed the CRF-induced PPI deficit, whereas the drug had no effect in control animals (fig. 1*b*). CRF significantly increased baseline startle (aCSF, 599.33 ± 51.20, h/r-CRF, 1016.42 ± 214.15) ($F[3,47]=3.1$, $p<0.05$), an effect that was not altered by valproate. Also, valproate did not affect baseline startle response.

Experiment 1.3: effect of carbamazepine, lithium, lamotrigine and topiramate on PPI deficits in CRFtg mice

Detailed results of these studies are reported in the supplementary material section.

Briefly, lithium and carbamazepine had no effect on percent PPI. The highest dose of lamotrigine (27 mg/kg) improved PPI but only at the highest prepulse intensity. This

Table 1 The effects of drugs that significantly affected baseline startle response. * p <0.05

Compound	Dose (mg/kg)	Startle (mV)	
		WT	TG
Valproate	0	576.0 ± 85.5	453.5 ± 73.5
	60	530.0 ± 42.1	468.0 ± 102.7
	120	477.9 ± 60.5	501.8 ± 67.9
	240	359.5* ± 41.5	543.3 ± 82.3
Muscimol	0	395.9 ± 223.9	523.8 ± 42.2
	0.33	410.9 ± 90.4	467.3 ± 49.0
	1.0	416.4 ± 68.2	442.2 ± 39.6
	3.0	175.6*± 75.0	282.2* ± 55.7
Vigabatrin	0	385.8 ± 55.6	409.9 ± 47.7
	200	344.3 ± 73.4	307.4 ± 99.2
	400	445.9 ± 123.6	397.5 ± 88.7
	800	74.8* ± 39.2	284.6* ± 97.1

WT, wild type; TG, CRFtg

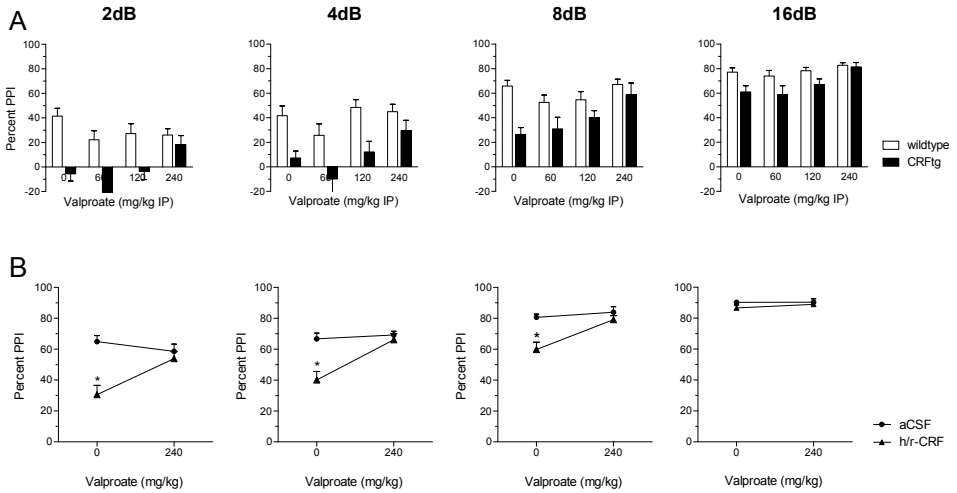

Fig. 1 (A) Effect of valproate on (A) percent PPI in WT (white bars) and CRFtg mice (black bars), and on (B) icv CRF-induced PPI disruption at different prepulse intensities. Group sizes: (A) WT n=12, CRFtg n=11; (B) aCSF-veh: n=14; h/rCRF-veh: n=12; aCSF-valproate: n=9; h/rCRF-valproate: n=6. aCSF: artificial cerebrospinal fluid, h/rCRF: human/rat corticotrophin-releasing factor. Data are expressed as mean + SEM.

effect was similar in WT and CRFtg mice. Topiramate (100, 300 mg/kg) improved PPI in CRFtg mice. This effect was only significant for prepulses of 4 dB. In WT mice, the highest dose of topiramate (300 mg/kg) significantly reduced PPI, independent of prepulse intensity.

Study 2: role of GABA-ergic neurotransmission in the effect of valproate on PPI deficits in CRFtg mice

Drug treatment effects were independent of prepulse intensity in all studies reported here. For the purpose of clarity, PPI was therefore collapsed across prepulse intensity.

Experiment 2.1: Effects of $GABA_A$ and $GABA_B$ receptor antagonists on beneficial effects of valproate in CRFtg mice

Valproate (240 mg/kg) significantly increased PPI in CRFtg mice in all four antagonist experiments (see figure 2*a-d*, table 2). As valproate did not alter baseline startle in CRFtg mice and as there were no significant interaction effects between antagonists and valproate with respect to baseline startle response (not shown), only effects on PPI are reported here.

Neither the $GABA_A$ receptor antagonist bicuculline, nor the $GABA_B$ receptor antagonists phaclofen and SCH-50911, nor combined administration of bicuculline with phaclofen blocked the PPI-effect of valproate in CRFtg mice (figure 2*a-d*), see table 2 for ANOVA results. As expected, PPI of CRFtg mice was significantly lower than in WT mice in all four experiments (planned comparison t-tests in vehicle-vehicle groups).

Experiment 2.2: effect of the $GABA_A$ receptor agonist muscimol on PPI in CRFtg mice

The effect of muscimol on PPI was dependent on genotype ($F[3,101]=8.7$; $p<0.001$). Analysis per genotype showed that muscimol did not alter PPI in CRFtg mice, while the

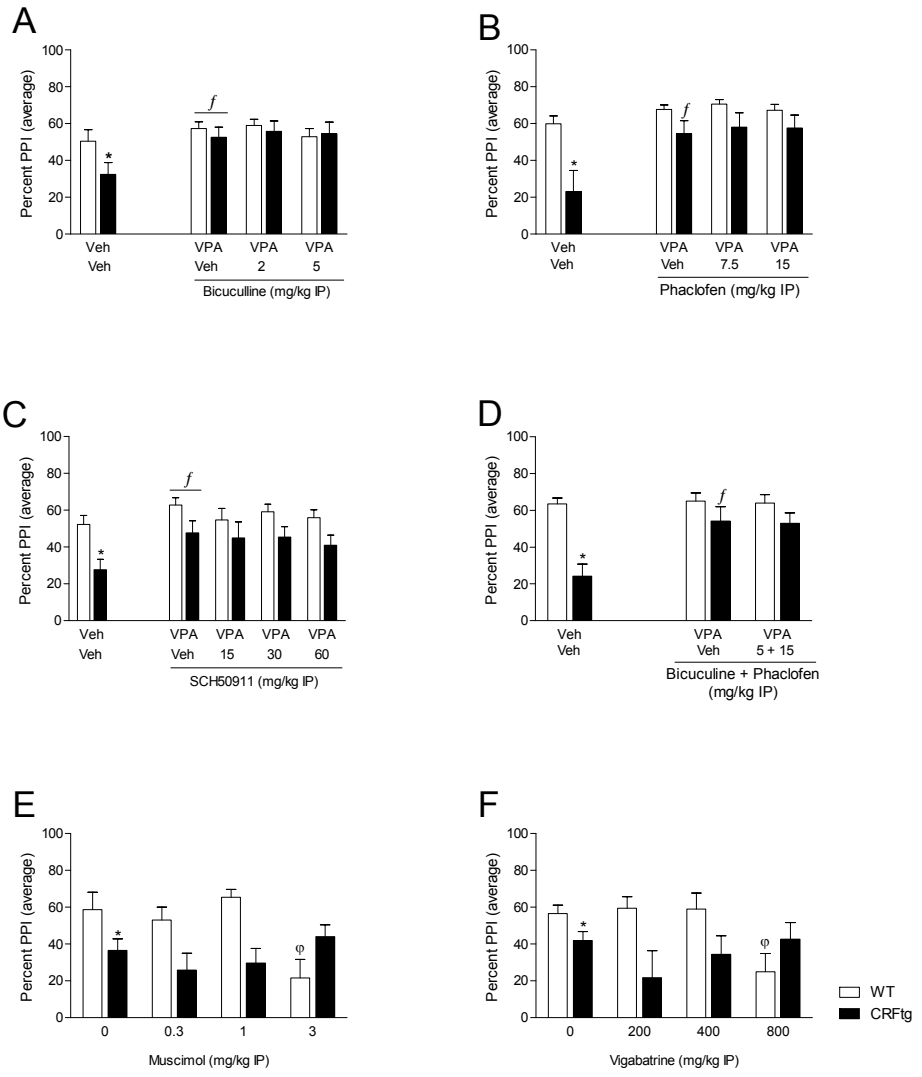

Fig. 2 Percent PPI in WT (white bars) and CRFtg mice (black bars) following treatment with valproate (A-D) co-administered with bicuculline (A), phaclofen (B), SCH-50911 (C) or the combination of bicuculline and phaclofen (D), muscimol (E), vigabatrin (F). Group sizes, valproate and bicuculline: WT, TG n=13; - phaclofen: WT n=14, CRFtg n=12; - SCH 50911: WT n= 9-19, CRFtg n=11-21; - bicuculline and phaclofen: WT n=11-17, CRFtg n=14-19, muscimol: WT, TG n=14; vigabatrin: WT, TG n=8 (drug groups), n=24 (vehicle groups). Data are expressed as mean + SEM. *p<0.05, genotype difference in control condition; f p<0.05, valproate treatment effect (VPA-Veh) vs. vehicle control group(s) (Veh-Veh); ϕ p<0.05, genotype difference in drug condition.

drug significantly reduced PPI in WT mice at the highest dose tested (Dunnett's after significant ANOVA $F(3,52)=9.4$; $p<0.001$) (figure 2*e*). Muscimol significantly reduced baseline startle in both genotypes at the highest dose tested (3 mg/kg) ($F[3,101]=5.2$, $p=0.002$).

Experiment 2.3: effect of GABA-transaminase inhibitor vigabatrin on PPI in CRFtg mice
The effect of vigabatrin on PPI was dependent on genotype (F[3,87]=4.9; p=0.003). Analysis per genotype showed that vigabatrin did not alter PPI in CRFtg mice, and significantly reduced PPI in WT mice at the highest dose tested (Dunnett's test after significant ANOVA F(3,44)=6.0; p=0.002) (figure 2*f*). Vigabatrin 800 mg/kg significantly reduced startle in both genotypes (Dunnett's after significant ANOVA F[3,87]=3.9; p=0.011).

Study 3: involvement of corticosterone in the effect of valproate

Experiment 3.1: effect of valproate on plasma corticosterone levels in CRFtg mice
The effect of valproate on plasma corticosterone levels was dependent on genotype (F[2,51)=6.0; p=0.005). Further analyses showed that in WT mice, valproate significantly increased plasma corticosterone levels only at the highest dose (F(2,26)=16.9; p<0.001), while in CRFtg mice, both 120 and 240 mg/kg valproate significantly increased plasma corticosterone levels (F(2,28)=30.6; p<0.001) (figure 3*a*).

Experiment 3.2.: involvement of HDAC inhibition in the effects of valproate
The HDAC inhibitor sodium butyrate had no significant effect on percent prepulse inhibition (F[3,63]= 1.5, p= 0.2), neither in CRFtg nor in WT mice. No significant interaction effects of sodium butyrate treatment with genotype or prepulse intensity were observed (genotype x treatment F(3,63)= 0.5, p=0.7; prepulse intensity x treatment F(9,189)= 1.3, p=0.25). Sodium butyrate did not alter the baseline startle response in either genotype (F[3,63)= 1.3, p=0.3) (figure 3*b*).

Table 2 Summary of the ANOVA results from the antagonism studies on valproate's effect on PPI in CRFtg mice. * p <0.05

	Main effect treatment	Main effect genotype	Interaction effect treatment x genotype
Bicuculline exp.			
Valproate	F [1,24]= 11.8*	F [1,24] =4.9*	F [1,24] =2.9
Bicuculline	F [2,48] =0.6	F [1,24]=0.3	F[2,48]=0.4
Phaclofen exp.			
Valproate	F [1,24]= 22.3*	F [1,24] =10.1*	F [1,24] =8.1*
Phaclofen	F [2,48]=1.6	F [1,24]=3.4	F[2,48]=0.5
SCH-50911 exp.			
Valproate	F [1,72] =11.5*	F [1,72] =19.6*	F [1,72] = 1.1
SCH-50911	F [3,138]= 0.5	F [1,128]= 15.5*	F[3,128]=0.1
Bicuculline + phaclofen			
Valproate	F [1,56]= 12.0*	F [1,56] =30.4*	F [1,56] =9.7*
Bicuculline + phaclofen	F [1,47]= 0.06	F [1,47]= 5.1*	F[1,47]=0.0

4. Discussion

Here we showed that valproate, in contrast to the other mood stabilizers tested, reverses CRF-induced PPI-deficits. This effect was not mediated by enhanced GABAergic signaling, reduced HPA axis activation or HDAC inhibition.

Valproate consistently improved the disrupted PPI response in CRFtg mice, whereas it had no effect in WT mice. Besides, valproate reversed PPI deficits induced by i.c.v. CRF infusion, indicating that the drug effect was directly related to elevated CRF signaling, rather than to adaptations caused by long-term CRF overexpression. Interestingly, valproate is inactive in most PPI disruption tests, including amphetamine, ketamine and dizocilpine induced PPI disruption [158, 159], although beneficial effects have been reported in DBA mice and following apomorphine-induced disruption [159, 160].

Enhancement of GABA-ergic signaling is the most marked difference between valproate and the other mood stabilizers we tested in PPI [147, 148]. Therefore, we investigated whether the PPI-normalizing effects of valproate are mediated by its effects on the GABA system. A major mechanism whereby valproate may increase GABA concentrations, is inhibition of GABA transaminase, which slows down GABA degradation [161].

In animals, the anxiolytic effects of valproate can be blocked by $GABA_A$ receptor antagonists [162]. Here we found that the $GABA_A$ receptor antagonist bicuculline did not diminish the normalizing effect of valproate on PPI in CRFtg mice. In line with this finding, beneficial effect of valproate on PPI in CRFtg mice was not mimicked by the $GABA_A$ receptor agonists muscimol. Several lines of evidence point to a role for $GABA_A$ receptor function in sensorimotor gating. Inactivation of alpha3-subunit containing $GABA_A$ receptors has been shown to induce deficits in sensorimotor gating [163]. Also, mutant mice exhibiting reduced hippocampal expression of alpha5-subunit containing $GABA_A$ receptors displayed a deficient PPI response [164]. However, based on the current findings, we conclude that $GABA_A$ –ergic mechanisms do not play a role in the effect of valproate on sensorimotor gating.

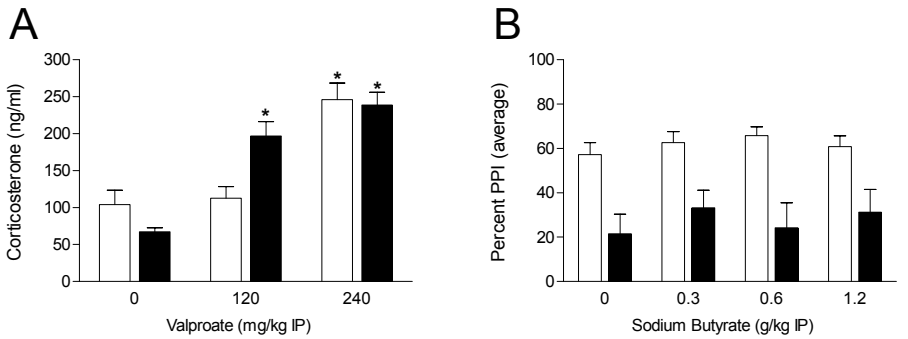

Fig. 3 (A) Effect of valproate on plasma corticosterone levels in WT (white bars) and CRFtg mice (black bars) (B) effect of sodium butyrate treatment on percent PPI in CRFtg. Group sizes: (A) WT-0,120; TG-0,240 *n*=9; WT-240 *n*=11; TG-120 *n*=10 (B) *n*=12 (all groups). Data are expressed as mean + SEM.,*p<0.001, compared to corresponding vehicle value.

Several reports showed that activation of $GABA_B$ receptors improves PPI, making $GABA_B$ another candidate to mediate the effects of valproate. Besides the finding that long-term valproate treatment increases $GABA_B$ receptor density, little is known about effects of valproate via $GABA_B$ receptors [165, 166]. Acute administration of the $GABA_B$ receptor agonist baclofen improved PPI-impairments induced by methamphetamine, phencyclidine or dizocilpine [167-169]. Also, baclofen normalized PPI in DBA/2J mice [170]. However, in the present study, the $GABA_B$ receptor antagonists phaclofen and SCH 50911 did not reverse the effect of valproate in CRFtg mice, demonstrating that $GABA_B$ receptor activation is not involved in the effect of valproate either.

Last, blocking both $GABA_A$ and $GABA_B$ receptors by co-administration of bicuculline and phaclofen did not reduce the effect of valproate on PPI, indicating that the effect of valproate is independent of its enhancement of GABA-ergic neurotransmission. To verify this conclusion, we determined if inhibition of GABA-transaminase by vigabatrin had beneficial effects on PPI in CRFtg mice. Although local activation of GABA-ergic neurotransmission has been shown to improve PPI [171], effects of enhancing GABA-ergic neurotransmission through systemic administration of compounds has not been reported before. The absence of effect of vigabatrin on PPI indicates that enhancement of GABA-ergic neurotransmisson is not sufficient to improve CRF-induced PPI deficits either. Although valproate targets several other systems in parallel with the GABA-ergic system, together, our data suggest that enhancement of GABA-ergic signaling is not the main mechanism through which valproate exerts its effect on CRF-induced PPI deficits.

Clinically, valproate has beneficial effects in subgroups of manic patients who are non-responsive to lithium [172]. The present data may suggest that effects of valproate on the CRF system contribute to the therapeutic effect in this particular subgroup of patients. Valproate is reported to inhibit glucocorticoid receptor function in cells [173], to inhibit CRF transmission in hypothalamic neurons *in vitro,* [152] and to reduce ACTH secretion in humans.

It has been suggested that HPA axis overactivity could induce psychotic symptoms by increasing mesolimbic dopaminergic activity and that treatments that block cortisol effects might be effective in the treatment of psychosis [24, 174], although this has been disputed by others [25, 26]. Thus, considering the reported in vitro effects of valproate, it could be hypothesized that the beneficial effects of valproate on CRF-induced PPI deficits are set about by a reduction in HPA axis activity. The present study however shows that valproate dose-dependently *increased* plasma corticosterone levels in both WT and CRFtg mice, demonstrating that valproate does not exert its effect on CRF-induced PPI deficits via a reduction in HPA-axis activity. Furthermore, it is unlikely that valproate exerts its effect by enhancing corticosterone secretion; increments in corticosterone are more likely to disrupt PPI [83], and we previously showed that enhancement of plasma corticosterone levels does not alter PPI in CRFtg mice [26].

This is the first study measuring corticosterone levels following valproate treatment in mice. In humans and rats, valproate was reported to either reduce, enhance or not to affect plasma corticosterone levels [150, 151, 175]. According to Schteingart

and coworkers [175], these different outcomes may be explained by differences in basal stress levels. Several other psychoactive drugs, including benzodiazepines and certain antipsychotics, also enhance plasma corticosterone levels [176-178] by directly interacting with the neural circuits involved in stress-induced hormone release [177, 178].

Under vehicle conditions, CRFtg and WT mice had similar plasma corticosterone levels, probably as a result of injection stress [143]. Following valproate administration, only the highest dose significantly enhanced plasma corticosterone levels in WT mice, whereas in CRFtg 120 mg/kg valproate already markedly enhanced corticosterone secretion. This finding extends the observed interaction between valproate and the CRF system from sensorimotor gating to neuroendocrine regulation.

Although it is highly unlikely that valproate improved PPI via enhancement of corticosterone levels per se, theoretically it could be that enhancement of corticosterone is involved in the PPI effect of valproate, when acting in concert with its HDAC inhibiting properties. Glucocorticoids bind to glucocorticoid and mineralocorticoid (GR and MR) receptors, which are both acetylated after agonist binding by histone acetyltransferase. Subsequently, these nuclear receptors can be deacetylated by HDAC2 [179]. In human lung cells, it has been shown that acetylated GR receptors trans-activate gene expression, while deacetylated GR receptors inhibit gene expression via trans-repression (e.g. via binding to NF-kB) [180]. In analogy with the lung, it could be that deacetylated GR in the brain bind to a different set of proteins than the acetylated forms of these receptors. Since valproate is an inhibitor of HDAC2 [181], a change in the balance between acetylated and deacetylated GR receptors may underlie the effects of this drug on PPI in CRFtg mice. However, sodium butyrate, which is a HDAC2 inhibitor with an IC_{50} value comparable to valproate [181], did not improve PPI in CRFtg mice in the present study. As other studies have shown acute beneficial effects of sodium butyrate on cognitive functioning [182, 183], our data indicate that HDAC inhibition by itself is not the determining factor in valporate's mechanism of action. To further study the potential relevance of a shift in balance between acetylated and deacyetylated GR receptors for behavioural processes, it would be interesting to determine the effects of HDAC inhibition with varying corticosterone levels in future studies.

In conclusion, this study extends findings regarding interactions between valproate treatment and the CRF system *in vitro* to the behavioral level. We demonstrated that the beneficial effects of valproate on CRF-induced PPI deficits are not mediated by $GABA_A$ or $GABA_B$ receptor activation, enhancement of GABAergic signaling, or reduced HPA axis activity; which probably reflects the fact that the mechanism of action of valproate in various psychiatric illnesses is still unclear after 45 years of clinical use [184]. Most likely, valproate's broad clinical activity cannot be accounted for by a single mechanism of action. However, our findings suggest that valproate may be particularly useful for the treatment of specific subgroups of bipolar patients that present with dysregulated CRF systems.

5. Supplementary material: effect of mood stabilizers on PPI deficits in CRFtg mice

5.1. Lithium

Lithium had no effect on PPI ($F[3,66]=1.6$, N.S.). Also, lithium treatment did not interact with genotype or prepulse intensity. Overall, percent PPI was markedly higher in WT than in CRF-tg mice ($F[1,22]=31.2$, $p<0.001$) (figure 1B). Lithium had no effect on baseline startle.

5.2. Carbamazepine

The effect of carbamazepine on PPI was dependent on prepulse intensity and genotype (3-way interaction; $F[9,180]=2.3$, $p=0.02$). Further analysis showed that carbamazepine significantly reduced percent PPI at 16 dB in WT ($F[3,30]=5.3$, $p=0.005$), but not in CRF-tg mice (figure 1C). Carbamazepine enhanced baseline startle ($F[3,60]=3.3$, $p=0.03$), equally in both genotypes. Post hoc analysis showed that this effect of carbamazepine on baseline startle was significant at 20 mg/kg.

5.3. Lamotrigine

The effect of lamotrigine was dependent on prepulse intensity ($F[9,189]=2$, $p=0.04$), but independent of genotype ($F[3,63]=0.8$, $p=0.5$, N.S.). Further analysis showed that lamotrigine facilitated PPI at 16 dB ($F[3,63]=9.9$, $p<0.001$), but not at other prepulse intensities. This effect was significant at a dose of 27 mg/kg. Overall, percent PPI in CRF-tg mice was significantly lower than in WTs ($F[1,21]=10.6$, $p=0.004$) (figure 1D). Lamotrigine enhanced baseline startle ($F[3,63]=4.7$, $p=0.005$), similarly in both genotypes, which was significant at the two highest doses, 9 and 27 mg/kg.

5.4. Topiramate

The effect of topiramate was dependent on genotype ($F[3,57]=4.9$, $p=0.004$). Analysis per genotype revealed that topiramate improved PPI in CRF-tg mice ($F[3,30]=3.1$, $p=0.041$). This effect was dependent on prepulse intensity ($F[9,90]=2.1$, $p=0.034$), and significant for the 100 and 300 mg/kg doses at 4 dB. In WT mice, topiramate reduced PPI ($F[3,27]=3.0$, $p=0.045$), independent of prepulse intensity. Further analysis showed that this effect was significant at the highest dose tested (300 mg/kg) (figure 1E). Topiramate decreased baseline startle ($F[1.9, 57]=23.7$, $p<0.001$), similarly for both genotypes. Further analysis revealed that this effect was caused by the two highest doses tested (100 and 300 mg/kg).

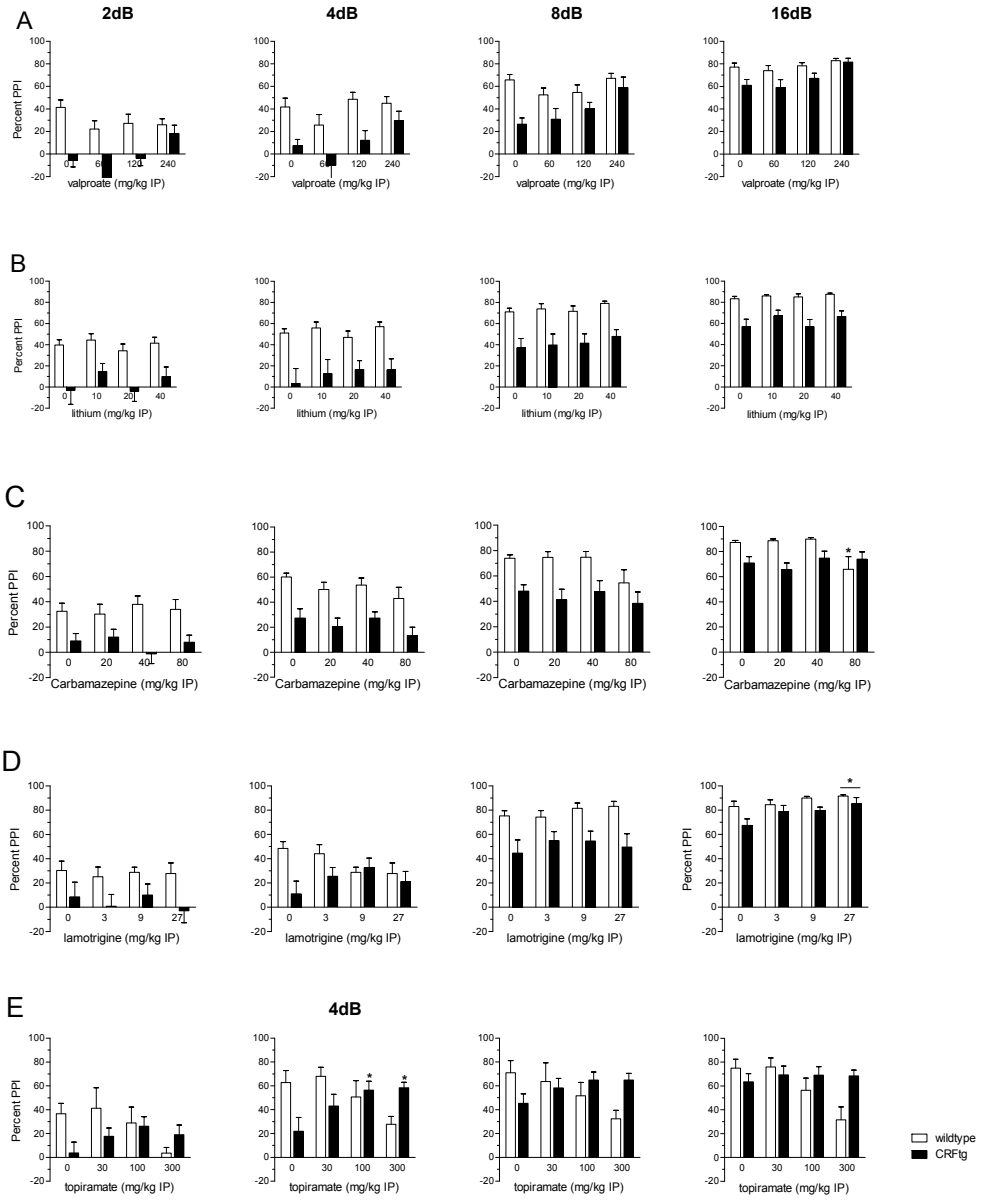

Fig. 1 Effects of VPA (A), lithium (B), carbamazepine (C), lamotrigine (D), topiramate (E) on PPI in WT (white bars) and CRF-tg (black bars) mice, for the different prepulse intensities (see text for details). VPA: WT n=12, CRF-tg n=11; lithium: WT n=12, CRF-tg n=12; carbamazepine: WT n=11, CRF-tg n=11; lamotrigine WT n=11, CRF-tg n=11; topiramate WT n=11, CRF-tg n=10. Data are expressed as mean + SEM. * indicates significant difference from corresponding vehicle

Table.S1 Effects of VPA, lithium, carbamazepine, lamotrigine, and topiramate on the baseline startle response in WT and CRF-tg (TG) mice. * $p < 0.05$

Compound	Dose (mg/kg)	Startle (mV)	
		WT	TG
Valproate	0	576.0 ± 85.5	453.5 ± 73.5
	60	530.0 ± 42.1	468.0 ± 102.7
	120	477.9 ± 60.5	501.8 ± 67.9
	240	359.5 ± 41.5 *	543.3 ± 82.3
Lithium	0	447.2 ± 62.8	340.1 ± 34.9
	10	477.2 ± 55.4	417.2 ± 57.7
	20	467.0 ± 54.0	365.7 ± 49.8
	40	467.0 ± 41.0	427.6 ± 53.2
Carbamazepine	0	518.0 ± 64.9	482.1 ± 53.1
	20	586.6 ± 69.9 *	665.8 ± 80.2 *
	40	630.7 ± 83.8	633.5 ± 72.7
	80	269.7 ± 80.6	582.9 ± 168.2
Lamotrigine	0	557.0 ± 77.4	466.4 ± 76.5
	3	579.5 ± 58.7	577.0 ± 71.1
	9	655.8 ± 57.7	698.1 ± 90.2 *
	27	644.0 ± 66.3 *	594.7 ± 87.7
Topiramate	0	420.3 ± 95.6	584.4 ± 81.7
	30	307.6 ± 65.7	515.7 ± 54.7
	100	151.5 ± 34.3 *	337.7 ± 60.1 *
	300	57.9 ± 8.1*	188.8 ± 35.5 *

Chapter 4

The amphetamine-chlordiazepoxide mixture, a pharmacological screen for mood stabilizers, does not enhance amphetamine-induced disruption of prepulse inhibition

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Abstract

In rodents, administration of a mixture of the psychostimulant D-amphetamine and the benzodiazepine chlordiazepoxide results in supra-additive hyperlocomotion, a phenomenon used to identify mood stabilizers. In an attempt to determine whether the D-amphetamine/chlordiazepoxide assay could extend to other behaviors that are affected in mania, we evaluated the effects of the mixture on prepulse inhibition. In addition, we combined chlordiazepoxide with the selective dopamine reuptake inhibitor GBR 12909 or the noradrenergic stimulant (-)ephedrine, and tested these alternative mixtures in locomotor activity and prepulse inhibition tests. Chlordiazepoxide (3 mg/kg) robustly potentiated amphetamine-induced hyperactivity, but did not change the amphetamine-induced disruption of prepulse inhibition. This indicates that the D-amphetamine-chlordiazepoxide-induced hyperlocomotion does not extend to other dopamine-driven behaviors. GBR 12909 (16 mg/kg) and (-)ephedrine (50 mg/kg) both enhanced locomotor activity and disrupted PPI, but combined treatment of either of these compounds with chlordiazepoxide had no significant additive effect on locomotor activity or prepulse inhibition. These findings suggest that the effect of the D-amphetamine-chlordiazepoxide mixture cannot be accounted for by the dopamine-enhancing properties of amphetamine alone. Last, valproic acid did not reduce the GBR-induced hyperactivity. Therefore, further pharmacological evaluation of the effects of GBR 12909 is warranted to determine its pharmacological potential to model mania-like behavior. Based on the current results, it is concluded that the utility of the pharmacological D-amphetamine/chlordiazepoxide assay as a tool to study brain mechanisms relevant to mania is limited.

1. Introduction

Bipolar disorder refers to a category of mood disorders that are diagnosed by episodes of mania, mostly accompanied by episodes of depression [185]. The etiology and pathophysiology of this complex disease is largely unknown, and to date, no animal model has been developed that exhibits spontaneously alternating episodes of mania- and depression-like behaviors [186]. Commonly, mania-like behavior is induced pharmacologically in normal rodents, and one assay employed to screen mood stabilizers, i.e. drugs used for the treatment of mania, is the D-amphetamine/chlordiazepoxide assay [187-191]. In this test, a mixture of the psychostimulant D-amphetamine and the benzodiazepine chlordiazepoxide is administered to a rodent, which results in heightened levels of hyperlocomotion relative to levels induced by the mixture components alone.

Increased motor activity, clinically manifesting as agitation, is one of the symptoms of bipolar disorder [192], which gives the assay some face validity. Thereby, albeit by an unknown mechanism, the pharmacological assay has proven successful in predicting efficacy of several clinically effective anticonvulsant mood stabilizers [187] and lithium [191]. As the neurobiology underlying bipolar disorder is poorly understood, and there is a current need for improved animal models preferably based on different facets of the disease [193, 194], we considered it worthwhile to further characterize the rodent D-amphetamine/chlordiazepoxide test.

During a manic episode, bipolar patients exhibit deficits in sensorimotor gating, as measured by prepulse inhibition of the acoustic startle response (PPI) [4]. PPI refers to the reduction in the magnitude of the startle reflex, which occurs when a weak sensory stimulus (the prepulse) is presented immediately before a startling stimulus [195-197]. Typically, PPI is diminished in psychotic illness, and impaired PPI is a well-accepted endophenotype of schizophrenia [9, 89, 198]. In rodents, administration of psychotomimetic drugs, such as D-amphetamine, readily disrupts PPI [199, 200]. Historically, the behavioral effects of D-amphetamine (i.e. PPI disruption and locomotor hyperactivity) have been attributed to dopaminergic substrates; however, noradrenergic effects may also be involved [201], which could be of particular interest for the study of bipolar disorder, as both catecholamines are classically associated with mania [202-205].

In this study, we aimed to assess if the D-amphetamine/chlordiazepoxide assay could extend to other behaviors that are affected in mania. To do so, we determined the effects of the D-amphetamine/chlordiazepoxide mixture on PPI, a measure found to be affected in acute mania [4]. In order to investigate which properties of amphetamine are needed to give a supra-additive hyperlocomotion in combination with chlordiazepoxide, we alternatively combined the benzodiazepine with GBR 12909, a selective dopamine reuptake inhibitor, and with ephedrine, a noradrenergic transporter substrate [206, 207]. For both mixtures, we tested for changes in locomotor activity and PPI. Finally, as GBR 12909 has also been proposed a model of bipolar disorder [208], we determined the influence of the mood stabilizer valproic acid on GBR-induced hyperactivity.

2. Materials and methods

2.1. Animals

Experiments were conducted on male C57Bl/6J mice, 8-21 weeks old, that were group housed in bedded plastic cages (EnviroDri; BMI, Helmond, the Netherlands), enriched with a piece of PVC-tubing and paper tissue, at constant room temperature (21 ± 2°C) and relative humidity (50-60%), on a 12:12 light/dark cycle (lights on: 06:00-18:00 h), with food and water available ad libitum. All experiments were performed according to the Guide for Care and Use of Laboratory Animals and were approved by the Ethical Committee for Animal Research of Utrecht University.

2.2. Drugs

In the mixture experiments, mice were treated with D-amphetamine sulphate (3.0 mg/kg, Fagron BV, Nieuwerkerk a/d IJssel), GBR 12909 dihydrochloride (16.0 mg/kg, Servier, France), and (-)Ephedrine sulphate (50.0 mg/kg, Chemiefarma NV, Maarssen); each compound alone, or combined with chlordiazepoxide hydrochloride (3.0 mg/kg, Pharbita, Zaandam). Valproic acid was obtained from Sigma, France. GBR 12909 was dissolved in distilled water, while the other compounds were dissolved in saline. All drugs were injected intraperitonalley (i.p.) in a volume of 10 mL/kg. Injection-test intervals were 5 min (D-amphetamine and chlordiazepoxide), 30 minutes (GBR 12909 and valproic acid) and 50 minutes (ephedrine). The longer injection test interval used for ephedrine was based on other studies that assessed locomotor activity following ephedrine administration [209, 210]. The doses of D-amphetamine and chlordiazepoxide were based on a study by Kelly et al., which showed, across different mouse strains, that combining amphetamine with increasing doses of chlordiazepoxide results in an inverted U-shaped response [211], reaching its maximum at the doses employed here. The dose of ephedrine was chosen on the basis of dose response curves in pilot experiments (not shown). Doses of GBR 12909 and valproic acid were based on literature [208] and previous findings with C57Bl/6J mice in our own laboratory [212], respectively.

2.3. Locomotor activity

Locomotion was recorded for 30 minutes in open-topped, square arenas with grey walls *(25 x 25 x 30 cm)*, under dimmed room light conditions. The session length was based on other studies employing the hyperactivity model [187, 211]. Data were collected in 1-min bins using Ethovision (Noldus, The Netherlands), and expressed as distance traveled (in cm) during 30 minutes. One week before drug testing, mice were exposed to the open field test in order to familiarize the animals to the testing procedure and to compose treatment groups with equal mean baseline activity.

2.4. Prepulse inhibition of the acoustic startle reflex

Startle reflexes were measured in eight identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA). Startle stimuli (110 dB, 50 msec) were presented alone, or preceded by noise prepulses (20 msec) of 2, 4, 8, or 16 dB above

background (70 dB), with 100 ms between onsets of the prepulse and startle stimuli. The test session started with a 5-min acclimation period followed by three consecutive blocks of test trials (block 1 and 3, startle-stimulus alone trials; block 2, startle-stimulus alone, startle + prepulse, and no-stimulus trials). Intertrial intervals ranged from 25 to 35 sec, and total test duration was 25 min (amphetamine study), or 45 min (ephedrine study). Apparatus and testing procedure have been described in detail elsewhere [26]. One week before drug testing, a baseline PPI measurement was performed, in order to familiarize the mice to the test procedure and to create treatment groups with equal mean percent PPI.

2.5. Statistical analysis

Summed locomotor activity in the open field (cm moved in 30 min) was analyzed using Two-way ANOVAs, with chlodiazepoxide and stimulant drug (mixture studies), or pre-treatment and valproic acid (GBR12909 - valproic acid study) as between-subject factors. Percent prepulse inhibition data were analyzed using repeated measures ANOVAs with prepulse intensity as repeated within factor, and chlordiazepoxide and drug as between factors. If there were no interactions between drug treatment and prepulse intensity, prepulse inhibition data were collapsed across prepulse intensity. Significant effects were followed by post hoc Bonferroni-corrected *t*-tests. Differences were considered to be significant if $p < 0.05$. Statistical calculations were carried out using SPSS 16. In the locomotor activity study, statistical outliers greater than two standard deviations from the mean were removed from further analysis (2 in the ephedrine-experiment and 3 in the GBR-valproic acid experiment). In the prepulse inhibition study, animals for which startle stimulus reactivity in startle-stimulus alone trials was below 100 units, were excluded from analysis (3 in the amphetamine study and 3 in the ephedrine study).

3. Results

3.1. Locomotor activity

Hyperlocomotion induced by the mixture of D-amphetamine (3.0 mg/kg, i.p.) and chlordiazepoxide (3.0 mg/kg, i.p.) significantly exceeded that induced by D-amphetamine (3.0 mg/kg, i.p.) alone (interaction: amphetamine * chlordiazepoxide, $F[1,55]=5.7$, $p<0.05$; post hoc, amphetamine vs. vehicle, $p<0.001$; amphetamine vs. mix, $p<0.05$) (figure 1A). Combined treatment of the same dose of chlordiazepoxide with GBR 12909 (16.0 mg/kg, i.p.) resulted in a significant interaction effect (GBR * chlordiazepoxide, $F[1,61]=5.1$, $p<0.05$). Post hoc analysis showed that treatment with GBR12909 and GBR-mix both significantly enhanced distance moved ($p<0.001$). However, the hyperlocomotion of GBR-mix treated mice did not differ significantly from that of mice treated with GBR alone ($p=0.196$). Valproic acid treatment did not reduce hyperactivity induced by GBR 12909 alone, nor by the mixture of GBR and chlordiazepoxide (interaction: valproate * pre-treatment, $F[4,98]=1.3$, N.S). Also, valproic acid did not significantly alter activity in any other treatment group ($F[2,98]=4.1$,

p<0.05; post hoc, N.S.). Treatment with GBR, both with and without chlordiazepoxide, significantly increased distance moved compared to vehicle treatment (post hoc, *p*=0.000 for both cases) Again, hyperactivity induced by the combination of GBR and chlordiazepoxide did not differ significantly from that induced by GBR alone (post hoc, p=0.3, N.S.) (figure 2). Like amphetamine and GBR 12909, ephedrine (50.0 mg/kg, i.p.) significantly increased total distance traveled (F[1,41]=14.2, *p*=0.001); however, addition of chlordiazepoxide (3.0 mg/kg, i.p.) did not significantly alter the ephedrine-induced hyperlocomotion (ephedrine * chlordiazepoxide, F[1,41]=1.5, N.S.) (figure 1E). Chlordiazepoxide administered alone did not affect locomotion in either experiment (figure 1A, C, E).

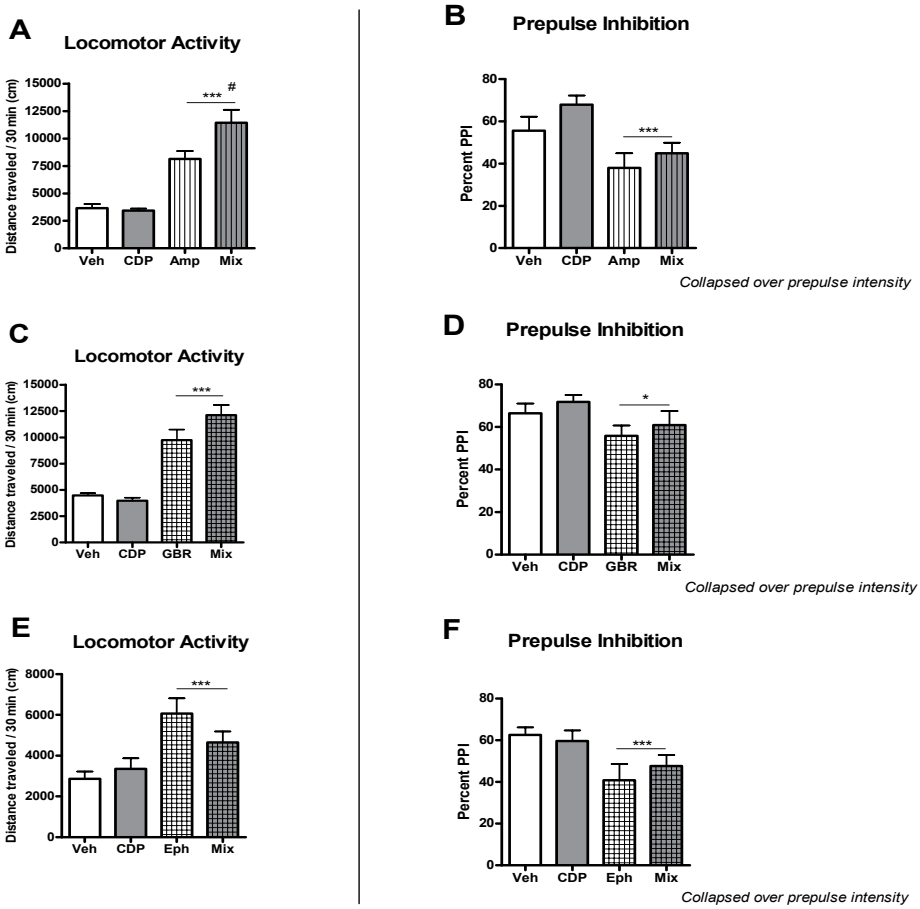

Fig. 1 (A, C, E) Total distance moved in the open field following treatment with (A) amphetamine and chlordiazepoxide (n=14-15), (B) GBR12909 and chlordiazepoxide (n=18-19), and (C) ephedrine and chlordiazepoxide (n=14-15). Data are shown as mean total distance moved + SEM. (B, D, F) Prepulse inhibition of the startle reflex, depicted as mean percent PPI + SEM, following treatment with (B) amphetamine and chlordiazepoxide (n=8-10), (D) GBR 12909 and chlordiazepoxide (n=14), and (F) ephedrine and chlordiazepoxide (n=10-12). * p<0.05, *** p≤0.001; # p<0.05, comparison vs. amphetamine. Veh, vehicle; CDP, chlordiazepoxide (3 mg/kg, i.p.); Amp, amphetamine (3 mg/kg, i.p.); GBR, GBR 12909 (16 mg/kg, i.p.); Eph, ephedrine (50 mg/kg, i.p.); mix, combination of chlordiazepoxide with respective stimulant (same dosing).

3.2. Prepulse inhibition of the acoustic startle reflex

D-amphetamine (3.0 mg/kg, i.p.) significantly disrupted PPI ($F[1,33]=18.6$, $p<0.001$), whereas the amphetamine-induced PPI-disruption was not significantly altered by chlordiazepoxide (3.0 mg/kg, i.p.) (amphetamine * chlordiazepoxide, $F[1,33]=0.3$, N.S.) (figure 1B). Also, chlordiazepoxide did not affect activity in no-stimulus trials, or baseline startle, in either group (not shown). GBR 12909 (16.0 mg/kg, i.p.) significantly disrupted PPI ($F[1,52]=6.1$, $p<0.05$), independent of chlordiazepoxide co-treatment (interaction: GBR 12909 * chlordiazepoxide, $F[1,52]=0.001$, N.S.) (figure 1D). Similarly, ephedrine (50.0 mg/kg, i.p.) significantly impaired PPI ($F[1,41]=14.2$, $p=0.001$), and ephedrine-induced PPI-disruptions were not changed by chlordiazepoxide (3.0 mg/kg, i.p.) (ephedrine * chlordiazepoxide, $F[1,41]=1.5$, N.S.) (figure 1F). Chlordiazepoxide given alone did not affect PPI in either experiment, when compared to vehicle (figure 1B, D, F).

4. Discussion

The present study was set up to further evaluate behavioral changes induced by a combination of D-amphetamine (3 mg/kg) and chlordiazepoxide (3 mg/kg), in order to assess the mixture's utility for modeling mania-like behavior.

As expected, hyperlocomotion induced by this mixture significantly exceeded that induced by D-amphetamine alone. Co-administration of chlordiazepoxide however, did not lead to exacerbation of amphetamine-induced PPI-disruptions, which indicates the involvement of distinct neural substrates for locomotor behavior and startle (modulation), respectively. Indeed, although the two behavioral responses are associated with partly overlapping neuronal circuitries (e.g. mesolimbic dopamine systems [22, 213], several studies have shown that drugs that induce locomotor activation, do not necessarily disrupt PPI (or vice versa) [214, 215]. Possibly, a combination of different doses of the psychostimulant and the benzodiazepine would have yielded a qualitatively different outcome; however, in this study we sought to further characterize the particular mixture which most potently induces hyperactivity.

The selective dopamine transporter inhibitor GBR 12909 (16 mg/kg) increased distance moved, consistent with other studies [208]. This hyperactivity was only

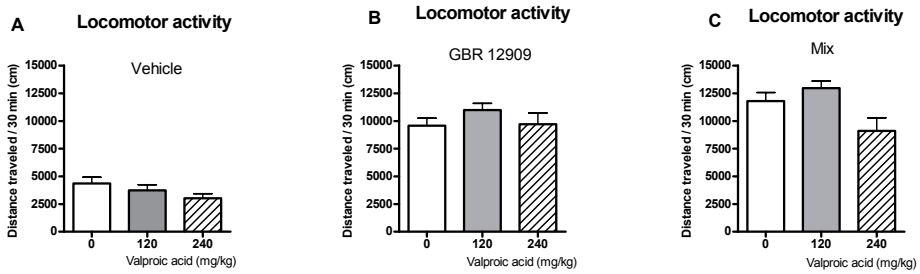

Fig. 2 Effect of valproic acid on GBR 12909-induced hyperactivity in the open field, shown as mean total distance moved + SEM. Mice were pre-treated with (A) vehicle (n= 9), (B) GBR 12909 (16 mg/kg, i.p.; n=13-15), or (C) GBR12909 (16 mg/kg, i.p.) and chlordiazepoxide (3 mg/kg, i.p.); n=13-16.

weakly, and nonsignificantly, strengthened by adding chlordiazepoxide (3 mg/kg), indicating that enhancement of dopaminergic signaling does not completely mimic the amphetamine effect. In addition, GBR-induced hyperactivity was not significantly attenuated by the antimanic agent valproic acid; nor was hyperactivity induced by the GBR-mixture. Treatment with GBR 12909 impaired PPI. To our knowledge, this effect has not been reported before. It is not surprising though, as enhancement of dopaminergic signaling is classically associated with PPI disruptions [10]. As with amphetamine, the PPI disruptions induced by GBR 12909 were unchanged by chlordiazepoxide. In mice, GBR 12909 has been shown to induce a behavioral profile that is more similar to that observed in manic bipolar disorder patients than amphetamine does, which supports the use of selective DAT inhibition in animal models of bipolar mania [208]. However, the GBR 12909 profile has not been pharmacologically validated. The current finding that hyperactivity induced by GBR 12909 alone was unchanged by the mood stabilizer valproic acid, warrants the need for a more careful examination of the sensitivity of GBR12909-induced effects towards different mood stabilizers.

Ephedrine, like amphetamine, is an indirect sympathomimetic [207]. While amphetamine possesses significant activity at dopamine transporters and to a lesser extent at noradrenaline transporters, the most potent pharmacological action of ephedrine is substrate activity at noradrenaline transporters, resulting in indirect release of noradrenaline. Besides, ephedrine also interacts with dopamine transporters but to a far lesser extent [206]. In the current study, ephedrine (50 mg/kg) significantly induced hyperlocomotion, consistent with previous reports [209, 210] . Thereby, ephedrine (50 mg/kg) was found to impair PPI, in line with the notion that increasing central noradrenergic transmission reduces PPI [216, 217]. However, neither locomotor hyperactivity, nor PPI was significantly altered by adding chlordiazepoxide (3 mg/kg), suggesting that the mechanism by which chloridazepoxide enhances the locomotor effects of amphetamine does not generalize to noradrenergic functioning. According to a recent pharmacological study, both amphetamine-induced hyperactivity and PPI disruption may be mediated by noradrenergic receptors, as the behavioral effects could be blocked by noradrenergic antagonists [201]. The current ephedrine results however, indicate that noradrenergic effects are not involved in the supra-additive hyperactivity that arises from combining D-amphetamine with chlordiazepoxide.

Considering the current findings, it remains unclear which properties of amphetamine are needed to induce the supra-addictive effect. As we only tested single doses of the mixtures' components, caution should be taken with the interpretation of these results. Without additional pharmacological or neurochemical evidence, extensive mechanistic considerations are therefore not appropriate. However, D-amphetamine increases extracellular concentrations of dopamine, noradrenaline and serotonin [218], and it may be noticed, that although the dopamine enhancing properties of amphetamine are likely to play an important role in the effect, they are not sufficient to give a full mechanistic explanation. First, the mixture of GBR 12909 and chlordiazepoxide did not result in enhancement of hyperactivity, suggesting the involvement of an unknown additional effect of amphetamine. In support of this finding, in rats it was found that the combination of amphetamine and chlordiazepoxide did not result in increased striatal dopamine levels, as compared to amphetamine

alone [188]. Furthermore, as reported here, the mixture did not strengthen amphetamine-induced PPI-disruptions, while PPI is known to be particularly sensitive to dopaminergic manipulations [22]. Besides enhancement of synaptic dopamine levels, it has been suggested that the increased hyperlocomotion induced by the amphetamine/chlordiazepoxide mixture could be ascribed to the anxiolytic properties chlordiazepoxide exerts in a novel environment [187] . However, based on the current results, this possibility seems unlikely, as chlordiazepoxide was unable to augment the stimulant effects of GBR12909 and ephedrine on locomotor activity.

In conclusion, we show here that whereas the combination of D-amphetamine and chlordiazepoxide robustly enhanced the stimulant-induced hyperlocomotion, it did not change prepulse inhibition; indicating that the mixture effects do not extend to other dopamine-driven behaviors, thereby limiting its utility as a tool to study mechanisms relevant to the psychopathology of mania. Concerns regarding the limitations of the assay have been raised before, for instance, Kelly et al. suggested that it may not be a useful model for the screening of novel drugs due to interpretive complexities [211]. Alternatively, GBR 12909 has been proposed as potential model of bipolar disorder [208]. However, here we found that valproic acid was unable to significantly diminish GBR-induced hyperactivity, indicating that further pharmacological evaluation of the effects of GBR 12909 is warranted to determine its pharmacological potential to model mania-like behavior.

Chapter 5

$GABA_B$ receptor agonists reverse prepulse inhibition deficits induced by acute but not chronic exposure to increased levels of corticotropin-releasing factor

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Abstract

Alterations in $GABA_B$ receptor and corticotropin-releasing factor (CRF) systems are reported in schizophrenia, a disorder characterized by deficits in prepulse inhibition (PPI) of the acoustic startle response. In rodents, PPI can be disrupted by CRF administration. Here we investigated the possible interaction between CRF and $GABA_B$ receptor signaling in the regulation of PPI. To this end, we tested the effects of the $GABA_B$ receptor agonists baclofen and SKF97541 on PPI disruptions induced by intraventricular (i.c.v.) administration of h/r-CRF in 129Sv mice, and on PPI deficits in transgenic mice overexpressing CRF (CRFtg). Baclofen and SKF97541 improved PPI deficits induced by acute i.c.v. infusion of CRF, but had no effect in CRFtg mice. The results indicate that $GABA_B$ receptor activation compensates for the PPI deficits induced by acute CRF infusion, and suggest that long-term exposure to elevated CRF levels causes adaptation at the level of mesolimbic dopamine neurons or in the nigroreticular pathway that renders these areas less sensitive to the PPI-enhancing effects of $GABA_B$ receptor agonists.

1. Introduction

Neurochemical deficits in the gamma-amino-butyric acid (GABA) system have been linked to schizophrenia [219-223], along with increases in $GABA_A$ receptor binding [224, 225]. In addition, a role for $GABA_B$ receptors in psychosis has increasingly been recognized. For instance, reduced expression of $GABA_B$ receptors was found in cerebellum, hippocampus and several cortical areas of post-mortem schizophrenia brains [226-229], and a recent transcranial magnetic stimulation study found that first-episode psychotic patients exhibit alterations in $GABA_B$ functionality [230].

The prototypical $GABA_B$ receptor agonist baclofen showed some therapeutic efficacy in the treatment of schizophrenia [231, 232]. However, negative results were also reported [233], suggesting that baclofen might be beneficial for specific subpopulations of patients. In animal models, baclofen improved prepulse inhibition (PPI) of the acoustic startle response, which is a validated tool for the screening of antipsychotic activity [9, 89]. PPI refers to the reduction in the magnitude of the startle reflex, occurring when a weak sensory stimulus (the prepulse) is presented immediately before a startling stimulus [195-197]. Clinically, reductions in PPI are characteristic of schizophrenia, where they are thought to be associated with symptoms of sensory flooding and cognitive fragmentation [22, 234].

In rodents, dopaminergic [199] and glutamatergic [235] psychotomimetics induce PPI deficits. In addition, PPI can be disrupted by corticotropin-releasing factor (CRF) [27, 236, 237]. Interestingly, in humans, dysregulation of CRF stress systems is implicated in psychotic disorders. For instance, male schizophrenic subjects showed decreased levels of CRF-binding protein in amygdala post-mortem tissue [64] and positive treatment response to the antipsychotic quetiapine in schizophrenia patients was associated with decreased cerebrospinal fluid CRF levels [140]. Moreover, genetic variations in CRF-related genes were associated with schizophrenia, schizoaffective disorder and bipolar disorder (reviewed by [141]).

Notably, interactions between CRF and $GABA_B$ signaling have been reported in frontal cortex and amygdala [238, 239], but effects of $GABA_B$ receptor agonists on CRF-disrupted PPI are currently unknown. Therefore, in the present study we investigated the possible interaction between CRF and $GABA_B$ receptor signaling in the regulation of PPI. To this end, we tested the effects of the $GABA_B$ receptor agonists baclofen and SKF97541 on PPI disruptions induced by intracerebroventricular (i.c.v.) administration of CRF in mice. To study the possible influence of chronically high CRF levels on $GABA_B$ signaling, we tested baclofen and SKF97541 in transgenic mice overexpressing CRF (CRFtg).

2. Materials and methods

2.1. Animals

CRF infusion studies were performed on male 129SvEvTac mice, 10-16 weeks old (Taconic, Denmark). CRFtg mice were locally bred as described previously [66]. Briefly, the CRF transgene, consisting of the complete coding sequence of rat CRF cDNA (.6-kb fragment), was inserted into a 8.2-kb genomic DNA-fragment encompassing

the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence. The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons. At the local breeding facilities (Utrecht, the Netherlands), heterozygous transgenic males were paired with C57Bl/6JIco females (Charles River, the Netherlands). For the current experiments, 8-16 weeks old male CRFtg mice (line 2122, 20th generation) were used. Littermate wild-type (WT) male mice served as control subjects. All mice were group-housed in bedded plastic type X cages, enriched with a piece of PVC-tubing and paper tissue, at constant room temperature (21 ± 2°C) and relative humidity (50-60%), on a 12:12-h light/dark cycle (lights on: 06:00-18:00 h), with food and water available ad libitum. Experiments were performed according to the Guide for Care and Use of Laboratory Animals and were approved by the Ethical Committee for Animal Research of Utrecht University.

2.2. Surgery

One week after arrival, mice were systemically anesthetized using isoflurane gas anesthetic (2-3%, Isoflo, Abbott) mixed with oxygen and nitrous oxide. In the wound space, an additional local anesthetic was applied (Lidocaïne 5%, Alfacaine, Alfasan). Each mouse was prepared with a 23 gauge 2.5-mm-length unilateral guide cannula into the lateral ventricle (flat skull; anteroposterior, -0.2 mm; mediolateral, + 1 mm; dorsoventral, -2.5 mm from bregma), which was secured with dental cement. To make sure the cement would be held in place, shallow lines were carved into the skull. Plastic dummies were used to close the cannulae. Before onset of testing, animals were allowed to recover for one week. At the end of the experiment, cannula placements were assessed by infusion of methylene blue dye and verification of dye in the ventricular system (no mice excluded).

2.3. Drugs

2.3.1. CRF infusions

Human/rat corticotrophin-releasing factor (h/r-CRF, Bachem, Weil am Rein, Germany) was dissolved in 0.05M acetic acid and stored at -80°C until use. Infusion samples, freshly prepared on each testing day, were diluted 1:10 in sterile artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$ x 2 H_2O, 25 mM $NaHCO_3$, 25 mM glucose and 0.5% albumin). Vehicle consisted of 10% 0.05 M acetic acid in aCSF. 1 µg h/rCRF or aCSF was infused 60 minutes before the test by means of an internal cannula projecting 0.5 mm beyond the tip of the guide cannula, in a volume of 2 µl (1 µl/min). The infusion cannulae were connected to Hamilton syringes, placed in a syringe pump (KdScientific 220 sersies, USA) via polyethylene tubing. During the infusion procedure, mice were lightly anesthetized using a cocktail of isoflurane gas anesthetic (2-3%), oxygen and nitrous oxide.

2.3.2. $GABA_B$ receptor agonists

R(+)-Baclofen hydrochloride and SKF97541 (3-aminopropyl(methyl)phosphinic acid) (gifts from Servier, Croissy/Seine, France) were dissolved in saline. Drugs were administered intraperitoneally (IP) in a volume of 10 mL/kg, 30 minutes before start of the test. In the infusion studies, testing of the higher doses of $GABA_B$ receptor agonists

(range: baclofen, 1.25 – 5.0 mg/kg; SKF, 0.05 – 0.2 mg/kg) was discontinued because of strong startle reducing effects in 129Sv mice.

2.4. Prepulse inhibition of the acoustic startle reflex

2.4.1. Apparatus and test procedure

Startle apparatus and testing procedure have been described in detail elsewhere [26]. In the current study, startle reflexes were measured in eight identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA). Startle stimuli (110 dB, 50 msec) were presented alone, or preceded by noise prepulses (20 msec) of 2, 4, 8, or 16 dB above background (70 dB), with 100 ms between onsets of the prepulse and startle stimuli. The test session started with a 5-min acclimation period followed by three consecutive blocks of test trials (block 1 and 3, startle-stimulus alone trials; block 2, startle-stimulus alone, prepulse + startle, and no-stimulus trials). Intertrial intervals ranged from 25 to 35 sec, and total test duration was 42 min.

2.4.2. Matching

One week before drug testing, a baseline PPI measurement was performed, in order to familiarize the mice to the test procedure and to create treatment groups with equal mean percent PPI.

2.4.3. Experimental design

In the infusion experiments, drugs were tested in a between subjects design, while in the CRF-overexpression study a within-subject Latin square design was used, with subjects receiving each dose and vehicle once. On any occasion, time between tests was at least one week. Body weight was determined weekly before testing.

2.5. Statistics

Percent PPI was calculated as the mean startle magnitude to startle stimulus-alone, minus the mean startle magnitude to prepulse + startle stimuli, all divided by the mean startle stimulus-alone trials, and multiplied by 100. For calculation of the mean startle magnitude, only data from block 2 were used.

CRF-infusions – Percent PPI was analyzed by repeated-measures ANOVA with prepulse intensity as within-subjects factor and treatment (CRF and drug) as between-subjects factors. Startle amplitude was analyzed by univariate ANOVA, with CRF and drug treatment as between-subjects factors.

CRF overexpression – Percent PPI was analyzed using repeated-measures analysis of variance (ANOVA) with prepulse intensity and dose as within-subject factors and genotype as between-subjects factor. Acoustic startle magnitude was analyzed using a repeated-measures ANOVA with dose as the within-subjects factor and genotype as the between subjects factor.

In case of significant main or interaction effects, subsequent ANOVAs were performed followed by Dunnett's test or paired t-tests with Bonferroni correction of α. The level of significance was set at $p < 0.05$. Statistical outliers greater than two standard deviations from the mean were removed from further analysis (3 in the infusion experiments). Statistical analyses were carried out using SPSS for Windows, version 20.

3. Results

3.1. h/r-CRF infusions (i.c.v.): dose-response curve

CRF infusion studies were performed in 129Sv mice, as this strain is more sensitive to the PPI-disrupting effects of CRF than C57Bl/6J [27], the background strain of the CRFtg mouse line used in the subsequent studies.

Intracerebroventricular infusion of h/r-CRF significantly disrupted PPI in 129Sv mice. The effect of CRF was dependent on prepulse intensity ($F[9,141]=6.4$, $p<0.001$) and significant for 2, 4, and 8 dB prepulses, but not 16 dB prepulses. Therefore, further analyses on the effects of $GABA_B$ agonists on CRF-disrupted PPI were based on 2, 4, and 8 dB prepulses. All CRF doses significantly reduced PPI, but the lower doses (0.3 and 1 µg) were most effective (figure 1A). Based on these results, the 1-µg h/r-CRF dose was chosen for further studies.

Baseline startle response was significantly increased following infusion of 1 µg CRF ($F[3,47]=3.1$, $p<0.05$) (figure 1B), while CRF infusion did not significantly alter no-stimulus amplitudes ($F[3,47]=1.8$, $p=0.16$). Therefore, activity data are not included in further results.

3.2. Effects of $GABA_B$ receptor agonists: i.c.v. CRF infusions

The effect of the prototypical $GABA_B$ receptor agonist baclofen on PPI deficits induced by h/r-CRF infusion was tested at 0, 1.25 and 2.5 mg/kg. A significant interaction effect between CRF and baclofen treatment was observed ($F[2,42]=6.8$; $p=0.003$). Further analysis showed that baclofen (2.5 mg/kg) improved PPI in CRF-treated animals ($F[2,21]=7.7$; $p=0.003$), whereas the drug decreased PPI in the aCSF group at 1.25 mg/kg ($F[2,21]=3.7$; $p=0.04$) (figure 2A)). The effect of baclofen treatment on the startle response just failed to reach significance [$F(2,45)=3.1$, $p=0.054$] (figure 3).

The effect of the potent and selective $GABA_B$ receptor agonist SKF97541 (0.05 mg/kg) on PPI was dependent on CRF infusion ($F[1,32]=5.1$; $p=0.03$). Further analysis showed that SKF treatment significantly reversed the CRF-induced PPI deficit (figure 2B). Furthermore, SKF87541 significantly diminished the effect of CRF on baseline startle, whereas the drug had no effect in aCSF-treated animals (interaction effect SKF x CRF, $F[1,31]=9.6$, $p<0.01$) (figure 3B).

3.3. Effects of $GABA_B$ receptor agonists: CRF overexpression

As expected, CRFtg mice showed significantly lower PPI than WT mice ($F[1,33]=29.3$; $p<0.001$) (figure 4A).The effect of baclofen (1.25-5 mg/kg) on percent PPI was dependent on genotype ($F[3,99]=3.2$, $p<0.05$), and independent of prepulse intensity ($F[9,297]=1.1$, $p=0.4$). Further analyses however showed that the effect of baclofen just failed to reach significance when analyzed for CRFtg ($F[2,48]=2.4$; $p=0.08$) and WT mice ($F[3,51]=2.6$; $p=0.06$) separately. Baclofen altered the baseline startle response, dependent on genotype ($F[3,99]=4.2$, $p<0.01$). Further analysis showed that baclofen reduced the startle response in WT mice at the highest dose tested ($F[3,51]=7.8$, $p<0.001$), whereas it had no effect in CRFtg mice ($F[3,48]=1.9$; $p=0.1$) (figure 5A).

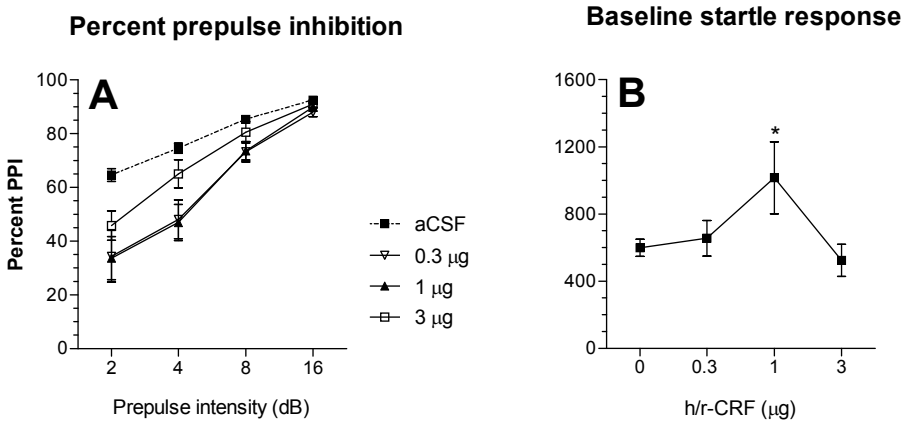

Fig. 1 Effects of h/r-CRF (0.3-3 μg, i.c.v.) in 129Sv mice on (A) prepulse inhibition (B) baseline acoustic startle. Group sizes: aCSF, n=27; h/r CRF 0.3 μg, n=10; h/r CRF 1 μg, n=10; h/r CRF 3 μg, n=4. Data represent mean ± SEM. *p<0.05, relative to vehicle value.

SKF97541, tested at 0.05, 0.1 and 0.2 mg/kg doses, did neither alter percent PPI, nor baseline startle in either genotype ($F[3,78]<1$, $p=0.85$; $F[3,78]<1$, $p=0.93$, resp.) (figure 4,5B). With respect to PPI, CRFtg mice differed significantly from wild type mice in all conditions tested ($F[1,26]=30.5$, $p<0.001$).

4. Discussion

This study shows that the $GABA_B$ receptor agonists baclofen and SKF97541 improve PPI deficits induced by acute infusion of h/r-CRF, but have no effect on PPI deficits in CRFtg mice. The results indicate that $GABA_B$ receptor activation attenuates the PPI-disrupting effects of CRF, and that boosting $GABA_B$ signaling is no longer effective after long-term exposure to elevated levels of CRF.

Acute i.c.v. infusion of h/r-CRF significantly disrupted PPI in 129Sv mice, which is in line with previous studies [142, 240]. As most robuste PPI deficits were induced by 1 μg h/r-CRF, this dose was used for subsequent CRF-$GABA_B$ interaction studies. Although this dose also significantly increased startle, the PPI-disrupting and startle-increasing effects of CRF are considered independent [30, 241, 242]. The current finding that all doses of h/r-CRF (0.3, 1 and 3 μg) disrupted PPI, whereas only the middle dose significantly enhanced baseline startle indeed confirms this assumption.

The $GABA_B$ receptor agonist baclofen dose-dependently improved PPI deficits induced by CRF infusion in 129Sv mice, which was significant at 2.5 mg/kg dose. In other studies, similar doses of baclofen (1-5 mg/kg) improved PPI deficits induced by NMDA receptor antagonists [167-169], methamphetamine [167] and the endogenously low PPI of DBA/2J mice [170]. In control animals, the lower dose of baclofen (1.25 mg/kg) decreased PPI in the present study. The meaning of this finding is difficult to interpret, as such a reduction was not observed with SKF97541, and neither of the $GABA_B$ receptor agonists reduced PPI in C57Bl/6J wild-type mice. Like baclofen, the

selective and highly potent $GABA_B$ receptor agonist SKF97541 significantly improved PPI deficits induced by acute i.c.v. CRF infusion, suggesting that the beneficial effect of baclofen on PPI is mediated through $GABA_B$ receptor activation. To our knowledge, no other studies have reported on the effects of SKF97541 on PPI. However, with respect to locomotor behavior, 0.1 mg/kg SKF97541 decreased sensitization to cocaine in rats, as did baclofen 2.5 and 5 mg/kg [243]. This is an interesting finding, as psychostimulant-induced hyperlocomotion and disrupted PPI are both responses

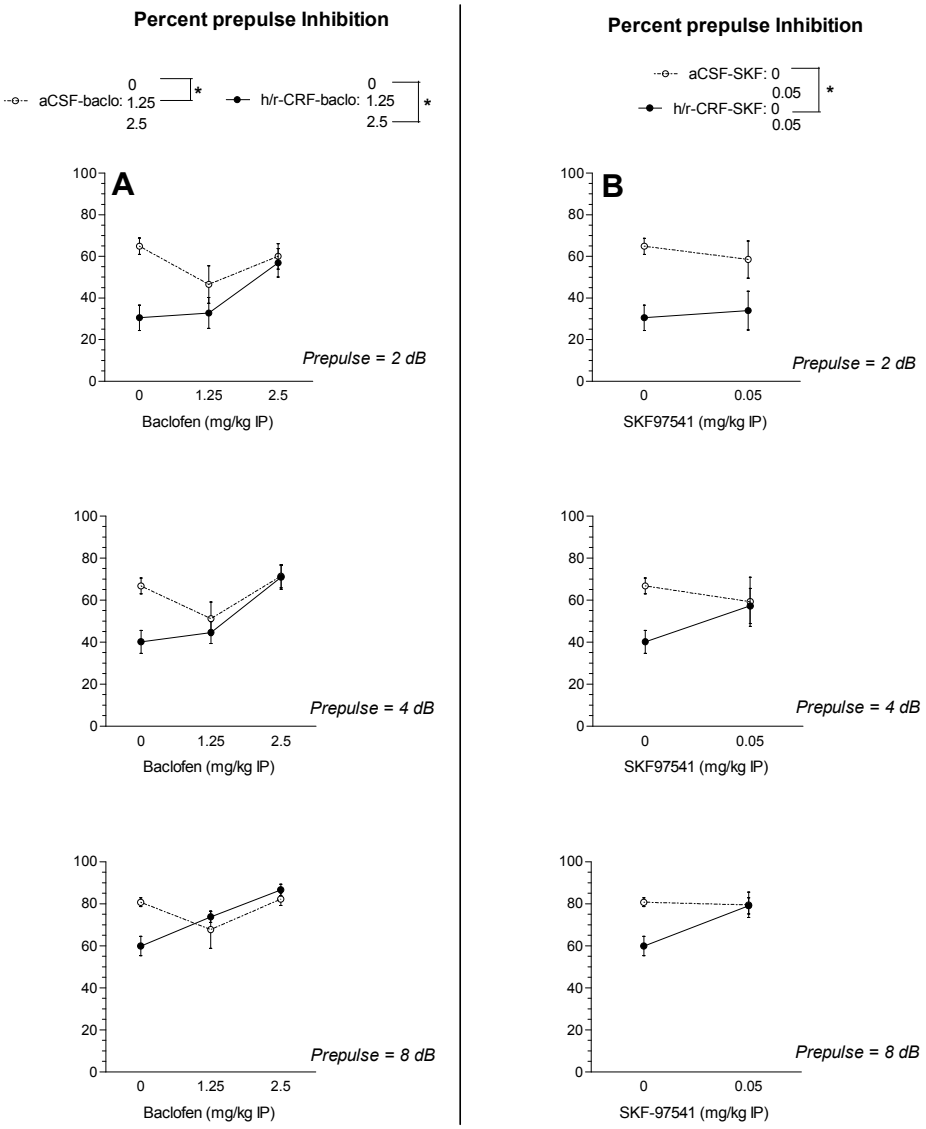

Fig. 2 Effects of baclofen (A) and SKF97541 (B) on percent prepulse inhibition in 129Sv mice following administration of h/r-CRF (1 µg, i.c.v., black circles) or artificial cerebrospinal fluid (aCSF, open circles). Group sizes: (A) aCSF-vehicle, n=12; aCSF-baclofen 1.25 mg/kg, n=6; aCSF-baclofen 2.5 mg/kg, n=6; CRF-vehicle, n=12; CRF-bacloflen 1.25 mg/kg, n=7; CRF-baclofen 2.5 mg/kg, n=5 (B) aCSF-vehicle, n=12; aCSF-SKF, n=5; CRF-vehicle, n=12; CRF-SKF, n=6. Data represent mean ± SEM. *p<0.05

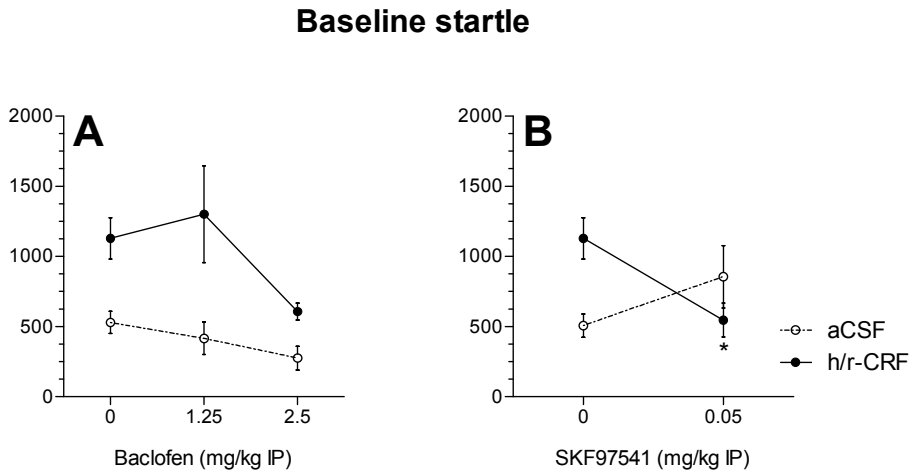

Fig. 3 Effects of baclofen and SKF97541 on baseline acoustic startle response in 129Sv mice following administration of aCSF and h/r-CRF. For group sizes see legend to figure 2. Data represent mean ± SEM. * p<0.01 compared to corresponding vehicle condition. See text for details.

associated with increased brain activity within mesolimbic dopamine pathways [22, 244, 245]. In agreement, SKF97541 and baclofen, locally applied in prefrontal cortex and striatum, reduced amphetamine-induced dopamine release in rats [246], and the authors propose a model that includes presynaptic $GABA_B$ receptors modulating the release of striatal dopamine. Several lines of evidence indicate that CRF affects the mesolimbic dopaminergic system as well [20, 247, 248], and CRF-$GABA_B$-dopamine interactions are reported in mouse dopaminergic neurons [249]. Combining these findings with our data, it is hypothesized that $GABA_B$ receptor agonists may improve CRF-disrupted PPI via effects on mesolimbic dopamine pathways (indicated as [1] in figure 6).

In addition to these effects in mid- and forebrain, $GABA_B$ receptors could also interact with CRF in the brainstem to influence PPI. In other studies, baclofen reversed PPI impairments induced by the NMDA receptor antagonists phencyclidine and dizocilpine [168, 169], and also normalized the spontaneous PPI-deficits of DBA/2J mice [170]. This broad efficacy of baclofen in PPI tests suggests additional actions on $GABA_B$ receptor signaling in brain areas that represent more downstream levels in the regulation of PPI. One such area could be the caudal pontine reticular nucleus (PnC). The PnC is thought to be a critical part of the primary acoustic startle pathway, where startle pulses are inhibited by prepulses [250]. PPI disruptions by methamphetamine and NMDA receptor antagonists were associated with stimulation of neurons in this brainstem nucleus, as measured by increased c-Fos expression. Baclofen not only improved the PPI-deficits in these models, but also significantly decreased the elevated c-Fos expression in PnC to basal levels [167]. The source of this $GABA_B$-ergic signal in PnC associated with PPI, is thought to be the substantia nigra pars reticulata [250]. Lesions of this basal nucleus profoundly reduced PPI, an effect that was not further amplified by amphetamine or the NMDA receptor antagonist MK801, indicating involvement of the substantia nigra pars reticulata in both the mediation and regulation of PPI [251].

Interestingly, in the substantia nigra and the PnC, CRF_1 receptors are expressed [17]. As activation of CRF_1 receptors can have inhibitory effects in neurons [249], i.c.v. CRF might partly disrupt PPI via reducing activity in nigroreticular $GABA_B$-ergic pathways. Possibly, this effect is rescued by the actions of $GABA_B$ agonists in the PnC, which could be more effective in the presence of CRF [249]. Thus, it is hypothesized that PnC represents a second level in the regulation of PPI where $GABA_B$ receptor agonists diminish the effects of i.c.v. CRF (indicated as [2] in figure 6). In addition to its role in

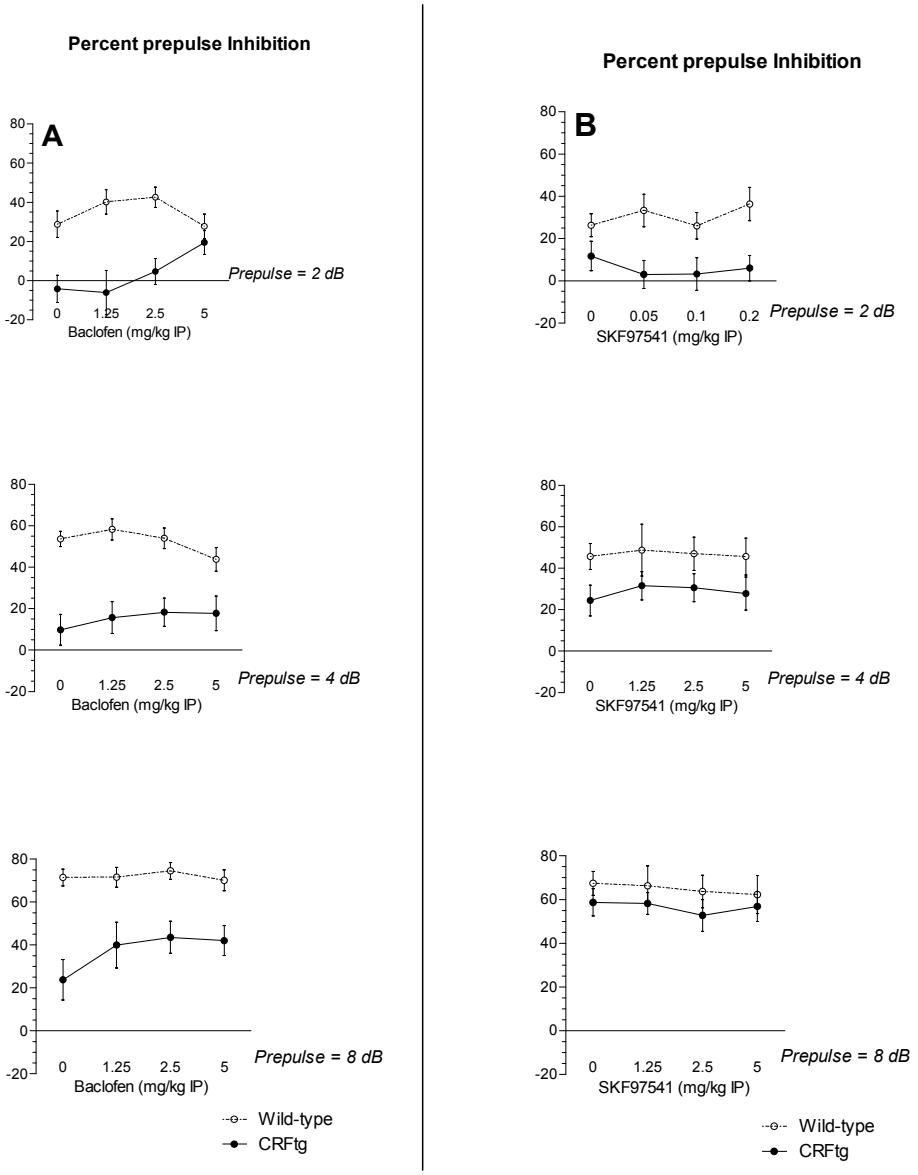

Fig. 4 Effects of (A) baclofen (wild type, n=18; CRFtg, n=17 per group) and (B) SKF97541 (wild type, n=14; CRFtg, n=14 per group) on percent prepulse inhibition for the different prepulse intensities, in corticotropin-releasing factor-overexpressing (CRFtg) and wild type mice. Data represent mean ± SEM.

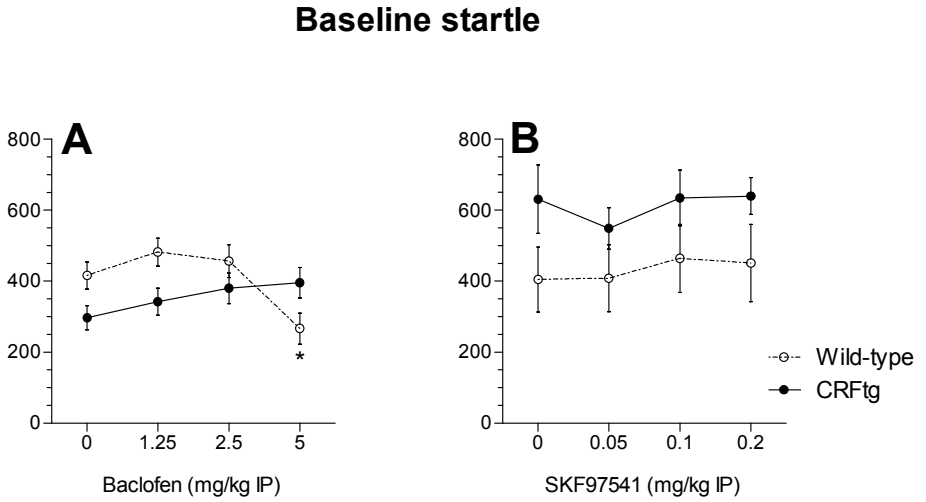

Fig 5 Effects of baclofen (wild type n=18, CRFtg n=17) and SKF97541 (wild type n=14, CRFtg n=14) on baseline acoustic startle response in CRFtg and wild type mice. Data represent mean ± SEM. *p<0.05 relative to corresponding vehicle controls.

PPI, PnC is involved in generation of the baseline startle reflex. With baclofen, a near significant decrease in startle response was observed in CRF infusion- and control groups, and SKF97541 decreased startle in the CRF infusion group, whereas it had no effect in controls at the doses used. Higher doses of SKF97541 however, markedly

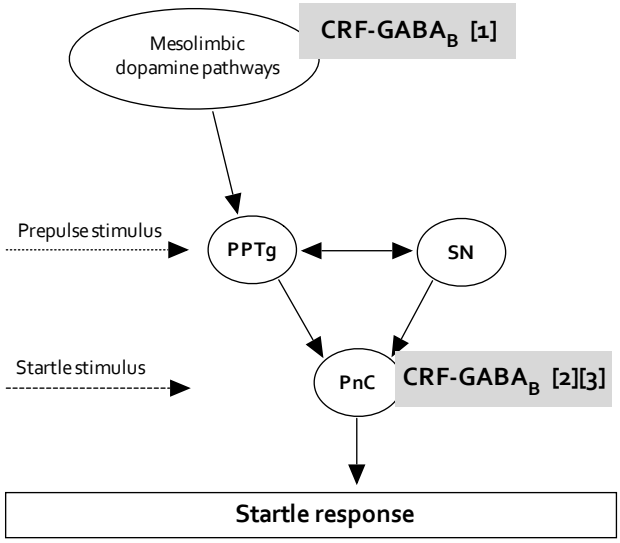

Fig. 6 Schematic diagram showing part of the connecting brain areas that regulate baseline startle and prepulse inhibition responses. Grey blocks indicate levels where CRF may interact with GABA$_B$ signaling. [1], [2] and [3] refer to further information in the discussion section. CRF, corticotropin-releasing factor; PPTg, pedunculopontine tegmental nucleus; SN, substantia nigra; PnC, caudal pontine reticular nucleus.

decreased startle in 129Sv control mice (not shown). Thus, possibly, the PnC is also involved in the startle-lowering effects of $GABA_B$ receptor agonists (see [3] in figure 6).

Contrary to results of CRF-infusion studies, $GABA_B$ receptor agonists had no effect on PPI deficits induced by long-term central CRF over-expression. It seems unlikely that this absence of effect in CRFtg mice is due to strain differences. Although the C57Bl/6J strain, which is the background strain of CRFtg mice appears less sensitive to $GABA_B$ receptor agonists than 129Sv mice, considerably higher doses of $GABA_B$ receptor agonists were tested in CRFtg mice than in 129Sv mice. Furthermore, behavioral effects of $GABA_B$ receptor agonists in C57Bl/6J have been reported using a similar dose range as in the present study [252, 253]. Alternatively, it could be that long-term exposure to elevated CRF levels caused adaptations at the level of CRF or $GABA_B$ receptors, resulting in altered sensitivity to $GABA_B$ receptor agonists with respect to CRF-induced behavioral alterations. Several findings point in this direction. First, compared to wild-types, CRFtg mice show differences in the expression of CRF_1 receptor mRNA in several brain areas, among which the substantia nigra [254], which could affect $GABA_B$-ergic neurotransmission (see figure 6, [2] and [3]). Second, effects of CRF on $GABA_B$ signaling and on mesolimbic dopamine neurons were attenuated by long-term or severe stress [20, 249]. Third, CRFtg mice also show altered sensitivity to the PPI-disrupting effects of the NMDA receptor antagonist MK801 (Douma et al., manuscript submitted for publication). NMDA receptors are reported to interact with CRF [255] and to act upstream of $GABA_B$ receptors in the modulation of PPI [167]. Thus, here it is hypothesized that long-term exposure to increased levels of CRF causes adaptation at the level of mesolimbic dopamine neurons or in the nigroreticular pathway that renders these areas less sensitive to the PPI-enhancing effects of $GABA_B$ receptor agonists. However, further pharmacological and neurochemical studies are needed to confirm this hypothesis.

In conclusion, the current study demonstrates that increasing $GABA_B$ receptor signaling reverses PPI disruptions induced by acute administration of CRF, and that this beneficial effect of $GABA_B$ receptor activation on CRF-induced PPI disruptions is abolished after long-term exposure to increased CRF levels. It is suggested that these processes could be mediated at the level of the brainstem PnC nucleus and mesolimbic dopamine pathways.

Chapter 6

CRF_1 receptor antagonists do not reverse pharmacological disruption of prepulse inhibition in rodents

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Abstract

Rationale Dysregulation of corticotropin-releasing factor (CRF) transmission is associated with psychotic states, and induction of sensory gating deficits.
Objectives Since schizophrenia is associated with impaired prepulse inhibition (PPI), and CRF-overexpressing (CRFtg) mice display PPI deficits, we examined actions of CRF_1 receptor antagonists in pharmacological models of PPI-disruption responsive to antipsychotics. Further, since dopamine (DA) release is implicated in perturbation of PPI, we evaluated their influence on amphetamine-induced DA release.
Methods The effects of CP154,526 (10-40 mg/kg), SSR125,543 (3-30 mg/kg) and DMP695 (40 mg/kg) were tested on PPI disruption provoked by D-amphetamine (2.5, 3 mg/kg), ketamine (5, 30 mg/kg) and MK801 (0.15, 0.5 mg/kg) in Wistar rats, C57Bl/6J and CD1 mice, and on spontaneously-low PPI in Iffa-Credo rats and DBA/2J mice. The interaction of CP154,526 with D-amphetamine and MK801 was examined on PPI disruption in CRFtg mice. Finally, we determined the influence of CP154,506 on D-amphetamine-induced dopamine outflow in nucleus accumbens (NAc) and prefrontal cortex (PFC) of CRFtg mice using microdialysis.
Results No CRF_1-antagonist improved PPI deficits in any behavioral model. CRFtg mice showed *blunted* rather than enhanced PPI-disruption in response to MK801, but not D-amphetamine. Further, amphetamine-induced dopamine release was *less* pronounced in CRFtg versus Wild-type mice, a difference absent following pre-treatment with CP154,526.
Conclusion The inability of CRF_1 receptor antagonists to block pharmacological disruption of sensorimotor gating or amphetamine-induced DA release does not support antipsychotic potential. It cannot be excluded though that CRF_1 receptor antagonists, alone or in combination with antipsychotics, would be useful in treating psychosis under stress conditions, or other aspects of schizophrenic disorders.

1. Introduction

Several lines of evidence point to dysfunction of corticotropin-releasing factor (CRF) systems in psychosis. A decrease in CRF binding protein was found in the amygdala of male schizophrenic and bipolar patients [64] and positive treatment response to the antipsychotic quetiapine was associated with decreased levels of CRF in CSF of schizophrenia patients [140]. Also, posttraumatic stress disorder (PTSD) patients with secondary psychotic features have significantly higher CRF levels in cerebrospinal fluid than non-psychotic PTSD and control subjects [63].

In addition to these psychotic states, CRF is implicated in the induction of sensorimotor gating deficits, as measured by prepulse inhibition (PPI) of the acoustic startle response [4, 9, 46]. The startle reflex is a quick, involuntary contraction of bodily muscles, evoked by sudden and intense acoustic stimuli. PPI is the reduction of the startle response which occurs when a weak, non-startling sensory stimulus is presented 30-500 ms before the startling stimulus [89]. Theoretically, deficient PPI is thought to reflect a dysfunction in the gating of sensory and cognitive information, possibly leading to symptoms of cognitive fragmentation and thought disorder in schizophrenia [46, 256]. Though, it should be noted that PPI deficits are not specific to schizophrenia [9]. Disruption of PPI in rodents is used as a tool for the screening of antipsychotic drug properties [9, 89]. PPI deficits are induced using dopamine receptor agonists [199] or NMDA receptor antagonists [235]. Both methods have construct validity with respect to schizophrenia [257].

Furthermore, PPI can be disrupted by exposure to stressors (reviewed by [258]), long-term CRF overexpression, and acute infusion of CRF into the brains of mice and rats [27, 29, 66]. The PPI deficits induced by long-term CRF overexpression are normalized by CRF_1 receptor antagonists [26]. In addition, CRF-induced PPI deficits can be reversed by typical and atypical antipsychotics [28, 29]. Neuroanatomically, interactions between CRF and dopamine that are relevant for PPI can be expected in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). First, CRF_1 receptors are located on dopaminergic neurons in the ventral tegmental area [17], a major source of dopaminergic input to NAc and mPFC. Second, mPFC and NAc also contain CRF_1 receptors [17] and are important neurochemical substrates in the dopaminergic regulation of PPI [22]. Third, CRF has been reported to increase dopaminergic activity in the ventral tegmental area [259], NAc [260, 261], and PFC [262, 263], although decreases have also been reported in the PFC [260, 264]. In addition to dopamine, CRF is reported to affect glutamatergic signaling in brain circuits that regulate PPI [265-268].

Considering the putative role of CRF in psychosis, and the PPI disruptive effects of increased levels of CRF in animals, here we studied the antipsychotic potential of CRF_1 receptor antagonists. To assess whether CRF_1 receptors are a potential target for schizophrenia, we evaluated the effects of CRF_1 receptor antagonists in D-amphetamine-, ketamine-, and MK801-induced PPI disruption, and also in spontaneously low PPI in the DBA/2J mouse strain [269]. As non-peptidergic CRF_1 receptor antagonists differ in their mode of interactions at CRF_1 receptors – which may possess multiple binding sites and/or various isoforms [270] – and species

differences have been reported with respect to anxiolytic effects of CRF_1-antagonists [271], we tested the effects of three CRF_1 receptor antagonists CP154,526, DMP695 and SSR125543 in different (sub)strains of Wistar rats and mice (C57Bl/6J, CD1 and DBA/2J mice) across two different labs. Last, to explain the behavioral effects within a mechanistic framework, we performed an in vivo microdialysis study to determine the effect of CP154,526 on D-amphetamine-induced dopamine overflow in NAc and mPFC of transgenic mice overexpressing CRF (CRFtg) and determined whether chronically elevated central CRF levels alter sensitivity to D-amphetamine and MK801 in PPI.

2. Materials and methods

See **Table 1** for a schematic overview of the experiments performed.

2.1. Prepulse inhibition of the acoustic startle reflex

2.1.1. Apparatus and test procedure

Studies 1.1., 1.2., 2.2., 3.1., 3.2., and 4.2.

Startle reflexes were measured in eight identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA). Startle stimuli (115 dB, 50 ms) were presented alone, or preceded by noise prepulses (20 msec) of 2, 4, 8, or 16 dB above background (70 dB), with 100 ms between onsets of the prepulse and startle stimuli. The test session started with a 5-min acclimation period followed by three consecutive blocks of test trials (block 1 and 3, startle-stimulus alone trials; block 2, startle-stimulus alone, startle + prepulse, and no-stimulus trials). Intertrial intervals ranged from 10 to 20 sec, and total test duration was 25 min.

Studies 2.1. and 4.1.

Animals were tested in four startle boxes (Med Associates, East Fairfield, VT, USA). Startle pulses (120 dB, 50 ms) were preceded by prepulses (rats: 30 ms, mice: 20 ms) of 7, 14, or 20 dB above background (65 dB). Onsets of pulses and prepulses were separated by 100 (rats) or 40 (mice) ms. The test session started with a 5-min acclimation period followed by 5 startle stimuli that served to accustom the animals to the startle pulses. These startle pulses were followed by a block of 40 stimuli, in which equal amounts of startle-stimulus alone, and startle + prepulse trials (with each of the three prepulse intensities), were presented in pseudorandom order. Intertrial intervals were variable (mice: 18-25 s, rats: 15-25 s). In study *2.1.*, mice were individually housed prior to the first injection.

2.1.2. Matching

One week before drug testing, or one day in study *4.1*, a baseline PPI measurement was performed, in order to familiarize the subjects to the test procedure and to create treatment groups with equal mean percent PPI. In study *2.1*, no matching session was included.

Table 1 Summary of animals, drugs and experimental procedures.

Study	Laboratory	Animals*			Induction				CRF_1 receptor antagonist			
		Species	Strain	Weight / age	Drug	Dose** (mg/kg)	Route	I.t.i.	Drug	Dose*** (mg/kg)	Route	I.t.i.
Dopamine models												
1.1.	UU	Rat	Wistar Wi	250-350 g	d-Amphetamine sulphate	2.5	IP	10 min	SSR125,543	30	IP	30 min
1.2.	UU	Mouse	C57Bl/6J	8-16 wks	d-Amphetamine sulphate	3	IP	10 min	CP154,526	40	IP	30 min
Glutamate NMDA models												
2.1.	Sanofi	Mouse	CD1	30-35 g	MK801 hydrogen maleate	0.15	IP	60 min	SSR125,543	30-Oct	IP	60 min
2.2.	UU	Mouse	C57Bl/6J	8-16 wks	MK801 hydrogen maleate	0.5	IP	acute	DMP695	40	IP	30 min
3.1.	UU	Rat	Wistar Wu	250-350 g	Ketamine hydrochloride	5	SC	acute	DMP695	40	IP	30 min
3.2.	UU	Mouse	C57Bl/6J	8-16 wks	Ketamine hydrochloride	30	IP	acute	CP154,526	40	IP	30 min
Low PPI animal strains												
4.1.	Sanofi	Rat	Wistar Iffa Credo	260-280 g	-				SSR125,526	30-Mar	IP	60 min
4.2.	UU	Mouse	DBA/2J	7-8 wks	-				SSR125,526	Oct-40	IP	30 min
5	UU	Mouse	CRFtg, wildtype (C57bl/6J)	9-16 wks	MK801 hydrogen maleate	0.3; 1.0	IP	acute	-			
6	UU	Mouse	CRFtg, wildtype (C57bl/6J)	9-16 wks	d-Amphetamine sulphate	2.5 ; 5.0	IP	10 min	-			

UU, Utrecht University; i.t.i., injection-test interval; IP, intraperitoneal; SC, subcutaneous.

* All experiments were performed according to the Guide for Care and Use of Laboratory animals and were approved by the Ethical Committees for Animal Research of Utrecht University and Sanofi-Aventis, resp.

** Doses were based on dose-response pilots (not shown).

*** Doses were based on previous experiments from our labs [26, 272] **Table 1** Summary of animals, drugs and experimental procedures.

2.2. Microdialysis

2.2.1. Probe implantation

Microdialysis probes were implanted in the medial prefrontal cortex (PFC, left probe, MAB 4.7.2. CU; AP +1.9, ML +0.9, DV -3.3 from bregma) and nucleus accumbens (NAc, right probe, MAB 4.7.1. CU; AP +1.5, ML +1.0, DV -5.0 from bregma). Probes were secured with dental cement. To make sure the cement would be held in place, shallow lines were carved into the skull. After microdialysis probe implantation, mice were housed individually for the duration of the experiment.

2.2.2. Experimental procedures

Two days after implantation, microdialysis experiments were performed in conscious freely moving mice. First, the system was perfused with Ringer solution (147 mM NAcl, 2.3 nM KCl, 2.3 mM $CaCl_2$, and 1 mM $MgCl_2$) with the use of a KdScientific Pump 220 series (USA) at constant flow rate of 1 mL/min. Mice were connected to a dual channel swivel (type 375/D/22QM) which allowed them to move relatively unrestricted. During microdialysis, the pump rate was set at 0.07 mL/h. Two hours after connection, ten 30-minute samples (i.e., s1-s11) were manually collected in vials containing 15 µL of 0.1 M acetic acid and frozen at -80ºC until analysis with HPLC. After two hours of baseline samples (s1-s4), mice were injected IP with CP154,526 (0, 40 mg/kg) (s4), followed by D-amphetamine (0, 5 mg.kg) 30 minutes later (s5), where after additional samples were collected for 3 hours (s5-s11).

2.2.3. Histology

After three days, mice were sacrificed and their brains were quickly frozen in isopentane and stored at -80ºC. For probe localization, brains were transferred to a 30% sucrose solution and after two to three days, frozen slices of 60 µm were made. These slices were stained with a cresyl violet staining for probe track verification. Data were discarded if the microdialysis probe was not in the PFC or NAc (1 animal removed from TG/NAc-amf group).

2.2.4. HPLC-ECD

For HPLC with electrochemical detection, an Alexyz 100 LC-EC system (Antec, The Netherlands) was used, consisting of two pumps, one auto-sampler with a 10 port injection valve, two columns and two detector cells. The mobile phase for column 1 (DA) consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 12% methanol and 500 mg/L 1-Octanesulfonic acid, sodium salt (OSA); and for column 2 (DOPAC) of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.2), 10% methanol and 500 mg/L OSA. From each microdialysis sample 5 µl was injected simultaneously onto each column. Mobile phases were pumped at 50 µL/min. Dopamine and the metabolites, DOPAC and HVA, were detected electrochemically using µVT-03 flow cells (Antec, The Netherlands) with glassy carbon working electrodes. Potential settings were for DA +0.30 V, and for DOPAC +0.59 versus Ag/AgCl. The chromatogram was recorded and analyzed using the Alexys data system. The limit of detection was 0.03 nM (S/N ratio 3:1). Methods have been described in detail elsewhere [273].

2.3. Statistics

Percent PPI was calculated as the mean startle magnitude to startle stimulus-alone, minus the mean startle magnitude to startle + prepulse stimuli, all divided by the mean startle stimulus-alone trials, and multiplied by 100. For calculation of the mean startle magnitude, only data from blocks that included prepulse trials were used. PPI data were analyzed using repeated-measures analysis of variance (ANOVA) with prepulse intensity as within-subjects factor and CRF_1 antagonist and drug pre-treatment (PPI disruption experiments), or CRF_1 antagonist (naturally low PPI experiments) as between-subjects factor(s). If there were no interactions between drug treatment and prepulse intensity, PPI data were collapsed across prepulse intensities. In study *2.1.*, three repeated-measures ANOVAs were performed; one to determine the effect of SSR125543 on MK801-induced PPI disruptions, the other two to determine the effects of respectively MK801 and SSR125543 on PPI under vehicle conditions. In studies *1.1, 1.2* and *2.2.*, MK801 and D-amphetamine only disrupted PPI at 2, 4 and 8 dB prepulses, so treatment effects of the CRF_1 receptor antagonist were analysed using these intensities. Acoustic startle magnitude was analyzed using one-way ANOVA with drug (pre-) treatment as between factor(s). Post hoc analyses were performed by Dunnett's and planned comparison t-tests.

In the microdialysis experiment, separate analyses were performed for mPFC and NAc, and for dopamine and DOPAC. Genotype differences in mean basal values were analyzed using students t-test on the mean value of samples 1 to 4, from all animals under study. Data on effects of combined drug treatment were analyzed using repeated-measures ANOVA with time (samples 5-11) as within-subjects factor and genotype and treatment as between-subjects factors, followed by separate repeated-measures ANOVAs for the three different treatment conditions. In the latter case, drug effects were analyzed relative to mean basal values (samples 1 to 4), over a period of 2.5 hours starting at 30 minutes post injection (CP154,526: samples 5-10; combined treatment CP154,526 and amphetamine: samples 6-11). Post hoc analyses for changes over time were analyzed with simple contrasts relative to mean basal value. Post hoc analysis for comparisons between genotypes at certain time points was performed with t-tests or multivariate ANOVA with basal and post injection samples as dependent and genotype as fixed factor. The area under the curve (AUC) was calculated using the trapezoid algorithm, for values from sample 4 (CP, combi) or sample 5 (amphetamine) onwards. AUC data were analyzed by one-way ANOVA followed by planned-comparison t-tests.

The level of significance was set at $p < .05$. Statistical analyses were carried out using SPSS for Windows, version 20.

2.4. Results

In every case reported here, percent PPI increased significantly with increasing prepulse intensity and this effect will not be described further. As drug treatment effects were independent of prepulse intensity in all cases, PPI was collapsed across intensity for the purpose of clarity. Data on the baseline startle response are summarized in table 2.

2.4.1. Experiment 1. Effects of CRF_1 receptor antagonists in D-amphetamine disrupted PPI

Study 1.1. SSR125543 in Wistar Wi rats

D-amphetamine (2.5 mg/kg, IP) significantly disrupted PPI in Wistar Wi rats ($F[1,44]=8.8$; $p=0.005$), independent of prepulse intensity ($F[2,88]<1$, $p=0.997$). SSR125543 (30 mg/kg) did not alter D-amphetamine-induced PPI deficits (interaction D-amphetamine x CRF_1-antagonist, $F[1,44]<1$; $p=0.9$), and SSR125543 had no effects on percent PPI by itself ($F[1,44]<1$; $p=0.7$) (figure 1A).

Study 1.2. CP154,526 in C57Bl/6J mice

D-amphetamine (3.0 mg/kg, IP) significantly disrupted PPI in C57Bl/6J mice ($F[1,50]=19.0$; $p<0.001$), independent of prepulse intensity ($F[2,77]<1$, $p=0.9$). CP154,526 (40 mg/kg) did not significantly alter D-amphetamine-induced PPI deficits (interaction, D-amphetamine x CRF_1-antagonist, $F(1,50)=2.5$; $p=0.12$), and CP154,526 had no overall effect on percent PPI ($F[1,50]<1$; $p=0.8$) (figure 1B).

2.4.2. Experiment 2. Effects of CRF_1-antagonists in MK801 disrupted PPI

Study 2.1. SSR125543 in CD1 mice

MK801 (0.15 mg/kg IP) significantly disrupted PPI in CD1 mice ($F[1,14]=8.5$; $p=0.01$), independent of prepulse intensity ($F[2,28]<1$; $p=0.4$). SSR125543 (10, 30 mg/kg) did not significantly alter MK801-induced PPI deficits ($F[2,21]<1$; $p=0.5$), and SSR125543 (30 mg/kg) had no significant effect on percent PPI by itself ($F[1,14]<1$; $p=0.5$) (figure 2A).

Study 2.2. DMP695 in C57Bl/6J mice

MK801 (0.5 mg/kg IP) significantly disrupted PPI in C57Bl/6J mice ($F[1,32]=9.1$; $p=0.005$), independent of prepulse intensity ($F[2,64]<1$; $p=0.6$). DMP695 (40 mg/kg) did not significantly affect MK801-induced PPI deficits (interaction, MK801 x CRF_1-antagonist, $F[1,32]<1$; $p=0.4$). DMP695 had no significant effects on percent PPI in C57Bl/6J mice per se (main effect DMP, $F[1,32]=1.4$; $p=0.2$) (figure 2B).

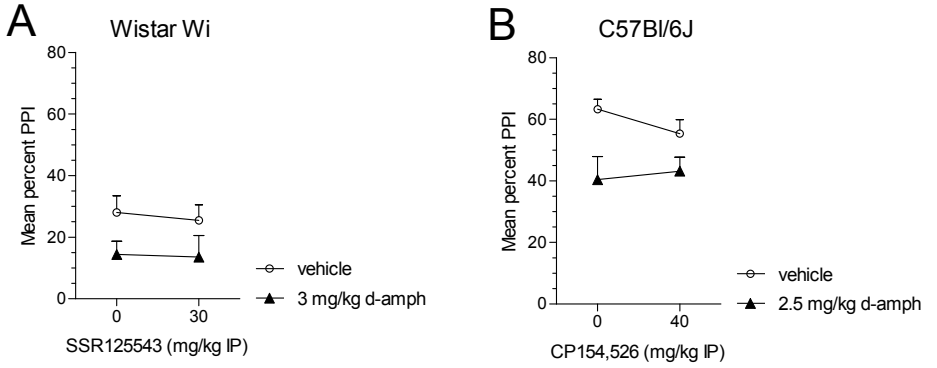

Fig. 1 Effects of CRF1 receptor antagonists on D-amphetamine-disrupted PPI. (A) SSR125543 in Wistar Wi rats, (B) CP154,526 in C57Bl/6J mice. Group sizes (A) n=12, (B) vehicle-vehicle, amphetamine-CP154,526: n=14; CP154,526-vehicle: n=13; vehicle-amphetamine: n=12. Data are collapsed over three prepulse intensities and expressed as mean ± SEM.

2.4.3. Experiment 3. Effects of CRF_1 receptor antagonists in ketamine-disrupted PPI

Study 3.1. DMP695 in Wistar Wu rats

Ketamine (5 mg/kg SC) significantly disrupted PPI in Wistar Wu rats ($F[1,43]=26.1$; $p<0.001$), dependent on prepulse intensity ($F[2,86]=4.0$; $p=0.02$). However, further analysis showed that PPI was significantly disrupted at each prepulse intensity, so data were collapsed across intensities. DMP695 (40 mg/kg) did not significantly alter ketamine-induced PPI deficits (interaction, ketamine x CRF_1-antagonist, $F[1,43]<1$; $p=0.5$). Also, DMP695 had no significant effect on percent PPI in Wistar rats on itself (main effect DMP, $F[1,43]=3.2$; $p=0.08$) (figure 3A).

Study 3.2. CP154,526 in C57Bl/6J mice

Ketamine (30 mg/kg IP) significantly disrupted PPI in C57Bl/6J mice ($F[1,53]=19.3$; $p<0.001$), independent of prepulse intensity ($F[2,106]=1.7$; $p=0.2$). CP154,426 (40 mg/kg) did not significantly alter ketamine-induced PPI deficits (interaction, ketamine x CRF_1-antagonist, $F[1,53]=3.4$; $p=0.07$), and it had no overall effect on PPI in C57Bl/6J mice ($F[1,53]<1$; $p=0.99$) (figure 3B).

2.4.4. Experiment 4. Effects of CRF1-antagonists on spontaneously low PPI

Study 4.1. SSR125543 in Wistar Iffa-Credo rats

The effect of SSR125543 (3-30 mg/kg) on percent PPI in Wistar Iffa-Credo rats was dependent on prepulse intensity ($F[5,64]=3.5$; $p=0.005$). However, the main effect of SSR125543 on percent PPI was not significant ($F[3,32]<1$; $p=0.7$), also when the effect was analyzed per prepulse intensity (figure 4A).

Study 4.2. CP154,526 in DBA/2J mice

CP154,526 (10-40 mg/kg) had no significant effect on percent PPI in DBA/2J mice ($F[3,44]=1.4$; $p=0.3$), regardless of prepulse intensity ($F[9,132]=1.1$; $p=0.3$) (figure4B).

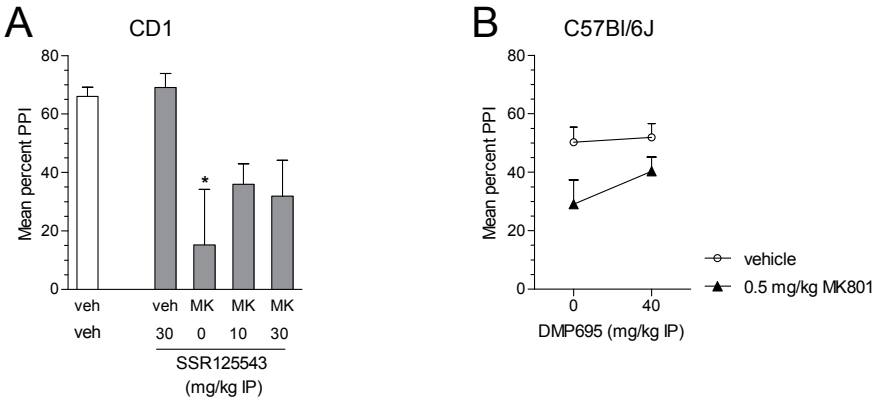

Fig. 2 Effects of CRF1-antagonists on MK801-disrupted PPI. (A) SSR125543 in CD1 mice; veh, vehicle; MK, MK801, 0.15 mg/kg (B) DMP695 in C57Bl/6J mice. Group sizes, (A) n=8, (B) vehicle-vehicle, DMP695-vehicle: n=8; vehicle-MK801, DMP695-MK801, n=10. Data are collapsed over three prepulse intensities and expressed as mean ± SEM. *p<0.05, compared to vehicle controls.

2.4.5. Experiment 5. Effects of chronically elevated CRF levels on sensitivity to D-amphetamine and MK801-disrupted PPI

Study 5.1. MK801 in CRFtg mice

The effect of MK801 was dependent on genotype ($F[2,79]=3.3$; $p=0.04$), and independent of prepulse intensity ($F[5,237]<1$; $p=0.7$). Further analysis showed that MK801 had no effect in CRFtg mice ($F[2,40]<1$; $p=0.5$), whereas it significantly disrupted PPI in wild-type mice both at 0.3 and 1.0 mg/kg ($F[2,39]=13,2$; $p<0.001$). When comparing CRFtg and wild-type mice, percent PPI of the former was significantly lower for the vehicle and 0.3 mg/kg MK801 conditions, and similar at the highest dose of MK801 tested (planned comparison t-test following significant ANOVA) (figure 5A).

Study 5.2. D-amphetamine in CRFtg mice

D-amphetamine significantly disrupted PPI ($F[2,86]=4.3$; $p=0.016$), independent of genotype ($F[2,86]<1$; $p=0.99$) and prepulse intensity ($F[4,258]=2.0$; $p=0.1$). Post hoc Dunnett's test showed that the D-amphetamine-induced PPI disruptions were significant only for the 2.5 mg/kg dose. PPI in CRFtg was significantly lower than in wildtypes for all treatment groups (figure 5B).

2.4.6. Experiment 6. Effects of CP154,526 on extracellular dopamine following D-amphetamine treatment in CRFtg mice

2.4.6.1. Dopamine in NAc

Basal dopamine levels were similar in CRFtg and Wild-type mice (students t-test $p=0.3$) (table 3).

The overall repeated-measures ANOVA on dopamine concentrations in NAc showed a significant treatment x time x genotype effect ($F[3,138]=4.0$, $p=0.001$). Analysis per treatment showed that CP154,526 significantly increased extracellular dopamine concentrations, at all time points measured (post hoc after significant ANOVA, $F[6,48]=117$, $p<0.001$). As shown in figure 6A, this effect was similar for both genotypes ($F[6,48]=2$; $p=0.08$).

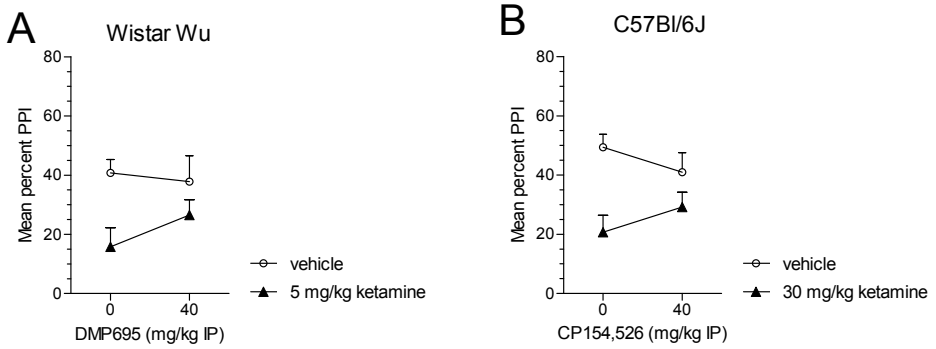

Fig. 3 Effects of CRF1-antagonists on ketamine-disrupted PPI. (A) DMP695 in Wistar Wu rats, (B) CP154,526 in C57Bl/6J mice. Group sizes, (A) n=12, (B) vehicle-vehicle: n=15, other groups, n=14. Data are collapsed over three prepulse intensities and expressed as mean ± SEM.

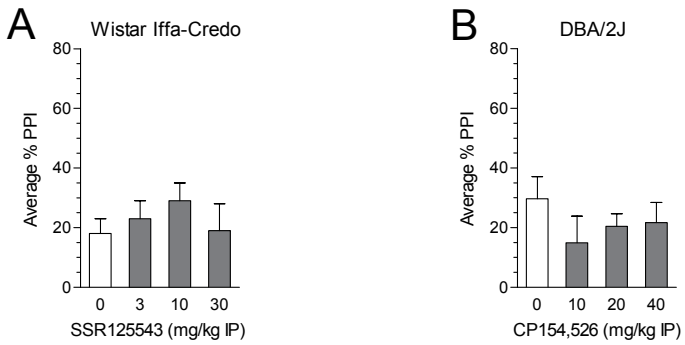

Fig. 4 Effects of CRF1-antagonists in models of spontaneously low PPI. (A) SSR125543 in Wistar Iffa-Credo rats, (B) CP154,526 in DBA/2J mice. Group sizes, (A) SSR125543 (30 mg/kg), n=7; SSR125543 (10 mg/kg), n=9; SSR125543 (3 mg/kg), vehicle: n=10, (B) N=9. Data are collapsed over three (A) or four (B) prepulse intensities and expressed as mean ± SEM.

D-amphetamine treatment significantly elevated dopamine concentrations, at all time points measured (post hoc after significant ANOVA, $F[1,48]=127$, $p<0.001$). This effect was dependent on genotype ($F[1,48]=12$, $p=0.005$), and further analysis showed that the increase of extracellular dopamine by D-amphetamine was *lower* in CRFtg mice than in wild-types from 30 to 150 min after D-amphetamine injection (figure 6B).

Relative to mean basal dopamine levels, treatment with D-amphetamine preceded by CP154,526 injection resulted in a significant increase in dopamine concentrations in the NAc at all time points measured (post hoc after significant ANOVA, $F(2,42)=43$, $p<0.001$). Importantly, however, this effect was independent of genotype ($F[2,42)<1$, $p=0.7$), indicating that CP154,526 abolished the differences between genotypes in the NAc with respect to the dopamine release after D-amphetamine treatment (figure 6C). In fact, apparently, CP154,526 increased the amphetamine-induced elevation in dopamine levels in CRFtg mice, while decreasing those levels in wild-types.

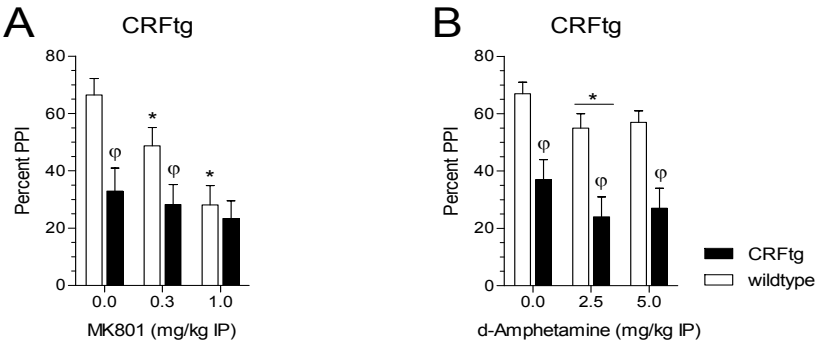

Fig. 5 Effects of (A) MK801 and (B) D-amphetamine on percent PPI in CRFtg and wildtype mice. Group sizes: (A) vehicle and MK801 (0.3 mg/kg) condition: wildtype n=14, CRFtg n=14; for MK801 (1.0 mg/kg): wildtype n=14, CRFtg: n= 15; (B) for vehicle and amphetamine (2.5 mg/kg) wildtype n=17, CRFtg: n=13; amphetamine (5.0 mg/kg): wildtype n=18, CRFtg: n= 14. Data are collapsed over four prepulse intensities and expressed as mean ± SEM. * $p<0.05$, compared to corresponding vehicle, ϕ $p<0.05$, genotype difference in treatment condition.

Table 2a Drug effects on baseline startle response (expressed as mean ± SEM)

Experiment	Startle (mV)			
	Vehicle-vehicle	Drug pretreatment	CRF1-antagonist	Drug pretreatment + CRF1-antagonist
1.1.Amph + SSR	1421 ± 228	1060 ± 99	1218 ± 249	842 ± 127
1.2.Amph + CP	303 ± 35	352 ± 86	293 ± 73	214 ± 52
2.1. MK801 + SSR	567 ± 77	389 ± 53	262 ± 48	(10) 491 ± 82 (30) 429 ± 59
2.2. MK801 + DMP	264 ± 45	439 ± 71	344 ± 64	318 ± 47
3.1. ket + DMP	676 ± 103	447 ± 74	450 ± 122	370 ± 48
3.2. ket + CP	448 ± 40	493 ± 75	525 ± 68	418 ± 62
4.1. Wistar low - SSR	1817 ± 54	-	(3) 1747 ± 76 (10) 1695 ± 61 (30) 1830 ± 60	-
4.2. DBA low - CP	218 ± 34	-	(10) 147 ± 22 (20) 160 ± 29 (40) 191 ± 34	-

	Drug treatment	Startle (mV)	
		Wild-type	CRFtg
5.1. CRFtg - MK801	0	336 ± 38	387 ± 66
	0.3	509 ± 82	410 ± 52
	1	571 ± 93	508 ± 86
5.2. CRFtg - Amph	0	486 ± 50	700 ± 62
	2.5	422 ± 73	380 ± 76
	5	464 ± 89	481 ± 35

Amph D-amphetamine, SSR SSR125543, CP CP154,526, DMP DMP695 ket ketamine

In table 4, the AUC values are given for NAc dopamine concentrations following treatment with CP154,526, D-amphetamine or the combination of both agents in CRFtg and wild-type mice. Univariate ANOVA showed that there was a significant genotype x treatment interaction ($F[2,23]=4.2$; $p=0.027$). According to post-hoc t-tests, D-amphetamine-induced dopamine overflow was significantly lower in CRFtg than in wild-type mice, and CP154,526 significantly increased the amount of dopamine released by D-amphetamine in CRFtg but did not alter the D-amphetamine effect in wild-types.

2.4.6.2. DOPAC in NAc

Basal DOPAC levels were similar in both genotypes (students t-test, $p=0.8$).

D-amphetamine significantly reduced NAc extracellular DOPAC levels, measured as AUC (post hoc after significant ANOVA ($F[2,22]=52$, $p<0.001$). This treatment effect

Table 2b Summary of the ANOVA results from startle data of table 2a. * p<0.05.

Experiment	Main effect drug pretreatment	Main effect CRF_1-antagonist	Interaction effect pretreatment x CRF_1-antagonist
Amph + SSR	F(1,44) = 3.9	F(1,44) = 1.3	F(1,44) < 1
Amph + CP	F(1,50) < 1	F(1,50) = 1.1	F(1,50) < 1
2.1. MK801 + SSR	F(1,35) < 1	F(2,35)= 1.4	F(1,35) < 1
2.2. MK801 + DMP	F(1,32) = 1.6	F(1,32) < 1	F(1,32) = 2.9
3.1. ket + DMP	F(1,44) = 2.9	F(1,44) = 2.8	F(1,44) < 1
3.2. ket + CP	F(1,53) < 1	F(1,53) < 1	F(1,53) = 1.5
4.1. Wistar low - SSR	-	F(3,32) = 2.4	-
4.2. DBA low - CP	-	F(3,48) = 1.1	-
Experiment	**Main effect** drug treatment	**Main effect** genotype	**Interaction effect** treatment x genotype
5.1. CRFtg - MK801	F(2,79) = 3.0	F(1,79) < 1	F(2,79) < 1
5.2. CRFtg - Amph	F(2,88) = 4.1*	F(1,88) = 1.0	F(2,88) = 1.9

Amph D-amphetamine, SSR SSR125543, CP CP154,526, DMP DMP695 ket ketamine

was similar for both genotypes (F[2,22]<1, p=0.7), and unaltered by CP154,526 (table 5).

2.4.6.3. Dopamine in mPFC

Basal dopamine levels were similar in wild-type and CRFtg mice (students t-test p=0.3) (table 3).

The overall repeated-measures analysis on dopamine levels in the mPFC showed a significant treatment x time x genotype effect (F[12,156]=3.4, p<0.001).

Further analysis showed that treatment with CP154,526 elevated extracellular dopamine concentrations over time (F[6,48]=23, p<0.001) compared to baseline, which was significant from 60 to 120 minutes after injection (post hoc). The effects of CP154,526 on dopamine concentrations were comparable for both genotypes (F[6,48]=1.8, p=0.11) (figure 6D).

Table 3 Basal levels of extracellular dopamine, DOPAC and HVA, in CRFtg and wildtype mice.

	Nucleus Accumbens		**Medial prefrontal** cortex	
	Wild-type (n=15)	**CRFtg (n=14)**	**Wild-type (n=14)**	**CRFtg (n=18)**
Dopamine (nM)	1.9 ± 0.1	1.5 ± 0.2	0.3 ± 0.03	0.2 ± 0.02
DOPAC (nM)	248 ± 15.0	223 ± 22.6	42.6 ± 5.8	29.1 ± 2.5 *
HVA (nM)	218 ± 8.8	235 ± 18.3	68.9 ± 17.6	72.1 ± 12.9

*p<0.01, compared to corresponding wildtype.

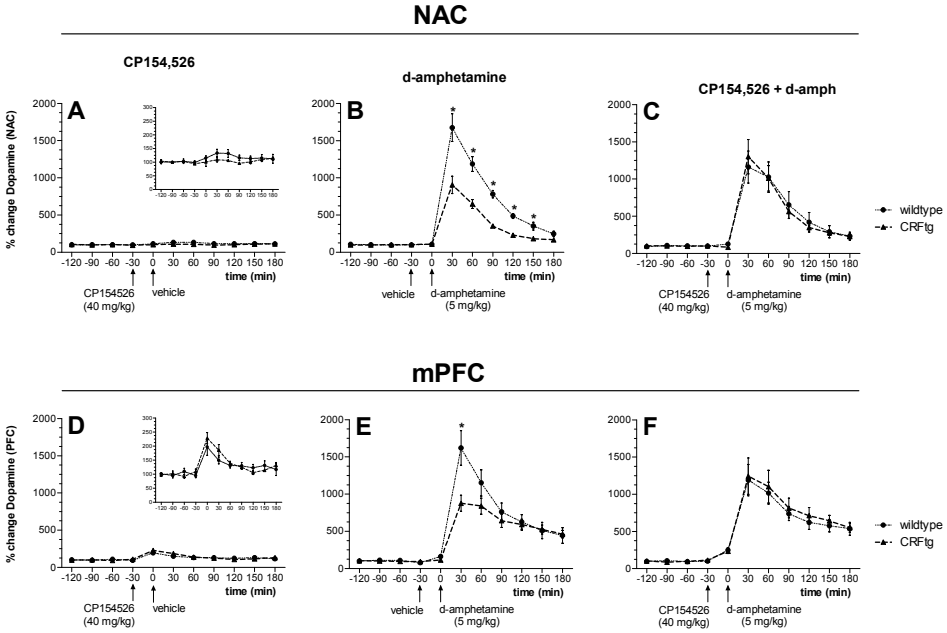

Fig. 6 Effect of treatment with CP154,526 (at t=-30 min) (A, D), D-amphetamine (at t=0 min) (B, E) or combination of CP154,526 and D-amphetamine (C, F) on changes in extracellular dopamine levels in wild-type and CRFtg mice. (A - C) Nucleus accumbens (NAc), (D - F) prefrontal cortex (PFC). Time points -120 to -30 min represent baseline measurements. Group sizes: NAc, n=4 (CRFtg-combi), n=5 (other groups); mPFC, n=6 (CRFtg-amphetamine, -combi; wild-type-combi), n=5 (CRFtg-CP154,526, wildtype-CP154,526), n=4 (wild-type-amphetamine). Data are expressed as percentage of baseline + SEM. *p<0.05, genotype difference within drug condition.

Treatment with D-amphetamine increased extracellular dopamine concentrations in the mPFC ($F[6,48]=81$, $p<0.001$), which was significant for all time points (post hoc). The changes observed over time were dependent on genotype ($F[6,48]=9.8$, $p<0.001$). Further analysis indicated that, similar to the NAc, peak dopamine concentrations were significantly lower in CRFtg mice than in wild-types, at 30 minutes post injection (post hoc) (figure 6E).

Treatment with D-amphetamine preceded by CP154,526 injection resulted in a significant increase in dopamine concentrations at all time points measured, relative to mean basal dopamine levels (post hoc following significant ANOVA, $F[6,60]=39.8$, $p<0.001$). This effect of combined treatment was independent of genotype ($F[6,60]<1$, $p=0.9$), indicating that CP154,526 abolished the differences between genotypes in the mPFC with respect to dopamine response after D-amphetamine treatment (figure 6F).

Table 4 depicts the AUC values of dopamine released after treatment with CP154,526, D-amphetamine or the combination of both agents, in CRFtg and wild-type mice. In the mPFC, the amount of extracellular dopamine was not significantly influenced by any of the agents in either genotype ($F[2,26]=<1$, $p=0.4$, N.S.).

2.4.6.4. DOPAC in mPFC

Basal DOPAC levels were significantly higher in wild-type than in CRFtg mice (students t-test $p=0.009$).

Table 4 Dopamine release (area under the curve (AUC), nM x min) after treatment with CP154,526 (40 mg/kg), D-amphetamine (5 mg/kg), or CP154,526 (40 mg/kg) + D-amphetamine (5 mg/kg) in CRFtg and wildtype mice.

	Nucleus Accumbens		**Medial prefrontal** cortex	
	Wild-type	**CRFtg**	**Wild-type**	**CRFtg**
CP154,526	114.7 ± 37.5	61.2 ± 16.5	180.8 ± 44.0	209.8 ± 26.7
D-amphetamine	2082.9 ± 205.4	982.0 ± 91.0 *	2252.2 ± 347.4	1636.0 ± 228.6
CP154,526 + D-amphetamine	1620.4 ± 397.1	1573.5 ± 244.8 #	2054.8 ± 338.5	2244.7 ± 403.9

# p<0.05, between genotype comparison relative to corresponding treatment

* p<0.05, between treatment comparison per genotype (D-amphetamine vs. D-amph+CP154,526).

D-amphetamine significantly reduced mPFC extracellular DOPAC levels, measured as AUC (post hoc after significant ANOVA ($F[2,26]=66.7$, $p<0.001$). This treatment effect of D-amphetamine was similar in both genotypes ($F[2,26]<1$, $p=0.9$), and unaltered by CP154,526 (table 5).

3. Discussion

In this study we evaluated the effects of the non-peptide CRF_1 receptor antagonists CP154,426 (10-40 mg/kg), SSR125543 (3-30 mg/kg) and DMP695 (40 mg/kg) in rodent tests of disrupted PPI. CRF_1 receptor antagonists did not improve PPI disruption induced by D-amphetamine, by NMDA receptor antagonists, or in animal strains displaying spontaneously low PPI.

3.1. CRF_1 receptor antagonists and dopamine interactions

To our knowledge, this is the first study reporting on the effects of CRF_1 receptor antagonists in dopaminergic models of disrupted PPI. We found that CP154,526 (40 mg/kg) and SSR125543 (30 mg/kg) had no effect on PPI disruptions induced by the

Table 5 DOPAC release (area under the curve (AUC), nM X min) after treatment with CP154,526 (40 mg/kg), D-amphetamine (5 mg/kg), or CP154,526 (40 mg/kg) + d-amphetamine (5 mg/kg) in CRFtg and wildtype mice.

	Nucleus Accumbens		**Medial prefrontal** cortex	
	Wild-type	**CRFtg**	**Wild-type**	**CRFtg**
CP154,526	54.2 ± 31.3	10.2 ± 20.0	150.9 ± 23.8	188.1 ± 19.1
D-amphetamine	-124.6 ± 8.1	-140.5 ± 8.9	-81.7 ± 2.1	-54.9 ± 8.2
CP154,526 + D-amphetamine	-102.6 ± 18.1	-143.7 ± 6.7	-7.8 ± 15.1	13.6 ± 29.8

Group sizes: Nucleus Accumbens (Wild-type, CRFtg; CP154,526, d-amphetamine, and combined treatment, each group n=5; CRFtg, combined treatment, n=4); Prefrontal cortex (CP154,526 in wildtype, CRFtg; n=5; D-amphetamine in wildtype, n=4; combined treatment in CRFtg and D-amphetamine in wildtype and CRFtg, n=6).

dopamine releaser D-amphetamine. D-Amphetamine-induced hyperlocomotion, another read-out associated with antipsychotic potential, was also not reversed by CRF_1 receptor blockade [274]. A few PPI studies investigated the opposite process, i.e., the role of dopaminergic activation in CRF-induced PPI deficits, and found that haloperidol attenuates the PPI deficits of CRFtg mice and intracerebroventricular (i.c.v.) CRF-infusion in Wistar-Kyoto rats [28, 236]. These findings may suggest that CRF-induced PPI disruption involves enhanced dopaminergic activity, although studies in knock-out mice suggest that neither dopamine D_1 nor D_2 receptors are necessary for the CRF-induced PPI effects [240].

The absence of effect of CRF_1 receptor antagonists on amphetamine-induced PPI deficits contrasts with observed effects of CRF on the dopaminergic system [20, 247, 248]. This is not likely explained by the studies of CRF_1 receptor antagonists. First, drugs were tested at doses previously demonstrated to be pharmacologically active [26, 272, 275]. Second, multiple and chemically distinct CRF_1 receptor antagonists yielded similar data in our study. Neurochemically, the PPI-disruptive effects of direct and indirect dopaminergic agonists are linked to dopaminergic hyperactivity in the NAc [22, 276]. In this key area of the mesolimbic dopamine system, CRF facilitates cue-elicited motivation [277] and social bonding [278], both behaviors that are thought to be dopamine dependent [279, 280]. Moreover, with respect to drug addiction – a composite behavior that involves changes in mesolimbic dopamine pathways – the decreased brain reward function associated with drug withdrawal and stress-induced reinstatement of cocaine-seeking have been found to be CRF_1 receptor dependent, as both behavioral components could be blocked by CRF_1 receptor antagonists [281, 282]. Also, CP154,526 reduced morphine withdrawal-induced increases in dopamine turnover and tyrosine hydroxylase phosphorylation in the NAc [283].

Thus, several lines of evidence indicate that CRF may modulate dopaminergic function and that CRF_1 receptor antagonists are likely to dampen behaviors that involve dopamine transmission. Yet only few studies have reported on the effect of CRF_1 receptor antagonists on extracellular dopamine concentrations. Here we showed that systemic administration of CP154,526 enhanced basal dopamine concentrations in mPFC and, to a smaller extent, in NAc. Comparable effects were observed in CRFtg mice. Also, wild-type and CRFtg mice had similar basal dopamine levels, showing that long-term elevated central CRF levels do not alter basal tone of the mesolimbic dopamine system. In other studies, CP154,526 administration had no significant effects on baseline dopamine levels in PFC and NAc [247, 272, 284, 285]. Possibly, these contrasting results may be explained by differences in basal stress levels.

CRFtg mice showed reduced D-amphetamine-induced dopamine overflow relative to wild-type mice both in mPFC and NAc. D-Amphetamine acts as a false substrate for the dopamine transporter and increases dopamine release by reversing transport to expel intra-terminal dopamine stores [286]. Thus, the attenuated extracellular dopamine response to D-amphetamine in CRFtg mice is likely to be a result of decreased intracellular dopamine stores, although this is not reflected in basal dopamine levels. Therefore, the lower basal DOPAC levels in CRFtg mPFC may reflect reduced dopamine metabolism without altered basal release. Notably, the effects of CP154,526 on D-amphetamine-induced dopamine release appeared to be dependent

on the basal tone of CRF. These results fit the recent findings of Lemos and coworkers [20], which show that CRF normally facilitates dopamine release in the NAc, and promotes a positive affective state. Upon chronic stress, this dopaminergic CRF effect is abolished, and affect is switched to a negative emotional state. Accordingly, it may be suggested that CRF_1 receptor antagonists normally reduce dopamine release, but that this effect may change to enhanced dopamine release in a stressed system, like CRFtg mice. Such a mechanism would explain why the initial differential dopamine response of CRFtg and wildtype mice to D-ampethamine can be normalized by prior treatment with CP154,526. This hypothesis is in line with findings from anxiety tests, in which effectiveness of CRF_1 receptor antagonists has been suggested to depend on the stress levels involved [272, 275].

3.2. CRF_1 receptor antagonists and NMDA-glutamate interactions

DMP695 (40 mg/kg), CP154,526 (40 mg/kg) and SSR125543 (30 mg/kg) had no significant effect on PPI disruptions induced by the NMDA receptor antagonists MK801 and ketamine; neither of which has been reported before to our knowledge. To date, the neurochemical basis of PPI disruptions driven by NMDA receptor antagonists remains unclear. Typically, the PPI disruptive effects of systemic treatment with NMDA receptor antagonists are diminished by atypical, but not typical antipsychotics, indicative for involvement of dopamine-independent mechanisms, such as alpha-1 adrenoceptor or 5-HT2A antagonism [287, 288]. However, this PPI-improving effect has not been reported in all studies, and observed PPI improvements were often not complete reversals, suggesting complex circuit interactions involving multiple receptor types, synapses and brain regions [22]. In addition to systemic treatment with MK801, local infusions into the basolateral amygdala, and ventral and dorsal hippocampus were capable of disrupting PPI in rats [289, 290]. It is conceivable that the PPI deficits induced by systemic administration of MK801 are mediated by those brain sites, and if CRF_1 receptor activation would play a role in the effect, interactions with glutamate transmission may be expected in these areas. In the ventral hippocampus of rats, infusion of both the glutamate receptor agonist NMDA and the NMDA receptor antagonist MK801 result in profound PPI reductions [291]. As CRF dose-dependently activates hippocampal principal cells and inhibits NMDA-induced currents in hippocampal neurons via a CRF_1 receptor dependent mechanism [248], it is feasible that CRF and glutamate indeed interact at this point.

The basolateral amygdala is a brain area where CRF_1 receptors are abundant, and it is a major source of extra-hypothalamic CRF release [16]. Local infusions of MK801 or the $GABA_A$ receptor antagonist picrotoxin into the basolateral amygdala disrupt PPI, probably by excitation of the basolateral amygdala. Interestingly, CRF-mediated excitation is involved in acute glutamate receptor activation [292], inducing long-term synaptic plasticity and increasing excitability of basolateral amygdala neurons [293]. Thus, CRF and glutamate are reported to interact at the level of the hippocampus and amygdala, which are brain areas involved in MK801-induced PPI disruptions. Thereby, the current study shows that CRFtg mice are less sensitive for the PPI-disruptive effects of MK801, as compared to wildtype mice, suggesting an interaction between chronically elevated CRF levels and NMDA-glutamate neurotransmission relevant for

PPI, as has also been described for emotional disorders [154]. Although it cannot be excluded that this genotype difference is due to a bottom effect, such an effect would not explain the differential sensitivity observed for amphetamine and MK801 between genotypes. Specifically, CRFtg mice were equally sensitive to the PPI-disruptive effect of amphetamine, whereas wild-type mice were relatively less sensitive to MK801. Thus, in the latter case, it is likely that underlying neurotransmitter systems were altered by chronically elevated CRF levels. The absence of effect of CRF_1 receptor antagonists on the PPI-disruptive effects of NMDA receptor antagonists however, shows that acute blockade of CRF_1 receptor signaling cannot improve PPI disruptions induced by NMDA receptor antagonists. Probably, this finding reflects the idea that PPI disruptions induced by systemic treatment with NMDA receptor antagonists involve multiple sites of action and different neurotransmitter systems, which are unlikely to be compensated for by blocking a single receptor (see [294]). It cannot be excluded though that *chronic* pharmacological blockade of the CRF_1 receptor would have beneficial effects. However, the acute effect of CRF_1 receptor antagonists on PPI deficits in CRFtg mice does not support this notion [26].

3.3. CRF_1 receptor antagonists and spontaneously low PPI

SSR125543 (3-30 mg/kg) and CP154,526 (10-40 mg/kg) had no effect on the spontaneously low PPI response of Wistar Iffa-Credo rats and DBA/2J mice, respectively. PPI in Wistar Iffa-credo rats has not broadly been characterized; some atypical antipsychotics, including clozapine, olanzapine, but not risperidone or the typical antipsychotic haloperidol improved PPI in this strain. [295]. DBA mice on the other hand, have been tested extensively in PPI [269]. Their low PPI response was improved by pharmacologically diverse compounds, including typical and atypical antipsychotics, mood stabilizers, metabotropic glutamate receptor ligands and glycine transporter inhibitors [160, 296-298]. As with Wistar Iffa-credo rats, the neurochemical basis of the low PPI response of DBA/2J mice is not understood; however, based on the current results, a role for increased CRF signaling is not likely to be involved.

4. Conclusions

In conclusion, we tested three different non-peptidergic CRF_1 receptor antagonists, using different PPI tests, in both mice and rats. CRF_1 receptor antagonists were without an effect on sensorimotor gating deficits, not supporting antipsychotic potential. However, we also found that CRF_1 receptor antagonists modulate D-amphetamine-induced dopamine overflow, and that CRFtg mice are less sensitive to MK801-induced PPI disruption, suggesting that chronic CRF_1 receptor blockade might improve sensorimotor gating in glutamatergic and/or dopaminergic models. Last, the current findings do not exclude the possibility that CRF_1 receptor antagonists, alone or in combination with antipsychotics, could be useful in treating psychosis under stress conditions, or other aspects of schizophrenic disorder.

Chapter 7

General discussion

In this thesis, the role of CRF in sensorimotor gating processes was studied. In the first section of this last chapter, we will evaluate the use of PPI as behavioral readout. Then, the data obtained in the experiments will be summarized and discussed in the context of the objectives stated in the Introduction.

1. PPI: a translational behavioral measure related to psychosis

As stated in the Introduction, PPI is considered a reliable, robust operational measure of brain inhibitory gating, which is deficient in psychotic disorders such as schizophrenia. Since similar underlying brain substrates and pharmacological mechanisms regulating PPI have been proposed in animals and humans, the measure is considered to reflect a homologue phenomenon. Though, it was also noted that rodent brains are obviously different from human brains, and species differences do exist with respect to the PPI regulatory neural circuitry and underlying pharmacological mechanisms. This latter aspect was encountered several times in this thesis. For instance, in chapter 3 it was noted that 129Sv mice are more sensitive to the PPI-disruptive effects of exogenous CRF than C57Bl6 mice. Possibly, this reflects differences in the endogenous activity of neurotransmitter systems in these animals, which is also suggested in chapter 5. In this chapter, CRFtg mice appeared to be less sensitive to the effects of $GABA_B$ receptor agonists than CRF-treated 129Sv mice, suggesting that long-term exposure to high CRF levels induced an alteration in neurons that rendered CRFtg mice less sensitive to the effects of $GABA_B$ activation. Similarly, differences in baseline PPI exist between different strains of mice or rats, as was observed in this thesis for 129Sv versus C57Bl/6J mice (chapters 3 and 5), and for Wistar-wu (and Wistar-wi) versus Wistar Iffa-Credo rats (chapter 6). According to the data summarized and reviewed in chapter 2 however, the link between stress and PPI appears to be congruent with neurodevelopmental theories of schizophrenia; giving PPI considerable translational validity with respect to the aims in this thesis.

Another major strength of PPI is its large predictive validity with respect to antipsychotic effects of drugs, as improvement of experimentally impaired PPI in rodents strongly predicts clinical potency of antipsychotic agents (see Introduction). However, PPI should not be considered as a straightforward approach to model psychosis. Instead, it could be more closely related to global functioning in schizophrenia patients [299]; being more sensitive to trait features of the illness, which are "hard-wired" determinants, as opposed to clinical state variables [9]. The latter authors proposed that PPI, rather than being a diagnostic tool, may be of particular value for the study of the biology of schizophrenia and its relationship to functional outcome. Indeed, the link between PPI deficits and positive and negative symptoms in schizophrenia is not very strong [300], whereas PPI reductions highly correlate with core cognitive symptoms, such as distractability [301] and thought disorder [302]. Accordingly, it has been suggested that PPI likely represents "the interface of psychosis and cognition" [303]. This interpretation is highly relevant for drug discovery, given the current call for a focus on the cognitive core of schizophrenia, rather than positive symptoms [304].

A related behavioral index used to assess the effects of antipsychotic treatments is psychotropic-drug induced locomotor hyperactivity. In mice and rats, treatment

with low doses of psychostimulant drugs such as amphetamine induces an increase in horizontal locomotion [305]. The locomotor-activating effects of amphetamine are mediated by the mesolimbic dopamine system (see chapter 4), a neural substrate that is believed to be the most relevant neurobiological substrate in schizophrenia. This similarity gives the model some construct validity. More importantly, amphetamine-induced locomotor hyperactivity possesses considerable predictive validity, as it has been a useful tool for predicting the antidopaminergic activity of neuroleptics. At the same time however, this property restricts the utility of the behavioral measure to the screening of known therapeutic effects, which can solely lead to the discovery of related ("me-too") therapeutic agents [306]. In chapter 4, we further examined a variation to this pharmacologic tool: supra-additive hyperlocomotion induced by a mixture of amphetamine and chlordiazepoxide. This drug-induced state has been used to predict 'antimanic' effects of mood stabilizers. Results showed that the supra-additive effects on locomotion of this mixture did not extend to PPI, indicating that the mechanism underlying the potentiation of D-amphetamine by $GABA_A$ activation is not mechanistically relevant to sensorimotor gating.

Thus, from these results it is concluded that PPI and locomotor hyperactivity represent different aspects of psychotic-like behavior. In addition to disrupting PPI, central administration of CRF also produces locomotor hyperactivity [307]. Based on the arguments described above however, in this thesis PPI rather than horizontal locomotion was chosen as a tool to generate and test hypotheses regarding the interplay of CRF and psychotic-like behavior. In fact, both locomotor hyperactivity and PPI have good predictive validity with respect to detecting antipsychotic-like activity. Though, where locomotor hyperactivity could be useful for a convenient and fast screening of potential interactions of CRF with other neurotransmitters, it does not correspond to pathological processes. PPI on the other hand, is a reliable and robust homologue operational measure of inhibitory brain functions that is deficient in psychotic disorders, and PPI has high translational validity with respect to the aims of this thesis. Last, it likely represents the interface of psychosis and cognition, which is relevant to current drug discovery. Therefore, PPI is considered a highly suited tool to model psychotic-like behavior in rodents.

2. Determine which mechanisms are involved in CRF-induced PPI deficits.

Historically, researchers have exploited drug-induced states that produced schizophrenia-like symptoms in healthy humans to develop animal models for schizophrenia. In this way, three distinct pharmacological PPI models were developed, based on manipulations to disrupt PPI: dopamine receptor agonists, serotonin receptor agonists and glutamate receptor antagonists. Each of these distinct models has been used to test the effects of antipsychotic agents, and each has been proven to be sensitive to at least some of these therapeutics [10]. In this thesis we focused on possible interactions of CRF with dopaminergic, glutamatergic and GABAergic neurotransmitter systems, as those have been most prominent in current theories for

the development of schizophrenia. In order to put the findings into perspective, these theories are outlined below, in addition to the respective interactions found.

2.1. Dopamine

The first hypothesis on the origin of schizophrenia focused on the dopaminergic system, mainly based on three observations [308]. First, antipsychotic drugs act by blocking dopamine-D2 receptors. Second, large doses of psychostimulants, i.e. drugs that increase dopaminergic activity, can produce psychotic episodes in healthy subjects that are similar to the positive symptoms of schizophrenia. Third, small doses of the same drugs can exacerbate psychotic episodes in psychotic individuals or trigger transient symptoms in patients in remission. Indeed, it appeared that dopaminergic transmission is increased in patients with schizophrenia [309]. According to later versions of the "dopamine hypothesis of schizophrenia" however, particularly the balance between different neural pathways is hypothesized to be disrupted; hyperactivity in mesolimbic dopamine pathways is thought to be responsible for positive symptoms, while hypoactivity in mesocortical pathways could underlie negative and cognitive symptoms [257]. The dopamine hypothesis of schizophrenia has dominated theorizing for more than 30 years, and antipsychotic medications that were based upon the mechanism of antagonizing dopamine-D2 receptors have continued to be developed [310].

As discussed in chapter 6, a body of evidence suggests that CRF may modulate dopaminergic function, thereby influencing dopamine-based behaviors. In this chapter, we investigated whether modulation of dopaminergic signaling could be involved in the disruptive effects of CRF on PPI. We found that compared to wild-types, CRFtg mice had alterations in basal dopamine metabolism and attenuated extracellular dopamine response to Damphetamine, the latter being normalized by CRF_1 receptor antagonists. Thus, chronically elevated CRF levels do influence dopamine metabolism. These modulatory effects of long-term CRF on dopamine signaling could be relevant to PPI, in particular since the dopamine-D2 receptor antagonist haloperidol improved PPI deficits in CRFtg mice [28]. In addition, dopamine may play a role in the PPI-disruptive effects of CRF, as PPI disrupted by acutely administered CRF is also improved by haloperidol in rats [236].

However, behaviorial data obtained in our study indicate that CRFtg mice are equally sensitive to the PPI-disruptive effects of Damphetamine. Thus, to further quantify the role of dopamine in the PPI-effects of CRF, it could be interesting to study the effects of CRF on PPI after manipulating various parameters of the dopamine system in rodents.

2.2. NMDA and $GABA_B$

A newer hypothesis on the origin of schizophrenia is the "NMDA receptor hypofunction hypothesis of schizophrenia" (reviewed by Stahl [257]). This theory attempts to explain the origin of the dopamine dysfunctions observed in the illness, and came about from the observation that when NMDA receptors are made hypofunctional by NMDA receptor antagonists such as phencyclidine (PCP), in healthy subjects PCP can

induce psychotic symptoms reminiscent of schizophrenia. In the following, the basic theoretical underpinnings of this hypothesis will be outlined (see [257]).

Under normal conditions, descending glutamate pathways from cortical areas tonically inhibit dopamine release in the mesolimbic dopaminergic pathway, via NMDA receptors on inhibitory GABA interneurons in the ventral tegmental area. In schizophrenia, these NMDA receptors are thought to be regionally hypoactive, which attenuates the GABAergic braking system, ultimately leading to mesolimbic dopamine hyperactivity. Interestingly, PCP mimics both positive and negative symptoms of schizophrenia, which might suggest that NMDA receptor hypofunction not only underlies mesolimbic dopamine hyperactivity, but also mesocortical dopamine hypoactivity. Theoretically this is plausible, as the same glutamate neurons that are responsible for inhibiting the mesolimbic dopamine pathway via GABA interneurons, also synapse directly on dopaminergic neurons of the mesocortical pathway in the ventral tegmental area, thereby exciting them tonically. Thus, local hypofunction of NMDA receptors exerts opposite effects in downstream dopamine neurons, simultaneously causing hyper- and hypoactivity in mesolimbic and mesocortical dopamine pathways, respectively.

In addition, Stahl hypothesizes that NMDA receptor hypofunction could contribute to psychotic states via effects on a second pathway [257]. From the prefrontal cortex (PFC), glutamatergic connections descend to the nucleus accumbens (NAc), where they innervate local GABA interneurons via NMDA receptors. These interneurons, in turn, tonically inhibit glutamate neurons in the thalamus, a brain center that receives and integrates sensory input from other areas, and projects back to the original PFC glutamate neurons. In this circuitry, thalamic GABAergic input functions to create a local sensory filter, preventing too much sensory information traveling through the thalamus from reaching the cortex. Hypofunction of NMDA receptors on NAc GABA interneurons reduces the effectiveness of the thalamic filter, thereby inducing psychotic states. To make it even worse, dopaminergic input from the mesolimbic pathway, which was already increased due to NMDA hypofunction in the VTA, functions in this circuitry to inhibit the GABA interneurons projecting to the thalamus. Together, combined effects of lower excitatory drive to and increased inhibition of GABAergic neurons that create the thalamic filter, is hypothesized to cause an overload of sensory information escaping diffusely into the cortex, clinically manifesting as psychotic symptoms.

Thus, according to this second theory for the origins of schizophrenia, a series of dysfunctioning NMDA receptors located on GABA interneurons ultimately allows the generation of hyperdopaminergic psychotic states outlined in the original Dopaminergic hypothesis for schizophrenia [257]. In this thesis we investigated whether CRF could influence this route. In chapter 6 it was shown that CRF_1 receptor antagonists do not improve PPI deficits induced by the NMDA receptor antagonists ketamine and MK801, indicating that acute blockade of CRF_1 receptors cannot improve PPI disruptions induced by NMDA receptor inactivation. It was argued though, that chronic pharmacologic blockade of CRF_1 receptors might be needed to take effect, since CRFtg mice were less sensitive to the PPI-disrupting effects of MK801. Thus, in the long run, elevated CRF levels may alter NMDA-glutamate neurotransmission relevant to PPI. This finding is in line with a study showing that CRF potentiates NMDA

receptor signaling in ventral tegmental area neurons [255]. Combining this study with our findings, it may be speculated that following long-term exposure to elevated CRF levels, NMDA receptors become desensitized, which could contribute to disease pathology. To gain some support for this inference, the effects of agents that activate the glycine modulatory site on the NMDA receptor, the glycine B receptor could be tested on PPI in CRFtg mice, for instance. Clinically, this drug class reduces some symptoms in chronic schizophrenia [311].

To determine whether CRF could interact with GABAergic signaling to affect PPI, in chapter 3 we tested several drugs acting on the GABA system in CRFtg mice. The reason for this approach was that valproate, a mood stabilizer acting on the GABA system, robustly improved CRF-disrupted PPI. However, first, neither of the $GABA_A$ or $GABA_B$ antagonists tested was found to influence the beneficial effect of valproate on PPI in CRFtg mice. Thereby, the effect of valproate was not mimicked by the $GABA_A$ receptor agonist muscimol, or by the GABA-transaminase inhibitor vigabatrin. Together, these results indicate that the effect of valproate in CRFtg mice is independent of its enhancement of GABAergic transmission, and that enhancement of GABAergic neurotransmission is not sufficient to improve CRF-induced PPI deficits, respectively. Alternative mechanisms of action that were also excluded as possibly underlying the effect of valproate were inhibition of HDAC and reduction of HPA axis activity. Thus, valproate's effect was not accounted for by a single mechanism of action. We further focused on CRF-$GABA_B$ receptors though, as according to theory, GABA interneurons connect to dopaminergic neurons via $GABA_B$ receptors [311]. In chapter 4, we found that $GABA_B$ receptor agonists reverse PPI-deficits induced by acute, but not chronic elevations of CRF. The former finding indicates that $GABA_B$ receptor activation compensates for the PPI-disrupting effects of acutely administered CRF. Theoretically this is possible, as both CRF and $GABA_B$ receptors are G protein coupled receptors, which could promiscuously link to other second messenger systems. In figure 6 of chapter 4, two possible levels have been proposed in the brain circuitry regulating PPI, where CRF could interact with $GABA_B$. One of those levels is the mesolimbic dopamine system, which may be relevant to the differential effects found in the two CRF exposure regimes. In fact, as was described in the previous section, CRFtg mice show neurochemical alterations in their mesolimbic dopamine systems, and CRF-$GABA_B$ interactions have been reported in mice dopaminergic neurons [249]. Actually, it was proposed that presynaptic $GABA_B$ receptors could modulate the release of striatal dopamine. Possibly, long-term exposure to elevated CRF levels caused some adaptation in this route, for instance at the level of NMDA receptors, leading to alterations in dopamine and $GABA_B$ signaling in CRFtg mice. In this respect, it may be interesting to study the effects of dopaminergic and $GABA_B$-ergic agents in systems with low endogenous NMDA receptor function.

3. Determine if CRF_1 receptor antagonists could have antipsychotic-like actions

From the findings discussed above, it can be concluded that unraveling the mechanisms involved in the effects of CRF on PPI is very complex, making it impossible to obtain conclusive answers within the scope of this thesis. Thus, as an alternative route towards our goal to investigate the role of CRF in sensorimotor gating processes, we chose a therapeutic approach. Specifically, if stress through CRF is important in the development of psychotic disorders, then it would be expected that targeting the CRF system could be beneficial for the treatment of psychosis. In chapter 6, we tested the effects of non-peptidergic CRF_1 receptor antagonists in different models of disrupted PPI, each having considerable construct and predictive validity with respect to schizophrenia [10]. We found that none of the CRF_1 antagonists improved PPI deficits in any of the models, thereby not supporting antipsychotic potential. This absence of antipsychotic properties makes it unlikely that targeting CRF as monotherapy would be beneficial in treating acute psychosis. On the basis of our studies, it cannot be excluded though that CRF_1 receptor antagonists could have beneficial effects in stressful conditions, or other symptom dimensions of the schizophrenic disorders. In fact, long-term elevated CRF levels interact with NMDA receptor signaling relevant to PPI (chapter 6), which are receptors that are thought to underlie both positive and negative symptoms. Therefore, it could be that therapeutic effects of CRF_1 receptor antagonists would require chronic treatment; though, the acute effects of CRF_1 receptor antagonists on PPI in CRFtg mice argue against this idea [26]. Despite this apparent lack of effect of CRF_1 receptor antagonists when used as monotherapy, it could be that targeting CRF_1 receptors may be effective for the treatment of positive symptoms of schizophrenia when used as add-on therapy with antipsychotic medication, having affinity for dopamine-D2 receptors. First, in this thesis it was found that chronically elevated CRF levels influence dopamine metabolism that is possibly relevant to PPI. Second, dysregulation of CRF stress systems is implicated in psychotic disorders (see chapters 3, 5, 6). A high CRF tone could enable efficacy of CRF_1-receptor antagonists. Therefore, for future studies it would be interesting to determine the effects of combined treatment with antipsychotics and CRF_1 receptor antagonists in PPI models.

4. Conclusions

From the pharmacological interaction studies in this thesis, it is concluded that chronically elevated levels of CRF influence dopamine, NMDA and $GABA_B$ systems. This finding supports the idea that CRF is an important component in the vulnerability-stress hypothesis for the etiology of psychotic disorder, and could thus be relevant to pathophysiological processes. Yet, based on our results, CRF is not considered a probable target for the treatment of psychosis.

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Samenvatting in het Nederlands

Algemene inleiding: stress en psychose, CRF en PPI

Stress speelt, naast genetische aanleg (ook: kwetsbaarheid), een belangrijke rol bij het vóórkomen van psychotische aandoeningen, zoals schizofrenie. Dit verband wordt beschreven volgens het kwetsbaarheid-stressmodel. Kort gezegd stelt het model dat hoe kwetsbaarder een persoon, hoe minder stress nodig is om een psychose tot stand te brengen. Hoewel het kwetsbaarheid-stressmodel voor het ontstaan van psychose algemeen wordt geaccepteerd, is in gezonde proefpersonen tot op heden geen oorzakelijk verband gevonden tussen de ervaring van een stressvolle gebeurtenis en het tot stand komen van een psychotische ervaring. Wel laten schizofreniepatiënten na een stressvolle periode een grotere kans op terugkeer van een psychotische episode zien. Naarmate er meer kennis beschikbaar komt, wordt het kwetsbaarheid-stressmodel volledi ger, maar nog altijd begrijpen we niet precies welke hersenmechanismen een rol spelen. Er is dus onderzoek nodig waarin de complexe factoren stress en psychose worden vereenvoudigd. Dit proefschrift doet dat, door de samenhang tussen corticotropin-releasing factor (CRF), een eiwitje dat vrijkomt bij stress, en prepulsinhibitie (PPI) van de akoestische schrikreactie, een maat voor bij psychose verstoorde informatieverwerking, te onderzoeken. Omdat CRF en PPI in dit onderzoek een centrale rol spelen, worden ze hieronder nader uitgelegd.

CRF is klein eiwitje met twee functies. Als hormoon reguleert en coördineert het verschillende lichaamsreacties op stress. Het wordt dan afgegeven door de hypothalamus. Daarnaast kan CRF op andere plaatsen in de hersenen worden afgegeven, het is dan actief als neurotransmitter. Dit is een chemische stof die fungeert als overbrenger van het signaal tussen zenuwcellen (neuronen). De biologische effecten van CRF komen tot stand via twee typen receptoren (ontvangende eiwitten in de cel-cel communicatie): CRF_1 en CRF_2 receptoren. CRF_1 receptoren zijn met name betrokken bij het activeren van de stressreactie. Daarom is er een scala aan CRF_1 receptor antagonisten (remmers) ontwikkeld in de hoop dat die zouden werken bij patiënten met stressgerelateerde psychiatrische aandoeningen, zoals angst- en depressieve stoornissen. Ondanks aanvankelijke successen in diermodellen, zijn deze medicijnen wegens schadelijke bijwerkingen in mensen niet op de markt gekomen. De ontwikkeling van nieuwe varianten van deze klasse medicijnen staat echter niet stil.

Uit dierstudies is bekend dat activatie van CRF_1 receptoren ook PPI verstoort. De antipsychotische potentie van CRF_1 antagonisten is nog onbekend. Dit proefschrift probeert hier inzicht in te verkrijgen.

De akoestische schrikreactie is een reflexmatige samentrekking van de skelet- en aangezichtsspieren in reactie op een harde geluidspuls. Als deze geluidspuls kort (30 – 500 milliseconden) wordt voorafgegaan door een zachter geluid (een prepuls), dan wordt deze akoestische schrikreactie verzwakt. Dit wordt de PPI respons genoemd, die door een computer kan worden omgezet in een elektrisch signaal. Conceptueel wordt PPI gezien als een maat voor informatieverwerking. Uit de grote hoeveelheid informatie die voortdurend binnenkomt uit de omgeving, selecteren de hersenen belangrijke elementen, bijvoorbeeld de signalen van een gesprekspartner. Minder belangrijke dingen, zoals het geluid van voorbij razende auto's, worden genegeerd. Dit filter zorgt ervoor dat de hersenen niet overvoerd worden met prikkels.

Bij verschillende psychiatrische aandoeningen, zoals bij schizofrenie, obsessief-compulsieve stoornis en het Gilles de la Tourette syndroom, is PPI verstoord. In schizofreniepatiënten wordt een verstoorde PPI respons in verband gebracht met cognitieve fragmentatie, denkstoornis en positieve en negatieve symptomen. Verondersteld wordt dat in de hersenen van knaagdieren soortgelijke chemische processen ten grondslag liggen aan de regulatie van PPI als in die van mensen. Hierdoor is het mogelijk om onderzoeksresultaten verkregen in knaagdieren te vertalen naar de mens. Hoewel het onmogelijk is om psychose na te bootsen in dieren, is een verbetering van experimenteel verstoorde PPI in muizen en ratten wel voorspellend voor de effectiviteit van humane antipsychotica. Daarom wordt de PPI test in dit proefschrift gebruikt als gereedschap om hypotheses te genereren en te testen omtrent psychotische processen.

Dit proefschrift stond in het teken van onderzoek naar de invloed van de neurotransmitter CRF op informatieverwerkingsprocessen, gemeten aan de hand van PPI van de schrikreactie. De experimenten waren onderverdeeld in twee onderzoekslijnen:

1. Nagaan welke mechanismen betrokken zijn bij CRF-geïnduceerde PPI verstoringen
2. Het onderzoeken van de antipsychotische activiteit van CRF_1 receptor antagonisten

In het volgende wordt per hoofdstuk een samenvatting gegeven van de inhoud van dit proefschrift.

Hoofdstuk 2

In hoofdstuk 2 wordt een literatuuronderzoek beschreven. Hierin worden studies besproken die de effecten van stress op PPI bestuderen. Interessant genoeg blijken deze effecten te passen binnen theorieën van schizofrenie als ontwikkelingsstoornis; toepassing van acute stress tijdens het volwassen leven leidt, zowel in mens als knaagdier, niet tot blijvende veranderingen in PPI. Echter, wanneer proefdieren tijdens een kritieke periode in hun ontwikkeling werden blootgesteld aan stress, of de dieren een genetische aanleg bezitten, kan een stressvolle episode wel degelijk blijvende veranderingen in PPI teweeg brengen.

Bij mensen is er bekend dat er een grote variatie bestaat in stressgevoeligheid; de ene persoon kan meer stress aan zonder ziek te worden dan de andere. Dit heeft waarschijnlijk te maken met genetische verschillen. Het is studies echter nog niet gelukt om in patiënten specifieke genen te koppelen aan het ontstaan van psychoses in relatie tot stress. Dit komt waarschijnlijk doordat schizofrenie wordt veroorzaakt door vele kleine effecten van individuele genen samen. Voor toekomstig onderzoek is het daarom aan te bevelen om humane risicogenen te bekijken in relatie tot stress in modeldieren, met PPI als uitleesmaat voor de effecten op informatieverwerking informatieverwerkingsdeficiënties in schizofrenie.

Bij dit hoofdstuk moet worden opgemerkt dat schizofrenie de bekendste psychotische ziekte is, waar het meeste onderzoek naar psychotische processen op is gericht. Wanneer in dit proefschrift gesproken wordt van schizofrenie, is het

belangrijk te beseffen schizofrenie méér is dan psychose alleen, en dat soortgelijke neurochemische processen ten grondslag liggen aan andere vormen van psychose.

Hoofdstuk 3

In dit hoofdstuk staat valproaat centraal. Dit medicijn uit de groep van 'mood stabilizers' kan dienen als alternatief voor lithium in patiënten met manische depressie, ofwel bipolaire stoornis. Oorspronkelijk werd valproaat op de markt gebracht als anti-epilepticum, maar nu wordt het middel ook voorgeschreven bij de behandeling van angst- en paniekstoornissen, posttraumatische stressstoornis, anorexia nervosa en migraine. Het werkingsmechanisme van valproaat is grotendeels onbekend, maar er zijn enkele oudere studies die beschrijven dat valproaat de activiteit van CRF remt, mogelijk via de neurotransmitter GABA.

Bipolaire stoornis wordt in verband gebracht met veranderingen in het CRF-systeem. Daarnaast laten patiënten tijdens manische periodes een verstoorde PPI respons zien. In dit hoofdstuk hebben we daarom gekeken naar de effecten van valproaat en enkele andere mood stabilizers op de verstoorde PPI respons van CRF transgene muizen, dat zijn muizen met verhoogde CRF-niveaus in hun hersenen. In deze studies bleek dat valproaat de verstoorde PPI van CRF transgene muizen compleet normaliseerde, maar de andere mood stabilizers geen effect hadden. Om te achterhalen of de werking van valproaat direct verband hield met CRF, en niet het gevolg zou zijn van mogelijke secundiare veranderingen veroorzaakt door de chronisch verhoogde CRF-niveaus, hebben we ook gekeken naar de effecten van valproaat op de PPI van gezonde muizen die acuut waren toegediend met CRF. In deze muizen bleek valproaat net zo goed te werken. We concluderen dat valproaat PPI verslechteringen geïnduceerd door CRF verbetert, via een vooralsnog onbekend mechanisme. Voor de kliniek betekent dit dat valproaat in het bijzonder waardevol kan zijn voor patiënten met een ontregeld CRF-systeem.

Hoofdstuk 4

Dit hoofdstuk staat net als het vorige in het teken van bipolaire stoornis. Er is weinig bekend over hoe deze aandoening ontstaat, en er is geen diermodel beschikbaar dat afwisselende episodes laat zien van depressie en manie. Om de werkzaamheid van nieuwe medicijnen te testen, wordt manie-achtig gedrag doorgaans farmacologisch opgewekt in knaagdieren. Zo wordt een mengsel van de psychostimulant D-amphetamine en de benzodiazepine chlordiazepoxide gebruikt om mood stabilizers te identificeren. Toediening van dit mengsel aan knaagdieren brengt een versterkte motorische hyperactiviteit tot stand. Deze respons kan worden genormaliseerd door mood stabilizers. Hoe het effect tot stand komt, is onbekend.

In deze studie hebben we het mengsel van de psychostimulant en benzodiazepine nader onderzocht. Omdat manische patiënten een verstoorde PPI laten zien, hebben we gekeken of PPI ook verstoord zou zijn na toediening van het mensel. Dit bleek niet het geval. Verder hebben we geprobeerd te achterhalen welke componenten van D-amphetamine verantwoordelijk zijn voor de interactie met chlordiazepoxide. Dit deden we door in het mengsel D-amphetamine te vervangen door stoffen die selectief werken op ofwel dopamine (GBR 12909), of noradrenaline (ephedrine); beide

zijn neurotransmitters waar D-amphetamine op inwerkt. De selectieve stoffen bleken niet hetzelfde effect te hebben als D-amphetamine, wat wil zeggen dat niet één van deze neurotransmitters verantwoordelijk was voor het effect. Tenslotte hebben we getest of valproaat ook de hyperactiviteit die wordt veroorzaakt door GBR 12909 kan normaliseren, omdat dit stofje in de literatuur is aangevoerd voor het modelleren van manie-achtig gedrag. In onze proef bleek valproaat dit niet te kunnen, wat aangeeft dat de geldigheid van GBR 12909 binnen dit kader verder moet worden onderzocht.

Hoofdstuk 5

In hoofdstuk 3 is gevonden dat valproaat CRF-geïnduceerde PPI verslechteringen kan normaliseren. Hoewel het precieze werkingsmechanisme van valproaat onbekend is, wordt wel verondersteld dat valproaat faciliterend werkt op GABA-neurotransmissie. In het GABA-systeem zijn verschillende types receptoren te onderscheiden, waaronder $GABA_B$ receptoren. Omdat deze receptoren betrokken zijn bij de regulatie van PPI, onderzochten we in dit hoofdstuk de interactie tussen CRF en $GABA_B$ signalering. We keken hiervoor naar de effecten van verschillende $GABA_B$ agonisten (activators) op PPI van gezonde muizen, die was verslechterd door acute toediening van CRF. We onderzochten ook welk effect de agonisten hadden op de verslechterde PPI respons van CRF-transgene muizen. Vreemd genoeg werkten de $GABA_B$ agonisten enkel in de muizen die acuut waren toediend met CRF, de transgene muizen waren ongevoelig. Wij concluderen dat activatie van $GABA_B$ receptoren de acute effecten van verhoogde CRF-niveaus op PPI compenseert. Echter, op de lange termijn leiden blijvend verhoogde CRF-niveaus tot adaptaties die zorgen voor een verminderde gevoeligheid voor activatie van $GABA_B$ receptoren. In de discussie stellen we twee mogelijke hersengebieden voor waar deze adaptaties zouden kunnen plaatsvinden.

Hoofdstuk 6

Omdat ontregelde CRF-systemen in verband worden gebracht met psychotische toestanden en informatieverwerkingsdeficiënties, hebben we in dit hoofdstuk gekeken naar mogelijke antipsychotische eigenschappen van CRF_1 receptor antagonisten, met PPI als uitleesmaat. We hebben hiervoor verschillende farmacologische modellen gebruikt om PPI te verstoren. Deze modellen waren gebaseerd op verstoringen van het dopamine- en glutamaat-neurotransmittersysteem, die in theorie betrokken zijn bij psychotische toestanden. De modellen zijn gevoelig voor bestaande antipsychotica. Aangezien CRF_1 receptor antagonisten onderling verschillen in de interacties die ze aangaan met CRF_1 receptoren, en muizen en ratten verschillend kunnen reageren op deze stoffen, hebben we verscheidene CRF_1 receptoren getest in verschillende muizen- en rattensoorten, in twee laboratoria. Opvallend genoeg bleek geen enkele CRF_1 receptor antagonist te werken in de modellen. Dit resultaat geeft geen ondersteuning voor eventuele antipsychotische eigenschappen van CRF_1 receptor antagonisten. Op basis van deze studie valt echter niet uit te sluiten dat CRF_1 receptor antagonisten bruikbaar kunnen zijn voor de behandeling van stress-gerelateerde psychoses, of andere aspecten van schizofrene stoornissen.

Hoofdstuk 7

In dit laatste hoofdstuk worden de belangrijkste resultaten van dit proefschrift besproken, aan de hand van de twee onderzoeksvragen uit de introductie. Ook wordt toegelicht waarom er is gekozen voor PPI als uitleesmaat voor informatieverwerking die is verstoord bij psychose.

Uit de studies beschreven in dit proefschrift is naar voren gekomen dat chronisch verhoogde CRF-niveaus invloed hebben op verschillende neurotransmittersystemen die betrokken zijn bij psychotische toestanden: dopamine-, NMDA-glutamaat- en $GABA_B$-systemen. Dit geeft aan dat CRF een belangrijke component kan zijn in het kwetsbaarheid-stressmodel voor het ontstaan van psychotische stoornissen. Gebaseerd op hoofdstuk 6 lijkt CRF echter geen waarschijnlijk doelwit voor de behandeling van psychose.

About the author

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Tessa Douma was born on October the 23th in 's-Heerenberg. In 1999 she received her atheneum diploma from St. Ludger college in Doetinchem. After her propaedeutics (*cum laude*) in Chemistry at Utrecht University in 2000, she subsequently commenced her studies towards an undergraduate degree in Biology at Utrecht University. Tessa graduated with a BSc degree in Biology in 2003 and proceeded her studies taking courses in animal biology at Wageningen University, and in communication and education of life sciences at Utrecht University.

In 2006 Tessa was admitted to the Behavioural Neuroscience track of the Master Neuroscience and Cognition at Utrecht University. During her first internship within this program, she conducted a research project entitled "Home cage behaviour of socially stressed rats as an animal model of depression" under supervision of Dr. Johanneke van der Harst at the department of Animals, Science & Society. Her second research project was entitled "Olfactory bulbectomy as induction model for Alzheimer's disease" and was supervised by Dr. Ronald Oosting at the department of Psychopharmacology. She completed her master's thesis "Linking psychosis and stress: a look at behavioural studies, neurochemistry and prepulse inhibition" under supervision of Dr. Lucianne Groenink. In 2008 she graduated cum laude from Utrecht University with an MSc in Behavioural Neuroscience.

Since October 2008 Tessa was employed as a PhD-student under supervision of Dr. Lucianne Groenink and Prof. Dr. Berend Olivier at the department of Pharmacology at Utrecht University. The research she conducted within her PhD-project resulted in this thesis.

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Douma TN, Millan MJ, Verdouw PM, Olivier B, Groenink L. GABAB receptor agonists reverse prepulse inhibition deficits induced by acute but not chronic exposure to elevated levels of corticotropin-releasing factor.

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